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Novozym 435: the "perfect" lipase immobilized biocatalyst?

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Novozym 435 (N435) is a commercially available immobilized lipase produced by Novozymes. It is based on immobilization *via* interfacial activation of lipase B from *Candida antarctica* on a resin, Lewatit VP OC 1600. This resin is a macroporous support formed by poly(methyl methacrylate) crosslinked with divinylbenzene. N435 is perhaps the most widely used commercial biocatalyst in both academy and industry. Here, we review some of the success stories of N435 (in chemistry, energy and lipid manipulation), but we focus on some of the problems that the use of this biocatalyst may generate. Some of these problems are just based on the mechanism of immobilization (interfacial activation) that may facilitate enzyme desorption under certain conditions. Other problems are specific to the support: mechanical fragility, moderate hydrophilicity that permits the accumulation of hydrophilic compounds (*e.g.*, water or glycerin) and the most critical one, support dissolution in some organic media. Finally, some solutions (N435 coating with silicone, enzyme physical or chemical crosslinking, and use of alternative supports) are proposed. However, the N435 history, even with these problems, may continue in the coming future due to its very good properties if some simpler alternative biocatalysts are not developed.

1. Introduction

1.1. Lipases in biocatalysis

Chemistry is being continuously (and vigorously) pushed to become more environmentally friendly and compatible, therefore green chemistry is nowadays the final goal in most chemical industry developments.[1–4] This is coupled with an ever-increasing demand for products with growing complexity, in many instances with multiple functions and chirality in many of them. In this environment, the use of enzymes as industrial catalysts is rising.[5–7] Enzymes have many properties that make them very interesting: they are the most efficient catalysts in nature, performing their function under very mild conditions (at low pressure and temperature) in aqueous media. Moreover, they are very selective and specific, saving many protection/deprotection steps.[8–13] However, enzymes are catalysts with a biological origin, and they have evolved under natural selection to be able to respond under stress conditions. Thus, enzymes are inhibited by diverse components, their stability is moderate even under physiological conditions and their excellent properties are only exhibited in physiological reactions and substrates.[14] Besides, they are usually water soluble. These properties, although physiologically necessary, are a problem if they are going to be used as industrial biocatalysts, where they are expected to perform their function under standardized conditions. In this respect, maximal stability and activity will always be desired, and the substrates, as well as reaction conditions, may be quite far from the physiological ones.

As a result, enzymes normally need to be improved in many instances before their industrial implementation. Thanks to the development of many different scientific areas, there are several ways of improving these enzyme limitations. Microbiological (metagenomics)[15–20] and genetic (site-directed mutagenesis,[21–23] directed evolution,[24–28] *etc.*) tools

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may provide high enzyme production with improved properties compared to the native enzyme. Immobilization was first a requirement to solve the issue of enzyme solubility, but it has recently become a powerful tool to improve many other enzyme properties like stability, activity, selectivity, specificity, purity, inhibition, or resistance to chemicals.[29–39] As such, enzyme immobilization has evolved from a requirement to use these expensive catalysts to a tool to greatly enhance enzyme features.

Lipases are among the most widely used enzymes in biocatalysis.[40,41] The biological function of lipases is the hydrolysis of triglycerides to produce free fatty acids and glycerol.[42] The heterogeneity of the natural substrate[43–48] has converted lipases in enzymes with a very broad specificity, accepting substrates very different from glycerides (even amides). Thus, lipases are used *in vitro* to catalyze reactions different from those of the natural hydrolase function,[49–54] such as esterification,[55–61] acidolysis,[62–67] interesterificaton,[68–71] transesterification,[72–76] aminolysis,[77–81] perhydrolysis,[82,83] *etc.*, together with a collection of the so-called promiscuous reactions.[84–92]

Lipases are usually quite stable, and so they have been used in diverse media, like aqueous media, organic solvents,[93–95] ionic liquids,[96–99] supercritical fluids,[100–102] and deep eutectic solvents.[103–106] This way, lipases have a huge range of possibilities in industrial biocatalysis. Besides, they have a peculiar mechanism of action called "interfacial activation", which will be explained below.

1.1.1. Interfacial activation of lipases: drawbacks and new opportunities. As previously stated, the natural substrates of lipases are glycerides.[49–54] These molecules have low solubility in water, therefore they form insoluble drops, where the lipase must act. For this purpose, lipases have a peculiar mechanism of action, called interfacial activation, which permits lipases to become adsorbed on the hydrophobic surface of the glyceride drops and act in the interface (this is why lipases are called "interfacial enzymes").[107] This mechanism is based on the existence of a large hydrophobic pocket surrounding the active center.[108–115] An enzyme molecule with this large hydrophobic pocket will be very unstable and will have low solubility in aqueous media. However, this problem is avoided because this hydrophobic pocket it is covered by a polypeptide chain called lid, which generally isolates the active center from the reaction medium (in this "closed" form, the lipase is inactive). The lid also has an internal hydrophobic face that interacts with the hydrophobic areas of the active center and a hydrophilic external face, which interacts with the reaction medium.[108–115] This lid can move, and when it is shifted, it forms a huge hydrophobic pocket exposing the active center to the medium, resulting in the "open" and active form of the lipase, with the hydrophilic phase of the lid interacting with the protein surface. Both conformational lipase forms are in equilibrium, but in the presence of oil drops, the open form becomes adsorbed on the hydrophobic surface of the drops, shifting the conformational equilibrium towards the open form of the lipase and permitting the attack of the glycerides by the enzyme[108–115] (Fig. 1).

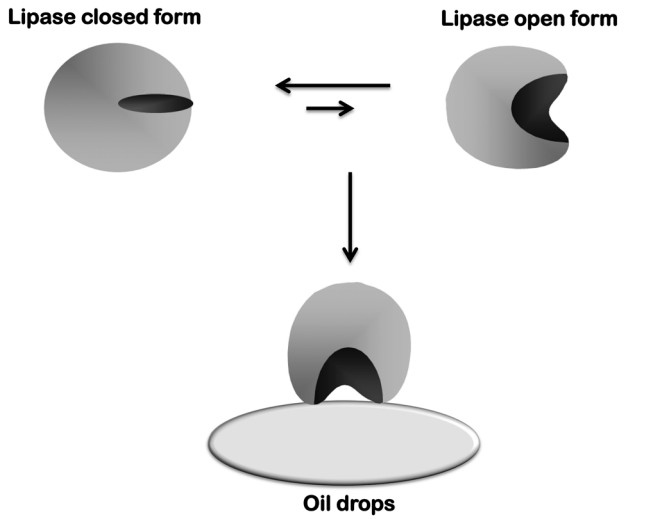

Fig. 1 Conformational equilibrium and interfacial activation of lipases.

This catalytic mechanism may be a problem for lipase handling.[116] Lipases are adsorbed on any hydrophobic surface. As an example, lipases tend to form dimeric aggregates by interaction between two open forms of lipases (giving altered properties)[117–119] (Fig. 2) or may interact with hydrophobic proteins on the crude extract (also altering enzyme properties).[120] This may also be a problem when immobilizing a lipase on a solid support, as the lipase molecules will be isolated from the external medium and external drops of the hydrophobic substrate can hardly interact with the enzyme, making their interfacial activation impossible.

However, once this phenomenon is known, it can be utilized for some purposes. For example, properly oriented immobilized lipases have been used as a chromatography matrix to purify other lipases *via* interaction between the open forms of two lipase molecules[120,121] (Fig. 3).

Moreover, as described later, this allowed development of one of the most utilized protocols for lipase immobilization: the immobilization of lipases on hydrophobic supports *via* interfacial activation.[122]

1.2. Lipase B from *Candida antarctica*

CALB has a molecular weight of 33 kDa, with a pI of 6.0. CALB is an α/β protein with many features similar to those of

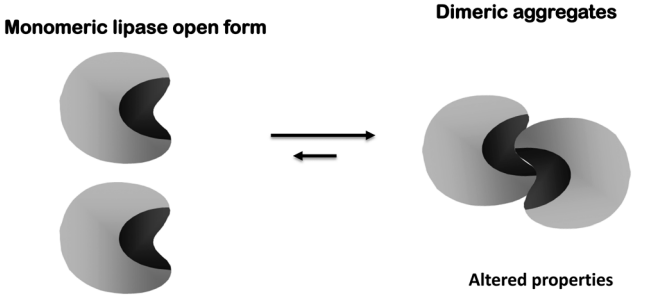

Fig. 2 Formation of dimeric aggregates between two open forms of lipases.

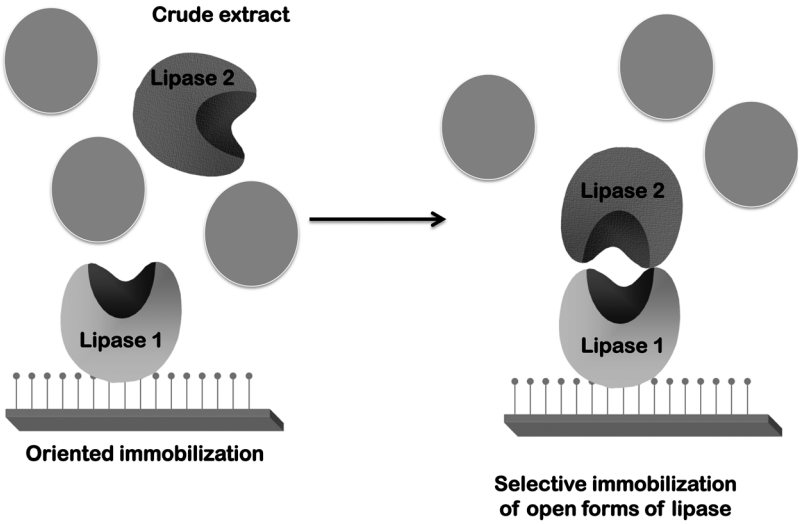

Fig. 3 Use of immobilized lipases as a chromatographic phase to purify lipases.

other lipases. The structure of CALB has been fully resolved and shows that the enzyme has a Ser-His-Asp catalytic triad in its active site, with a very small lid that is unable to fully seclude the active center.[123,124] Even with that small lid, CALB retains its capacity to be adsorbed on hydrophobic surfaces, that is, it remains an interfacial enzyme. For this reason it is considered a true lipase, although the closed form is not really closed as in other lipases.[125,126] This may make its handling simpler than using other lipases, as it does not have the strong tendency of other lipases to form dimeric aggregates.

This enzyme is among the most stable commercialized lipases[127,128] and has been used in a wide range of reactions; it is in fact very likely the most widely used lipase.[129–131] This lipase has been utilized in almost all areas of lipase utilization, from triglyceride modification to biodiesel production, from resolution of racemic mixtures to regioselective reactions, production and degradation of polymers, promiscuous reactions, *etc.*[132–152]

This good stability has made CALB one of the most intensively researched enzymes in ionic liquids.[153–155] CALB properties have been improved *via* genetic tools.[156–159] Some papers are based on the comparison of commercial CALB and some other recombinant CALB expressed in different hosts (mainly *Pichia pastoris*) presenting in some cases very different properties.[160–166]

1.3. Immobilization of lipases

As previously stated, immobilization of enzymes is a requirement for most industrial enzyme uses, and the same thing occurs when using lipases. As discussed above, immobilization may be a tool to improve enzyme features; the objective should be to have a reusable, active and, if possible, improved biocatalyst.[29–39] This way, the costs associated with immobilization may be fully compensated. Here, we will not review the immobilization; there are some excellent review papers that may be used for that purpose.[29–39,167–172] However, in the case of lipases some points must be carefully considered.

The first point is that an enzyme immobilized on porous supports will not be exposed to external interfaces. This is positive because enzyme inactivation is not possible by interaction with these interfaces,[33] but if the enzyme is inside a porous support, interfacial activation is not possible, (except when using nearly anhydrous media that can penetrate the support porous system).

Second, as previously explained, lipases tend to form bimolecular aggregates involving the open forms of two lipase molecules.[117–119,121,173–175] If immobilization is performed under conditions where this is favored, the effect may be quite negative because these dimers will be immobilized together with the monomeric enzyme molecules (Fig. 4). These dimeric lipase forms generally presented altered properties and lower activity than the monomeric enzyme.[117,173] Moreover, the percentage of dimers and monomers will depend on the exact immobilization conditions, making it difficult to reproduce the results when using different enzyme batches. Additionally, if only one of the enzyme molecules forming the dimer is attached to the support, some enzyme leakage may be produced contaminating the final product (Fig. 5). The use of detergents during immobilization may solve this problem: it will break the dimers and allow the immobilization of monomeric enzymes.[117–119,121,173–175] (Fig. 6) Moreover, if immobilization is *via* an intense enough enzyme–support interaction, the presence of detergent during immobilization can also improve enzyme activity by maintaining the open form of the lipase when the detergent is eliminated[176–183] (Fig. 6).

On the other hand, the active center of lipases is very flexible, and it has been shown in many instances that lipase properties may be strongly modulated *via* immobilization.[184–204] However this also has a counterpart: if the enzyme already has the desired specificity of selectivity, keeping the properties of the lipase after immobilization may

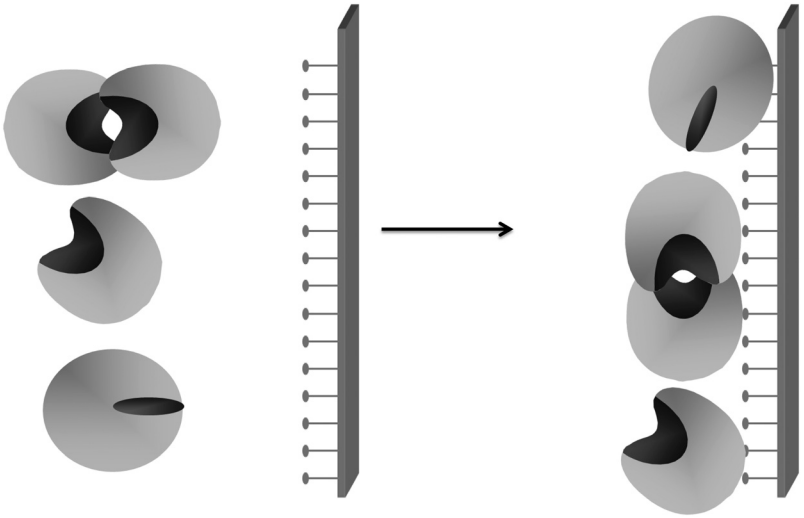

Fig. 4 Problems generated in lipase immobilization by the tendency of lipases to form enzyme dimeric aggregates.

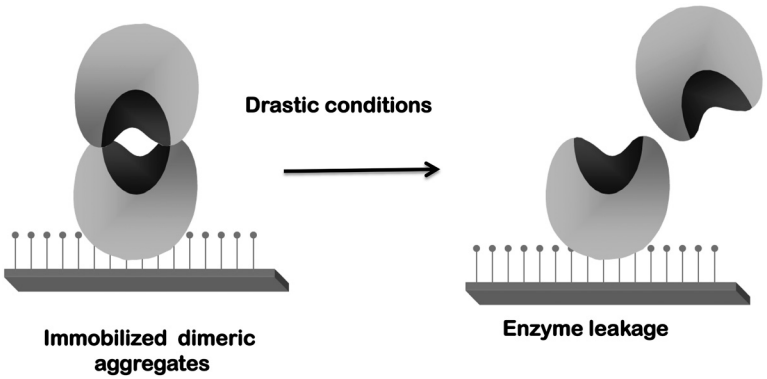

Fig. 5 Risk of enzyme desorption if dimeric aggregates are immobilized *via* one enzyme molecule only.

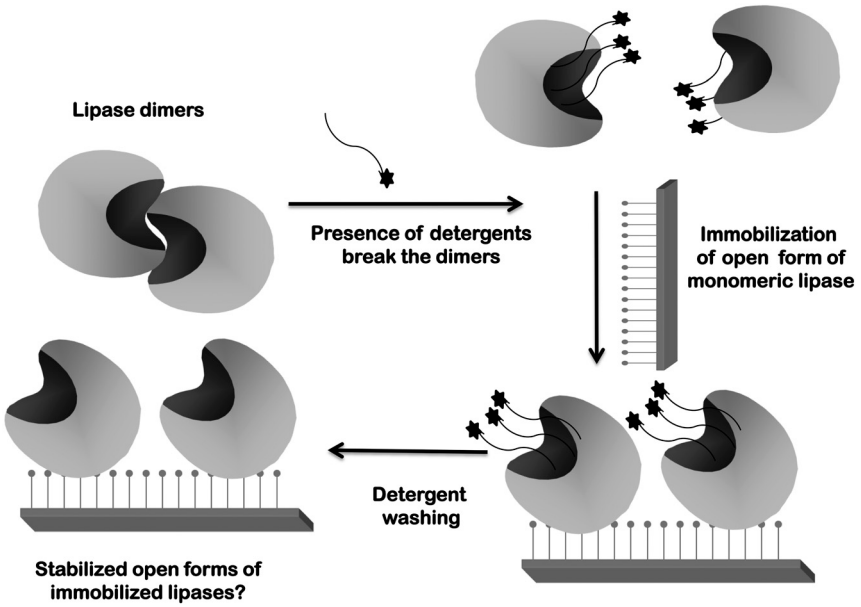

Fig. 6 Immobilization of lipases in the presence of detergents to break the dimers ensures immobilization of monomeric forms.

be quite difficult.[32] Thus, lipase immobilization must consider some points that are not required for other enzymes.

1.3.1. Immobilization of lipases *via* interfacial activation. As stated above, the ability of lipases to become adsorbed on any hydrophobic surface makes immobilization of lipases on hydrophobic supports a very popular strategy.[122] There are many advantages for this protocol: immobilization, purification and stabilization of the lipases become a one-step process, the immobilized enzyme is stabilized (because the open and adsorbed form of the lipase is more stable),[205–207] the open and monomeric form of the lipase become fixed (that is, lipases are less dependent on the immobilization conditions) (Fig. 7)[122] and the immobilized lipase is less sensitive to the experimental conditions (because there is no conformational equilibrium to be shifted).[122,208,209]

Thus, lipases immobilized by interfacial adsorption may almost fully retain their activity even under very high ionic strength, while a conventional immobilized enzyme will have the active center closed under these conditions.[122] However, the method also has some problems. Immobilization is reversible and based on hydrophobic interactions, which means that lipase molecules may be released to the medium at high temperatures, in the presence of co-solvents or detergents.[210] Special care must be taken with some substrates or products with detergent properties,[211] for example triglyceride hydrolysis will release free fatty acids and di- or monoglycerides, which have recognized detergent properties and can favor enzyme release. It has been reported that even diacylglycerols from short chain carboxylic acids such as diacetin or dibutyrin may be enough to favor enzyme release from the support.[212,213] This problem may be reduced if some intermolecular crosslinking (covalent or physical) is performed,[214–218] or using heterofunctional supports (supports having the acyl moiety and some reactive groups able to give rise to an additional covalent or ionic immobilization).[210,219–227] Later on, this will be discussed in detail in this review.

Thus, the immobilization of lipases *via* interfacial activation may be the most popular immobilization strategy, using silica (and other inorganic materials) nanoparticles or natural hydrophilic supports coated with long acyl groups, hydrophobic polymeric supports coated (or uncoated) with hydrophobic groups, *etc.*[191,228–247]

1.3.2. Novozym 435. Novozym 435 (N435) is an immobilized preparation of CALB supplied by Novozymes, and the first manuscript reporting its use dates from 1992.[248] The utilized support is Lewatit VP OC 1600, a macroporous acrylic polymer resin, where CALB is adsorbed *via* interfacial activation, although apparently the way the enzyme is immobilized results in the immobilization of some aggregates.[249–251] The features of CALB immobilized on other hydrophobic supports is, in some instances, quite different from that of N435, suggesting some particularities on the immobilization during N435 preparation, although the enzyme may be fully released from the support using detergents.[252]

This is the most used lipase preparation in the literature. A search in Scopus on 27th November 2018 revealed 1500 papers related to this commercial immobilized enzyme. This review will present some examples of successful use of this biocatalyst (focused from 2016 onwards), very likely one of the most commercially available stable and active preparations that have permitted many studies to be performed. Later, a deep discussion on the problems in the use of this biocatalyst will be performed; some will be related to the immobilization mechanism of the enzyme and shared with any other lipase immobilized just by the lipase interfacial activation mechanism. Other problems will

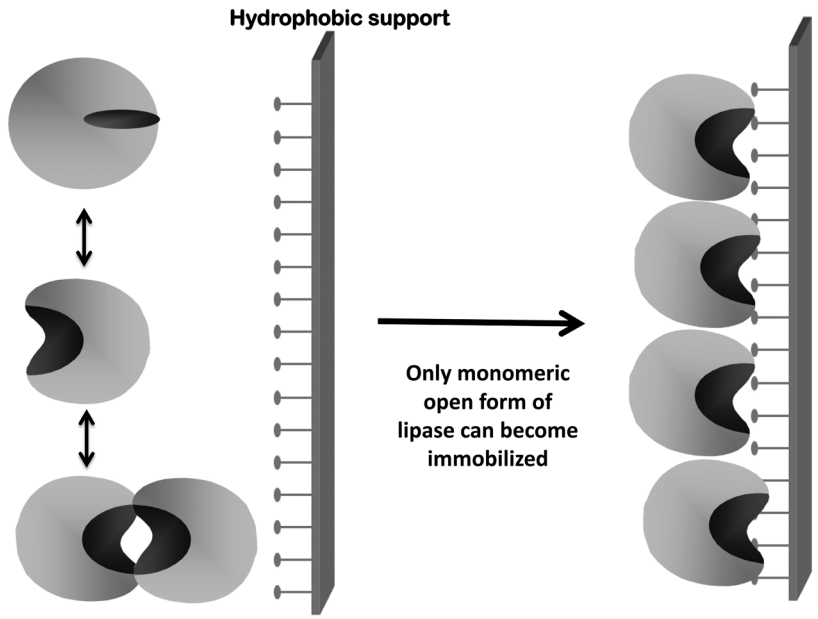

Fig. 7 Immobilization of lipases on hydrophobic supports at low ionic strength *via* interfacial activation: immobilization of monomeric and open forms of lipases.

be related to the support. Finally, some alternatives to solve the problems will be presented.

2. Lewatit VP OC 1600

The N435 support, Lewatit VP OC 1600, is a macroporous matrix with a spherical bead morphology. This organic carrier is made of macroporous poly(methyl methacrylate) crosslinked with divinylbenzene, and it is currently marketed by Lanxess (Germany) as Lewatit VP OC 1600.[249,253]

The chemistry of the synthesis of the Lewatit support has not been described in detail. However, it is presumed that the polymethacrylate divinylbenzene copolymer matrix is produced through condensation polymerization reactions or by addition between methacrylic esters and divinylbenzene.[254] Lewatit VP OC 1600 has an average particle size, surface area, and pore diameter of 315–1000 μm, 130 m^2 g^{-1}, and 150 Å, respectively (product information Lewatit VP OC 1600, Lanxess, edition: 2011-10-13). These characteristics have allowed its application to enzyme immobilization, especially for immobilizing lipases due to its relative hydrophobicity. This support has not been exclusively used by Novozymes to produce N435.

For example, CALB has been immobilized on this support in some laboratories. Thus, the enzyme was immobilized on different hydrophobic supports, including Lewatit VP OC 1600. The home-made preparation obtained using this support did not differ in its properties from N435, suggesting that industrial immobilization did not modify the enzyme.[252] However, both preparations differ in the enzyme catalytic properties from CALB immobilized on other hydrophobic supports (giving inverse enantiospecificity in the hydrolysis of rac-2-*O*-butyryl-2-phenylacetic acid and different selectivity in the hydrolysis of 3-phenylglutaric acid dimethyl diester).[252]

Considering the success of N435, it could be expected that many other lipases have been immobilized on the same support. However, only some examples may be found.

For instance, a lipase from *R. arrhizus* was immobilized on Lewatit VP OC 1600, Duolite A568, Amberlite X. A.D 761 and *O*-pentynyl dextran and used to prepare geranyl octanoate in organic solvent. The results showed that *O*-pentynyl dextran was clearly superior compared to adsorbents like Lewatit VP OC 1600, for which only a loading of 1.1% protein was obtained, additionally it was 3 times less active in the esterification of geraniol and octanoic acid.[255]

In another instance, phospholipase A1 was immobilized on Lewatit VP OC 1600, showing a good immobilization efficiency (79% global immobilization yield) and a very high specific activity (6.7×10^{-3} μmol per g protein per min) in the modification of phosphatidylcholine with *n*-3 polyunsaturated fatty acids.[256] These results surpassed those obtained using other hydrophobic supports such as silica coated with octyl groups, Accurel MP 1000 or Celite 545, among others.[256]

Rhizopus oryzae and *Carica papaya* lipases were also immobilized on Lewatit, and used in biodiesel production by transesterification of Jatropha oil with methanol. The results obtained showed that the highest immobilization yield was achieved with the lipase from *Carica papaya* (98%), while for lipase from *Rhizopus oryzae* the immobilization yield was only 77.2%.[257] However, the percentage of methyl ester production was 65%, w/w when using the lipase from *Rhizopus oryzae* while for *Carica papaya* lipase it was 51.7%.[257]

In a last example, the lipase from *Penicillium sp.* (CBMAI 1583) was immobilized on a battery of hydrophobic supports including agarose-butyl, agarose-phenyl and agarose-octyl, acrylic Toyopearl, octadecyl Sepabeads and Lewatit VP OC 1600. The biocatalysts were used in the hydrolysis of fish oil to get omega-3 fatty acids and ethanolysis to produce the respective ethyl esters. Immobilization yields were very high in all cases (over 75%) and the expressed activity ranged from 54.2% to 144.9% compared to the free enzyme. However, the least stable preparation at drastic pH values was that prepared using Lewatit.[258]

As such, the great success of Lewatit VP OC 1600 with CALB cannot be extrapolated to other lipases. It has been clearly showed that the selection of the "optimal" support may depend on the enzyme, the specific reaction (*e.g.*, to determine the activity, selectivity or specificity) and the experimental conditions (*e.g.*, to determine the enzyme stability, activity, selectivity or specificity).[185,191,234,252,259]

3. Novozym 435: a history of success

3.1. Use in chemistry and fine chemistry

3.1.1. Esterification reactions. The production of esters is one of the main applications of lipases in fine chemistry. Even though they can be synthesized chemically, the use of enzymes permits labelling the products as green and thus increases the price. N435 has been used in many examples. Esters can be produced *via* esterification, using unmodified substrates (that is, a thermodynamically controlled synthesis), or an activated acyl donor (*i.e.*, a kinetically controlled process).[260]

Many simple esters have been produced *via* esterification catalyzed by N435 using different media. As an advantage of enzyme specificity, the specific acylation of primary alcohols in the presence of secondary alcohols and phenols was demonstrated using ethyl acetate as a medium and acylating agent and N435 as a catalyst.[261] For example, butyl butyrate was produced by esterification in a solvent-less medium.[262] Optimization *via* the response surface methodology gave a yield of butyl butyrate near 100%. In a very interesting paper, N435 was used to esterify 1-butanol and butyric acid using diesel as the reaction medium, with a 90% yield.[263] The diesel thus modified has some improved properties. Another paper shows that butyric acid and ethanol were esterified in *n*-hexane using N435.[59] In another paper, the esterification of L-ascorbic acid and *n*-octanoic acid or *n*-caprylic acid catalyzed by N435 permitted yields above 85% to be reached.[264] N435 was used to modify Konjac glucomannan *via* esterification with oleic acid in isooctane.[265] Butyl caprylate was produced from caprylic acid and butanol in a solvent-free system

employing a stirred batch reactor catalyzed by N435 (yield was 92%).[266] N435 was used to study the thermodynamics in the esterification of succinic acid with ethanol.[267] More than 95% citronellyl palmitate ester from palmitic acid was produced by esterification catalyzed by N435 using hexane as a solvent.[268] Azelaic acid was diesterified with lauric alcohol using N435 and used as a bacteria growth inhibitor.[269] Another paper shows that methyl caffeate production *via* esterification catalyzed by N435 was improved *via* a microfluidic strategy in a continuous-flow reactor (yields surpassed 98% in 2.5 h).[270] N435 was also used in the esterification of oleic acid and kojic acid in a stirred tank reactor with a yield near 43%.[271] Using a fluidized tank reactor the yield decreased, but the operational stability of the biocatalyst was enhanced. Esters of some free fatty acids and picolinol were produced using N435 in toluene as the biocatalyst for the determination of free fatty acids, but they could not be applied for some epoxy free fatty acids, fatty wax, or parinaric acid.[272] The esterification of furfuryl alcohol and castor oil fatty acid catalyzed by N435 in a solvent-free system gave a yield of 88.64% (% w/w) at 5 h.[273]

Ionic liquids, deep eutectic solvents and supercritical fluids have been used in some instances as reaction media in N435 esterification reactions. Several alkyl dihydrocaffeates were synthesized in ionic liquids by the esterification of dihydrocaffeic acid with methanol, hexanol octanol and dodecanol catalyzed by N435.[274] Lauryl ferulate was produced *via* esterification of ferulic acid and lauryl alcohol catalyzed by N435 in ionic liquids (yields were higher than 90%).[275] This biocatalyst was used in the esterification of *n*-butanol and D,L-lactic acid in supercritical trifluoromethane/ionic liquid and supercritical carbon dioxide/ionic liquid medium.[276] Butyl stearate and ethyl stearate were produced *via* esterification catalyzed by N435 (92% yield).[277] Enzyme reuses were not satisfactory (activity decreased after 3 or 5 reuses). The esterification of dihydrocaffeic acid with hexanol in ionic liquids was statistically optimized using N435 as a catalyst (yield of 84.4%).[278] Oleic acid was esterified with different alcohols in supercritical carbon dioxide using N435.[279]

Ultrasound has been used to intensify some of these esterification reactions. The solvent-free ultrasound-assisted synthesis of citronellol laurate *via* esterification catalyzed by N435 gave more than 95% conversion and allowed the enzyme to be reused for 5 cycles.[280] N435 was used under ultrasonic irradiation to produce L-ascorbyl fatty acid esters as antioxidant materials.[281] Ascorbyl linoleate was obtained by the esterification reaction between linoleic acid and ascorbic acid catalyzed by N435, using an ultrasound bath.[282] The yields reached 90% using *tert*-butanol as an organic solvent.

Microwave irradiation has been used in many examples as a heating strategy. *n*-Butyl propionate was synthesized by esterification of propionic acid with *n*-butanol under microwave irradiation by N435, Lipozyme TL-IM and Lipozyme RM-IM.[283] N435 was the most active biocatalyst, reaching 92% conversion. In another paper, esterification of valeric acid and ethanol in solvent-free medium was intensified by microwave irradiation of N435, Lipozyme TL-IM and Lipozyme RM-IM.[284] N435 gave almost 70% conversion and could be reused. The solvent-free microwave-assisted production of isoamyl acetate *via* esterification (acetic acid) or transesterification (acetic anhydride and ethyl acetate) catalyzed by N435 and Lipozyme RM-IM has been studied.[285] N435 was found to be the optimal catalyst, using acetic anhydride; however it did not exhibit a good operational stability.

N435 has been compared with other lipases in some instances. For example, benzyl propionate was synthesized by lipase catalyzed esterification, comparing N435, Lipozyme TL-IM, Lipozyme RM-IM and a home-made biocatalyst from CALB.[286] Among them, N435 exhibited the best performance in solvent-free medium, with a yield of more than 40%. Esterification of geraniol with some acids was performed using different catalysts, with N435 being the most active one.[144] Geraniol and butanoic acid were esterified by N435 in a solvent-free system with a yield of over 95%, higher than the yield observed using a home-made biocatalyst.[287] N435 was also the best enzyme preparation among the assayed ones in the synthesis of several geraniol esters *via* an esterification process in a continuous-flow packed-bed reactor (*e.g.*, 87% of geranyl propionate).[288] Ethyl lactate was produced *via* esterification catalyzed by N435, with a yield near 90%.[289] N435 presented better results than some homemade biocatalysts or Lipozyme RM-IM. 5-Hydroxymethylfurfural and levulinic acid were used to produce 5-hydroxymethylfurfuryl levulinate by esterification catalyzed by N435 (the best enzyme among several assayed).[290] A yield of 85% was achieved using 2-methyltetrahydrofuran as a reaction solvent. Levulinic acid was esterified with 1-butanol, ethanol, and methanol catalyzed by N435.[291] In another paper, *n*-butyl levulinate was synthesized by esterification using Lipozyme RM-IM, Lipozyme TL-IM, and N435 in both a stirred tank batch reactor and a continuous flow packed bed tubular microreactor.[292] N435 was the best catalyst (yields of 85%). Solvent-free production of cetyl laurate, myristate, palmitate and stearate has been also reported to be catalyzed by N435 (conversion was higher than 98.5%).[293] The same groups reported that a cetyl ester mixture was obtained *via* esterification of myristic acid, palmitic acid or stearic acid (95%) and cetyl alcohol.[294] The reaction was catalyzed by different enzyme preparations, with N435 being the reference, and not overtaken by any of the new biocatalysts (with a product cost of 56.5 € per kg *versus* 58 € per kg for the best new catalyst).[287]

In other cases, a transesterification reaction has been used to get the target product. For example, eugenyl acetate has been synthesized in supercritical carbon dioxide by transesterification of eugenol and acetic anhydride catalyzed by N435.[295] More than 55% of feruloyl oleins were produced using N435 in toluene with ethyl ferulate and triolein as substrates.[296] Methyl gallate was produced using N435 as a catalyst and propyl gallate and methanol as substrates, in a deep eutectic solvent.[297] N435 was found to be the most efficient among the analyzed enzymes in the production *via* transesterification of the sorbitol ester of norbixin (50% total reaction yield).[298] N435 was used to catalyze

the synthesis of L-ascorbyl phenolates *via* a transesterification reaction using the corresponding vinyl phenolates.[299] The transesterification of butyl acetate with hexanol was studied with N435 to analyze the causes of catalyst activity loss in enzymatic catalyzed reactive distillation.[300] The transesterification of epoxidized soybean oil catalyzed by N435 permitted the production of epoxidized soybean oil methyl esters with a 95.7% yield.[301] The biocatalyst was reutilized in 10 cycles maintaining the activity. Vinyl acetate and 2-phenethyl alcohol have been used to produce 2-phenylethyl acetate in hexane employing N435 as a catalyst.[302] Aromatic aldehyde oximes were acetylated by reaction with vinyl and isopropenyl acetates catalyzed by N435 (the best one among the assayed ones) to produce aromatic aldehyde oxime esters, with the conversion being almost quantitative.[303] N435 was used in the production of wax esters using microbial oils *via* transesterification with behenyl or cetyl esters, with conversion yields up to 87.3% and 69.1%, respectively.[304]

Methacrylated trimethylolpropane cyclic carbonates were produced by two-step transesterifications catalyzed by N435 followed by thermal cyclization.[305] Six-membered cyclic carbonates with methoxycarbonyloxy and hydroxyl functionalities were obtained *via* transesterification of trimethylolpropane or dimethyl carbonate in a solvent-free medium flow reaction using N435, followed by thermal cyclization (yields over 80%).[306] Octyl ethanoate has been produced *via* ultrasound-assisted transesterification catalyzed by N435 and using vinyl acetate as an activated acyl donor in a solvent-free medium (yield over 97%).[307]

Other papers compared both esterification and transesterification routes. The synthesis of phenethyl acetate was studied using a free acid and different activated acyl donors, utilizing N435 as a catalyst.[308] The authors obtained yields of 99.12% and 98.44% employing acetic anhydride and vinyl acetate as the activated acyl donors, respectively (results were only slightly lower after 20 cycles). L-Ascorbyl flurbiprofenate was produced by both esterification and transesterification catalyzed by N435.[309] Using flurbiprofen as an acyl donor, 61.0% of L-ascorbic acid was converted, while only 46.4% was obtained by employing a flurbiprofen methyl ester (very likely due to the competition of methanol with L-ascorbic acid).

In some cases, two lipases have been combined to reach the desired result. An isosorbide diester was synthesized using a mixture of N435 and Ylip2 (77.4% of the diester).[310] In another case, N435 is coupled to other kinds of enzymes. For example, chiral diols and siloxane were coupled (66% yield) using dioxygenase and N435 (Lipozyme RM-IM and Lipozyme TL-IM were not active in this reaction).[311]

3.1.2. Production of optically pure products. The use of enzyme enantioselectivity (capacity of producing just one enantioisomer) or enantiospecificity is among the most relevant uses of enzymes, and N435 is not an exception. This can be produced by hydrolysis, synthesis or transesterification, or a combination of some of them.

Ethyl 2-((4*R*,6*S*)-2,2-dimethyl-6-((*E*)-styryl)-1,3-dioxan-4-yl)-acetate and the hydrolyzed (4*S*,6*R*)-acid have been produced by enantiospecific hydrolysis of racemic *syn*-ethyl (*E*)-2-(2,2-dimethyl-6-styryl-1,3-dioxan-4-yl)acetate catalyzed by Novozym-435.[312] Enantiomerically pure β-halohydrin (1*S*)-2-chloro-1-(2,4-dichlorophenyl)-1-ethanol was produced *via* kinetic resolution of the corresponding racemic acetate catalyzed by Lipozyme TL-IM or N435.[313] N435 was more efficient in producing (*S*)-β-halohydrin (ee of 99%). The kinetic resolution of flurbiprofen (*R*,*S*)-[2-(3-fluro-4-phenyl)phenyl] propionic acid using N435 as a catalyst and microwave irradiation has been reported.[314] The reaction permitted the conversion of the *R*-enantiomer into an ester with high enantioselectivity (ee_P was 98.9%).

Benzoxazole derivatives were synthesized using chiral alcohols and esters which were previously resolved by N435 catalyzed transesterification or hydrolysis.[315] N435 was used in the resolution of *trans*-2-phenylcyclopropyl azolides *via* hydrolysis or alcoholysis in methyl, giving *trans*-2-phenylcyclopropyl 1,2,4-azolide (*trans*-2-PCPT) of high optical purity.[316]

N435 sequential acetylation/hydrolysis has permitted the production of (*S*)-C5- lipidic dialkynylcarbinols in 97% ee and (*R*)-C5- lipidic dialkynylcarbinols in 99% ee from racemic mixtures.[317]

2-Phenylpropionic acid was esterified by N435 in bio-based solvents (*e.g.*, *p*-cymene) in a continuous flow reactor, enabling its kinetic resolution.[318] Racemic octahydroindolizine (indolizidine) was resolved using N435 to produce (7*R*, 8a*S*)-octahydro-5,5-dimethylindolizin-7-amine and (7*S*, 8a*S*)-octahydro-5,5-dimethylindolizin-7-ol 9, amine.[319] The resolution of (*R*,*S*)-1-(4-chlorophenyl)ethylamine was achieved employing N435.[320] The target unreacted product (*S*)-1-(4-chlorophenyl)ethylamine was obtained with an ee of 99% after a conversion of 52%. N435 was used as an example of the use of CO_2-expanded bio-based liquids.[321] The model reaction was the resolution of *rac*-1-adamantylethanol *via* esterification, which failed using standard solvents but gave very good results using CO_2-expanded methyltetrahydrofuran (enantiospecificity was 200).

Transesterification is perhaps the most popular strategy. N435 was the optimal catalyst in the resolution of (±)-1-methyl-3-phenylpropylamine (almost absolute specificity) using methyl benzoate as an activated acyl donor.[322] N435 was used in the enantioselective resolution of (*R*,*S*)-α-methyl-4-pyridinemethanol *via* transesterification.[323] The kinetic resolution of racemic-2-pentanol using vinyl butyrate as a co-substrate was investigated, comparing several enzymes.[324] N435 gave 50% conversion and 99% enantiomeric excess of (*S*)-2-pentanol after only 30 min. The kinetic resolution of (*R*,*S*)-α-tetralol *via* transesterification with vinyl acetate catalyzed by N435 was carried out in a packed-bed and a stirred-tank batch bioreactor.[325] While the continuous-flow packed-bed reactor needed a residence time of only 3 minutes to reach a 50% conversion for (*R*)-α-tetralol, after 8 h the conversion obtained using the stirred-tank batch reactor was 43.6%, although in both cases $ee_p \geq 99.99\%$ was achieved. Enantioresolution of 1-phenylethanol in reaction with corn germ oil ($E > 1000$) has been performed using N435 in supercritical carbon dioxide with a conversion near 90%.[326] 3-(*RS*)-Hydroxy-2-

methylenebutanenitrile was resolved using several lipases *via* transesterification, and N435 offered the best results.[327]

In some instances, two lipases allowed access to both enantiomers. The opposite enantioselectivities of N435 lipase and lipase AK in the acetylation reaction of (2,6,6-trimethyltetrahydro-2*H*-pyran-2-yl)methanol have permitted the production of the two enantiomeric forms of the alcohol.[328] In other cases, a metal-enzymatic combocatalysis was proposed. Pd/C and N435 were used in the dynamic kinetic resolution of 1,1,1-trifluoroisopropylamine, optimized *via* the response surface methodology to give a conversion higher than 95% under optimum conditions.[329]

In other instances, the enantioselectivity of the enzyme was exploited using prochiral substrates. For example, cryptocaryalactones were chemically produced after Novozym-435 enantioselective hydrolysis of the prochiral *anti*-ethyl-(*E*)-2-(2,2-dimethyl-6-styryl-1,3-dioxan-4-yl)acetate.[330]

3.1.3. Regioselective modifications of multifunctional substrates. The regioselectivity of N435, that is, the capacity of modifying just one group of a multifunctional substrate, is a very interesting feature of lipases, mainly explored in sugar chemistry. However, it is not possible to forget that simpler polyols, like glycerin, are also multifunctional compounds. These modifications may also involve hydrolysis, esterification or transesterification reactions.

N435 was used in the regioselective hydrolysis 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl hydroxybenzoate or 2,3,4-tri-*O*-acetyl-α-D-xylopyranosyl hydroxybenzoate.[331] Using β-D-glucopyranosyl hydroxybenzoates, the hydrolysis involves C-4 and C-6 positions; if β-D-xylopyranosyl hydroxybenzoate is the substrate, the deacetylation takes place at the C-4 position. In another paper, 92% yield of isosorbide-2-acetate was obtained *via* N435 catalyzed hydrolysis of isosorbide-2,5-diacetate, thanks to the high regioselectivity of the biocatalyst.[332] N435 was the best among the studied lipases for the hydrolysis of the C-6′ acetoxy group of macrolactonic sophorolipid.[333] This product was later acetylated.

Glucose was modified with palmitic acid *via* esterification catalyzed by N435 in different ionic liquids with a 77% yield in the best case.[334] Xylose caproate has been produced by esterification catalyzed by N435 (yield 64%).[335] Direct esterification of methyl glucoside with fatty acids has been studied using N435 with ionic liquids or deep eutectic solvents as reaction media.[336] 1,6-Di-*O*-octanomannitol was produced with a purity of 90% using N435 as a catalyst of the esterification reaction between mannitol and octanoic acid in a reaction medium composed of acetone and *n*-hexane.[337] N435 catalyzed the acylation of flavonoid glycosides from bamboo leaves with oleic acid, giving isoorientin-6″-oleate and isovitexin-6″-oleate.[338] N435 catalyzed the selective production of 5-*O*-acetyl-4-C-hydroxymethyl-1,2-*O*-isopropylidene-α-D-ribo- and xylofuranose which can be utilized for the convergent synthesis of two different types of bicyclic nucleosides.[339] Moreover, N435 has been utilized to produce glucosyl monoester surfactants using *N*-fatty acyl amino acid and D-glucose.[340] N435 has been used to produce 2′,3′,5′-tri-*O*-acetyl-4′-C-*p*-toluenesulfonyloxymethyl-β-D-xylofuranosylthymine and 2′,3′,5′-tri-*O*-acetyl-4′-C-*p*-toluenesulfonyloxymethyl-β-D-xylofuranosyluracil was used to produce C-4′-spiro-oxetano-α-L-ribonucleosides.[341] Three citrus fruit-derived flavonoids (grapefruit extract, naringin, and neohesperidin dihydrochalcone) have been esterified with different fatty acids (*e.g.*, omega-3 polyunsaturated fatty acids obtained from fish oil) in a reaction catalyzed by N435.[342] The conversions were over 85%, and the modification was in the primary alcohol of the glucose moiety of the flavonoids. Different chain length saturated fatty acids were used to acylate cyanidin-3-*O*-galactoside using N435, with the best results obtained using lauric acid.[343] The product was identified as cyanidin-3-*O*-(6″-dodecanoyl)galactoside. Quercetin-3-*O*-β-D-glucopyranoside was acylated using phenyl propanoate, phenyl acetate, benzoate and cinnamate vinyl esters with N435, which failed when using hydroxyaromatic acids, but with good results in the other cases.[344,345] In another research effort, quercetin-3-*O*-glucoside and phloretin-2′-glucoside were regioselectively esterified with several fatty acids under sonication employing N435.[346] 12-Vinyl dodecanedioate-23-*O*-silybin was regioselectively produced using N435.[347] 6-*O*-Acylglucose esters have been produced by N435 catalyzed esterifications between D-glucose and seven different fatty acid vinyl esters which were used as emulsifiers.[348] N435 was one of the few biocatalysts with activity in the production of (3*R*,6*R*)-6-acetoxy-7-hydroxylinalool or (3*R*,6*S*)-6-acetoxy-7-hydroxylinalool *via* esterification of 6,7-dihydroxy-linalool stereoisomers.[349]

Transesterification has also been used for this purpose. D-Xylose and L-arabinose lauryl mono- and diesters have been produced using N435 and the transesterification reaction was performed in an organic medium. The reaction used vinyl laurate and L-arabinose or D-xylose.[350] Using L-arabinose, a 57% overall yield of one monoester and one diester was achieved. Using D-xylose, a 74.9% global yield of modified products was achieved, but the reaction regioselectivity was lower and two monoesters and two diesters were synthesized.[350] Modification of lactulose with vinyl laurate was studied with 10 lipases.[351] N435 modified mainly the 1-*O*-position, while Lipozyme TL-IM and Lipozyme RM-IM mainly modified the 6-*O*-position. The regioselective synthesis of 3-*O*-acyl monoester lutein using N435 and vinyl propionate or vinyl stearate as activated acyl donors has been also successfully reported.[352] N435 was found to be the most efficient lipase to catalyze the production of Agave fructans mono- and diacylated with lauric acid *via* a transesterification reaction.[353] 1,3-Di-*O*-benzyl-myo-inositol was selectively acetylated using vinyl acetate.[354] When Lipozyme RM-IM and Lipozyme TL-IM were utilized, l-(+)-6-*O*-acetyl-1,3-di-*O*-benzyl-myo-inositol was obtained. However, N435 produced the non-chiral 5-*O*-acetylated product.[354] Fluorescent glycolipids were produced using vinyl esters and functionalized sugars employing N435 as a catalyst.[355]

Modification of glycerol is also performed, usually *via* glycerolysis of esters of the target acid. Glycerolysis of

phenolic acid ethyl esters under ultrasound irradiation and in a solvent-free system was used to get monoglyceryl phenolic acids using N435 (yields over 97%).[356] Glyceryl monocaffeate has been produced by glycerolysis of ethyl caffeate catalyzed by N435, and after optimization, the yields were over 95%.[357]

3.1.4. Polymer production and modification. Another popular application of lipases and N435 is in the production or modification of polymers. Polymer production is one of the most studied areas, as it can use very different materials and give biodegradable products. For example, the synthesis of polymers based on lactone derivatives is quite successful. Thus, a polyester derived from ω-pentadecalactone was produced employing N435 in a closed variable volume reactor and different organic solvents, with yields of around 90 wt% and molecular weights of the polymer over 50 000.[358] This group later studied the use of supercritical fluids in this reaction, with lower yields and molecular size (around 60 wt% and 33 000 g mol^{-1}).[359] Another paper shows the polymerization of ε-caprolactone using lipase N435 as a catalyst.[360] The successful enzymatic copolymerizations *via* ring opening of ε-thiocaprolactone and ε-caprolactone catalyzed by N435 have been reported.[361] The polyester poly(ω-pentadecalactone) was synthesized using N435 and a bifunctional initiator/chain transfer agent, with ω-pentadecalactone as the substrate.[362] Polycaprolactone was produced *via* enzymatic ring-opening polymerization. Irgacure-2959 was utilized as the nucleophilic initiator and N435 as the catalyst.[363] N435 exhibited better activity than NS 88011 (a commercial product also from CALB) in the production of aliphatic polyesters using globalide and ω-pentadecalactone as substrates, although the length of the polymer was smaller.[364] Poly(ε-caprolactone) has been synthesized *via* ring-opening polymerization of ε-caprolactone catalyzed by N435 in ionic liquids.[365]

Other polymers are based on 2,5-furandicarboxylic acid. For example, 2,5-furandicarboxylic acid-based semi-aromatic polyamides have been produced by polycondensation of dimethyl 2,5-furandicarboxylate and aliphatic diamines of diverse lengths, catalyzed by N435.[366] The best results were obtained using 1,8-octanodiamine. 2,5-Furandicarboxylic acid-based semi-aromatic polyamides have been produced *via* polymerization of dimethyl 2,5-furandicarboxylate catalyzed by N435.[367]

In other instances, glycerol based polymers are produced. For example, N435 was used to produce polyglycerol fatty acid esters of different-chain-length fatty acids in a solvent-free system.[368] In another instance, poly-(glycerol adipate) was produced from divinyl adipate and glycerol by N435 catalyzed condensation, controlling the branching of the polymer with the temperature.[369]

Sugar-based polymers may also be found. Sugar-polyethylene glycol amphiphilic copolymers were synthesized using N435 as a catalyst for the transesterification, introducing decanoic and myristic acids.[370] D-Fructose (99%) and D-glucose (34%) were modified with 2,2,2-trifluoroethyl methacrylate in *tert*-butanol using N435, and methacryloyl-D-fructose was polymerized using as the crosslinker ethylene glycol dimethacrylate.[371]

Glycerolysis of certain oils are also important to get monomers useful in polymer production. For instance, andiroba oil was subject to glycerolysis catalyzed by N435 to produce a polyol in a tubular fixed bed reactor using *t*-butanol as the reaction medium (monoacylglycerol yield was over 65%).[372] This was used later for polyurethane foam production. In another study, enzymatic glycerolysis of castor oil catalyzed by N435 in a solvent-free system gave a mixture of mono and diglycerides that were used in the stabilization and synthesis of poly(urea-urethane) nanoparticles *via* miniemulsion polymerization.[373] Later, a polyurethane foam was produced by this research group using mono- and diacylglycerols obtained by the glycerolysis of castor oil (64% yield).[374] A polymer was prepared from glycerol and oleic di-acid using N435 or classical thermochemical methods.[375] The enzyme polymerized product was found to be more biodegradable than the chemical one.

However, the range of materials used to produce polymers using N435 is very wide. Trimethylolpropane, 1,8-octanediol, and adipic acid were first pre-polymerized *via* the automatic catalytic effect of the reactants themselves to obtain an appropriate reaction substrate mixture.[376] Dimer acid cyclocarbonate was produced by the N435 catalyzed esterification of glycerol carbonate and dimer acid from *Sapium sebiferum* oil.[377] This compound could be used in the synthesis of bio-based non-isocyanate polyurethane ions. Chiral poly(ester amide) polymers were produced using N435, and the polymer successively presented hydroxyhexanoic and aspartate acids.[378] The reactions started using L or D *N*-(6-hydroxyhexanoyl) aspartate diesters, and methyl or benzyl ester groups at the α or β-carbonyl positions of the aspartic acid.

N435 has been used also to modify some polymers, mainly starch. For example, N435 was used to modify starch with myristic acid to alter its physicochemical properties.[379] In another paper from the same group, N435 was utilized in the production of myristic acid starch ester in a solvent-free system.[380] The modified starch exhibited good hydrophobicity and emulsion stability, and the gel strength was reduced. In another paper, lauric acid was esterified with starch using ionic liquids as reaction media and N435 as a catalyst, in order to improve the starch hydrophobicity.[381] Octenyl succinic anhydride starch production in ionic liquids using N435 has been also reported.[382]

Novozym 4435 does not always afford the best results. For example, CALB immobilized on polypropylene beads was compared to N435 in the polymerization of dimethyl adipate and 1,4-butanediol (BDO) using an optimized preparation.[383] In this case, higher molecular weight polyesters (4 kDa *versus* 3.1 kDa) were obtained using the home-made catalyst.

In other cases, N435 was combined with a chemo-catalyst. For example, this biocatalyst, the 1,5,7-triazabicyclo [4.4.0] dec-5-ene biocatalyst and an organocatalyst were employed in the polymerizations of ε-caprolactone, δ-valerolactone, L-lactide and trimethylene carbonate.[384] N435 did not work using L-lactide while the organocatalyst had low activity using caprolactone. The enzyme and organocatalyst were combined in an assembled tandem microreactor system, producing different well-defined triblock copolymers.[384]

Finally, N435 can be used to degrade some polymers. As an example, N435 was used in the successfully degradation of poly (butylene succinate-*co*-diethylene glycol succinate) and poly (butylene succinate-*co*-butylene diglycolic acid).[385]

3.1.5. Biolubricant production. The production of biolubricants is nowadays an important topic in lipase use, as the use of mineral oils may be dangerous for the environment. However, there are few examples using N435, as the features of the free fatty acids and alcohols useful for this objective may not be adequate for the specificity of CALB. Nevertheless, since 2016 there have been some examples. Thus, monoricinolein and diricinolein have been obtained by the glycerolysis of castor oil in a solvent-free system using N435, Lipozyme RM-IM and Lipozyme TL-IM.[386] N435 was found to have the best performance in this reaction, giving a biolubricant yield of almost 55%. However, in other cases N435 was not so adequate. The esterification of free fatty acids (obtained from soybean-oil hydrolysis) with neopentyl glycol, trimethylolpropane or pentaerythritol) was used to produce biolubricants, comparing the performance of Lipomod 34MDP, Lipozyme RM-IM and N435.[387] N435 gave the worst results using all alcohols.

3.1.6. Amidation reactions. Although lipases are esterases, they have been described to catalyze some amidation reactions, *via* direct amidation or a kinetically controlled process. For example, the ammonolysis catalyzed by N435 in a packed bed reactor permitted the conversion of (5-*S*)-*N*-(*tert*-butoxycarbonyl)-5-(methoxycarbonyl)-2-pyrroline to its corresponding amide.[388] Another paper shows that elastin-like recombinamers containing carboxylic groups may be modified by introducing different aminated compounds (aminophenylazobenzene, amino phenylboronic acid or aminopolyethylene glycol), using N435 as a catalyst, to produce photoresponsive, glucose-responsive or PEGylated elastin-like recombinamers.[389] N435 was also used as a catalyst for the transesterification/amidation of ethyl dihydroferulate.[390] Arachidonoyl ethanolamide was produced by amidation of arachidonic acid with ethanolamine catalyzed by N435 (95.6% yield), showing that the enzyme has an excellent chemoselectivity, even being an esterase.[391]

3.1.7. Promiscuous reactions. Enzyme promiscuity is nowadays confused with enzyme broad specificity, but at the beginning it was the capacity of some enzymes to catalyze reactions far from their natural function, in many instances not involving the lipase active center.[92]

Novozym has been shown in the last few years to exhibit some of these promiscuous activities. For example, the Morita–Baylis–Hillman reaction between 2, 4-dinitrobenzaldehyde and cyclohexenone was catalyzed by N435 with isonicotinamide as a necessary co-catalyst and β-cyclodextrin as an additive to improve the enzyme activity with a yield of 43.4% in 2 days.[392] In another example, the amidation of anilines with 1,3-diketones *via* C–C bond cleavage has been reported using also N435 as a catalyst.[393] The yields ranged from 64.3% to 96.2%, retaining more than 80% of the initial yield after seven reuses.

Lipase-mediated Dakin reactions have been reported. A broad variety of hydroxylated benzaldehydes were oxidized with high yields (from 90% to 97%) using N435, which could be reutilized in 10 cycles while maintaining its activity intact.[394]

Epoxidation of unsaturated oils is one of the most popular promiscuous reactions catalyzed by lipases[249,395] and this reaction has been also studied using N435. In this reaction, the lipase is responsible for the perhydrolysis of the oil using as the nucleophile hydrogen peroxide, forming a peracid that is actually responsible for the epoxidation reaction. For example, a research study shows how different lipases were assayed in the chemoenzymatic epoxidation of Karanja oil and N435 was found to be the most efficient one (epoxide conversion of 80%,) but hydrogen peroxide compromises the biocatalyst reuse due to enzyme inactivation.[396] In a similar way, monoepoxidated linoleic acid was produced by employing N435 with a reaction yield of 82.14%.[397] N435 epoxidation of acid sunflower oil was improved by introducing butyric acid as an active oxygen carrier (reaching an oxirane conversion of 96.4 ± 3.0%).[398,399] Finally, ultrasonic irradiation was used for enhancing N435 activity in the epoxidation of soybean oil.[400] A relative percentage conversion to oxirane oxygen of 91.22% was achieved within 5 h. The lipase was reused six times to produce epoxidized soybean oil. The functionalization of lignin from Organosolv and Kraft pulping processes to obtain oxirane rings was analyzed using N435 as a catalyst for the peroxidation of caprylic acid to peroxycaprylic acid (90% yield).[138] This peracid reacted with the unsaturated C–C bonds to form the oxirane ring, with a yield of 55% after optimization.

3.2. Food technology: glyceride modifications and production

3.2.1. Hydrolysis of glycerides. This reaction is the physiological function of lipases. However, the *in vitro* oil hydrolysis capability of lipases has some applications such as in the production of free fatty acids.[42,49–54]

Thus, the hydrolysis of waste cooking oil under solvent-free conditions was performed using N435 under ultrasound irradiation to produce free fatty acids.[401] After 2 h, a yield of 75.19% was obtained. Novozym-435 and Lipozyme TL-IM lipases were used to hydrolyze anhydrous milk fat and anhydrous buffalo milk fat and to enhance the flavor of milk, with N435 giving the higher production of butanoic and hexanoic acids.[402]

In another paper, virgin coconut oil (very rich in lauric acid and myristic acid) was subjected to glycerolysis catalyzed by N435 to produce mono- and diacylglycerols (MAGs and DAGs) to reinforce its antibacterial functionality.[403] Another example is the hydrolysis of triacylglycerols from anhydrous milk catalyzed by Novozym-435 to decrease the percentage of two short or medium chain fatty acids while the percentage of triglycerides with at least two long-chain fatty acids (CN 44–54) enhanced the melting and crystallization profiles of the product.[404] In another instance, hydrolysis of oils by

using some organic co-solvents produced a monophasic system that reached 88% using N435, while using Lipozyme TL-IM or Lipozyme RM-IM the reaction reached only 66%.[405]

N435 and *Thermomyces lanuginosus* lipase were compared in the hydrolysis of anhydrous milk cow fat or anhydrous buffalo milk fat using ultrasonic microwave-assisted extraction to eliminate short chain fatty acids.[406] N435 produced a significant decrease of triglycerides with short-chain fatty acids, altering the melting point of the products.

3.2.2. Alcoholysis of glycerides. The alcoholysis of glycerides is a kinetically controlled synthesis, where the activated acyl donor is the glyceride and the nucleophile is an alcohol. The yields depend on the enzyme kinetic properties, and this is the base of biodiesel production.[407] Here, we will focus on oil modification *via* alcoholysis, in many instances using glycerin as an alcohol.

N435 was also used in the glycerolysis of ratfish liver oil to produce bioactive lipid carriers with potential self-emulsifying properties.[408] The same group reported later that the N435 catalyzed glycerolysis of ratfish liver oil allowed the process to be studied in a pilot plant, showing a catalyst half-life of 145 h (enzyme activity cannot be fully restored by hexane washings), and that glycerolysis of triacylglycerol was 1.5 times faster than that of diacylglycerol.[409] N435 was used for the glycerolysis of *n*-3 polyunsaturated fatty acid-rich ethyl oil as the first step in a new two-step process designed for the production of pure triacylglycerols enriched in *n*-3 polyunsaturated fatty acids.[410] Diacylglycerol-enriched soybean oil was produced *via* glycerolysis of soybean oil catalyzed by N435 in a solvent-free system using a modified bubble column reactor.[411] Almost 50% diacylglycerol content was obtained and the enzyme can be reused in 10 cycles. Sardine oil was subjected to glycerolysis to produce mono and diglycerides rich in unsaturated fatty acids using supercritical CO_2 and organic solvents.[412] 47.6% of monolaurin was produced by glycerolysis of methyl laurate, with N435 being more efficient for this reaction than the lipase from *Aspergillus oryzae*.[413]

Polyunsaturated fatty acids in the form of 2-monoacylglycerols were prepared by ethanolysis catalyzed by N435 (yield of 27% of 2-monoacylglycerols).[414] Low temperature crystallization allowed 90% of 2-monoacylglycerol to be obtained, while molecular distillation gave a polyunsaturated fatty acid concentration of 72% while decreasing the content of 2-monoacylglycerols to 69.81%.

Soybean oil and ethanol were used in a reaction catalyzed by N435 to produce 2-monoacylglycerols and then, after esterification again catalyzed by N435 (yield around 65%) with acetic acid, a low energy lipid was produced having 55% of the energy of the initial oil.[415] The molecular distillation gave 94.3% purity of the desired low energy oil. The synthesis of 2-docosahexaenoylglycerol was performed *via* ethanolysis of algal oil using several lipases, with N435 being the most efficient biocatalyst and giving 27–31% of monoglycerides. In this case the catalyst could be reused for 7 cycles without any significant inactivation.[416]

Monoacylglycerols rich in ω-3 polyunsaturated fatty acids were obtained by glycerolysis of sardine oil catalyzed by N435 (67% MAGs) and further purified *via* short path distillation.[417] In another example, the performances of N435, Lipozyme TL-IM, and Lipozyme RM-IM in producing 2-monoacylglycerols rich in ω-3 polyunsaturated fatty acids (PUFAs) *via* ethanolysis of supercritical carbon dioxide extracted Pacific oyster oil were compared.[418] N435 gave a yield of 43.03%, very similar to that obtained using Lipozyme TL-IM (45.95%).

In another case, short- and medium-chain 1,3-diacylglycerols were synthesized as products with very low-calorie features *via* transesterification reactions between short- and medium-chain fatty acid ethyl esters and glycerol.[419] Different enzymes were assayed and N435 did not need the previous adsorption of glycerol on silica gel to form acylglycerols and gave a yield just behind that of Lipozyme RM-IM (52 *versus* 60.7%). Using N435, the reaction rate can be increased by adding 1% (w/w) of lecithin.

In some cases, N435 is combined with other lipases to achieve the objective. Ethanolysis of low-grade fish oil was subsequently performed using Novozyme NS 81006 and N435, to flexibly produce fatty acid ethyl esters or concentrated polyunsaturated fatty acids.[420] First, most of the fatty acid glycerides were transformed into ethyl esters (yield of 70–80%), while less than 20% of docosahexaenoic fatty acids were modified using Novozyme NS 81006. Using molecular distillation to eliminate the esters, the amount of glycerides containing polyunsaturated fatty acids increased from ~18% of crude fish oil to 34%. A second ethanolysis step catalyzed by N435 converted these glycerides to ethyl esters with 80–100% yield.[420]

In other reactions, N435 was not the best catalyst. For example, N435, Lipozyme TL-IM, Lipozyme RM-IM, and Lipase DF were evaluated in the synthesis of 2-monoacylglycerol enriched in omega-3 polyunsaturated fatty acids in supercritical carbon dioxide using salmon frame bone *via* ethanolysis.[421] In this instance, Lipozyme TL-IM showed the highest activity. Also, in the glycerolysis of lard, Lipozyme RM-IM worked better than N435.[422]

Eicosapentaenoic and docosahexaenoic acid enriched fish oil triacylglycerols were prepared by a two-step process.[423] Using AY "Amano" 400SD, fish oil was partially hydrolyzed, increasing the content of the target acids in the acylglycerols from 19.30% and 13.09 wt% to 25.95 wt% and 22.06 wt%, respectively. Subsequently, N435 was used in a transesterification reaction of the product with a stock enriched in eicosapentaenoic and docosahexaenoic ethyl esters. The final products prepared presented more than 95% of triacylglycerols, with high content of eicosapentaenoic and docosahexaenoic acids (28.20% and 25.61%, respectively).[423]

3.2.3. Esterification of glycerol. This is the opposite of the hydrolysis reaction, and it is defined as a thermodynamically controlled synthesis using unmodified substrates. The yields are determined by the thermodynamics of the process.[407] However, as glycerol is a pro-chiral and multifunctional substrate, enzyme selectivity or specificity may affect the final results, as some products may not be obtained if the enzyme is unable to produce them.

Caprylic acid and glycerol were esterified *via* ultrasound-assisted intensification, comparing Lipozyme RM-IM and N435; both enzymes showed their (yields just under 95%) applicability and were reused for 10 cycles.[424] Caprylic acid/polyunsaturated fatty acids/caprylic acid structured lipids were synthesized *via* esterification of omega-3 concentrate fatty acids with dicaprylic glycerol catalyzed by N435.[425] N435 was used to improve the camellia seed oil quality by esterification of the free fatty acids of the oil with epicatechin (the main products were epicatechin oleate and epicatechin palmitate).[426]

In another paper, medium chain fatty acids from different sources and glycerol were used to produce structured mono- and diacylglycerols using N435, Lipozyme RM-IM and Lipozyme TL-IM as catalysts.[427] N435 gave the highest incorporation of free fatty acids into glycerol (90% conversion of medium chain fatty acids into glycerol was obtained in 30 min). Diacylglycerols were rapidly synthesized in a solvent-free system *via* esterification of glycerol with a palm oil deodorizer distillate and 40 wt% oleic acid using N435 in a bubble column reactor.[428] The content of diacylglycerols was near 60%, while around 25% of monoglycerols and less than 3% of free fatty acids were found in the products.

N435 has been described as the most efficient catalyst in the esterification of glycerophosphorylcholine and conjugated linoleic acid.[429] However, an immobilized mutant lipase (MAS1-H108A) improved the results from 70 mol% to 89.10 mol%. In another paper, the esterification of glycerol and caprylic acid in supercritical carbon dioxide catalyzed by N435 and Lipozyme RM-IM has been studied.[430] N435 exhibited better performance, with a conversion of free fatty acids to tricaprylin of 97.3% in 6 h reaction time at 50 °C; 15 reuses had a small effect on the enzyme activity.

In some cases, several lipases are sequentially used to get the desired product. For example, a stearidonic acid rich triacylglycerol was produced *via* hydrolysis, followed by a two-step lipase-catalyzed esterification under vacuum, each catalyzed by a lipase.[431] A stearidonic acid rich stock was obtained by the hydrolysis of echium oil by using *Candida rugosa* lipase. For the esterification, N435 was used to esterify the stearidonic acid rich stock with glycerin and later, Lipozyme TL-IM continued this esterification. This gave an 86.4% yield. In another example, triglycerides enriched in *n*-3 polyunsaturated fatty acids were prepared using a multistep process. N435 was used to esterify *n*-3 polyunsaturated fatty acids and glycerol and these partial glycerides were subjected to hydrolysis using an immobilized lipase from *Malassezia globosa*.[432]

Linoleic, conjugated linoleic and pinolenic acids were esterified, using N435 as a catalyst, with a solvent-free system to prepare triacylglycerols with anti-obesity effects. A triglyceride content of 98.9% was obtained.[433] N435 showed pronounced selectivity to pinolenic acid > conjugated linoleic acid > linoleic acid.

In some cases, other components of the oils are used in the esterification to get the target products. For example, esterification of tyrosol and hydroxytyrosol extracted from olive mill wastewater with various fatty acids (caprate, laurate, and palmitate) catalyzed by N435 was successfully performed and shown to be efficient to avoid lipid oxidation.[434]

3.2.4. Interesterification of glycerides. This is a quite complex reaction, using two esterified substrates (such as glycerides or simple esters). The reaction proceeds *via* hydrolysis of the triglyceride to release a free hydroxyl group, and release a free fatty acid to the medium. The hydrolysis step is followed by the formation of a new ester bond by the reaction of the newly created hydroxyl group with a free fatty acid released from the other substrate, or by the use of this hydroxyl group in the alcoholysis of a free fatty acid released from the other ester.[435] Obviously, the whole process is strongly dependent on the enzyme features, including the immobilization strategy.[32]

For example, different oil blends have been interesterified. Thus, different lipases (Lipozyme RM-IM and N435) were used in the interesterification of mixtures of lard and rapeseed oil containing 35 and 25% of lard.[436] The reaction was performed faster and at higher temperature using N435 but the S_n-2 and S_n-1,3 distributions of the product were nearly random when N435 was used, while Lipozyme RM-IM did not modify the S_n-2 position. In another research study, the increase in omega-3 content at the S_n-2 position of high oleic sunflower and sardine oil *via* enzymatic interesterification was studied using Lipozyme TL-IM and N435, with Lipozyme TL-IM being more adequate.[437] N435 stood out from other catalysts (solid acid, sodium hydroxide and methoxide) in the production of low *trans* margarine fat analogs by interesterification of soybean oil and fully hydrogenated palm oil.[438] Medium-chain triacylglycerol rich structured lipids were synthesized by lipase-catalyzed interesterification of ARASCO with medium-chain triacylglycerols, comparing four commercial immobilized lipases, with N435 being the most efficient one.[439] Glycolipids were produced using α-chloralose and various vinyl esters as substrates and N435 as a catalyst.[440]

In other cases, oils and simple free fatty acid esters have been used. For instance, medium and long chain triacylglycerols were synthesized in a solvent-free system by interesterification of soybean oil with medium chain esters using N435 as a catalyst.[441] In another case, menhaden oil and ethyl caprate were used to produce structured lipids using two different lipases as catalysts. Results were better than those using a free acid.[442] N435 (almost 31%) gave better results than Lipozyme RM-IM (almost 20%). Feruloylated shea butter and feruloylated coconut oil were produced by interesterification of vegetable oil/fat with ethyl ferulate employing N435 as a catalyst in a packed-bed bioreactor.[443]

As in all other reactions, N435 did not always present the best properties. A blend of palm stearin and vegetable oil was interesterified to enhance the plastic range, comparing N435 and Lipozyme TL-IM.[444] In this case, the Lipozyme TL-IM product favored more the formation of β′ crystals.

In some cases, combi-lipases have been used (see the biodiesel section for the concept of combi-lipases). High S_n-2 docosahexaenoic and arachidonic acid oils were produced independently *via* enzymatic interesterification of S_n-2 docosahexaenoic and arachidonic acid oil rich single cell oils using a mixture of immobilized lipases, Lipozyme TL-IM and N435.[445]

3.2.5. Acidolysis of glycerides. In this case, an oil and the desired free fatty acid are mixed. The mechanism involves hydrolysis of an ester bond in the glyceride, followed by esterification using the substrate fatty acid.[435] Again, the results are very dependent on the enzyme specificity and selectivity.[32]

Thus, moringa oil (formed mainly long chains) was subjected to acidolysis with different medium chain fatty acids in supercritical CO_2, comparing the performance of N435 and Lipozyme RM-IM.[446] N435 gave the highest yield (63.2%). The biocatalyst could be reused for 15 cycles.

Acidolysis of camellia oil by lauric acid revealed that N435 was nearly non-selective due to its susceptibility to solvent systems (the enzyme was more selective in hydrolysis in aqueous medium).[447]

Human milk fat substitutes with four types of *n*-3 fatty acids for infant formula were produced *via* acidolysis of *Nannochloropsis oculata* rich oil by free fatty acids from *Isochrysis galbana* in a solvent-free SYSTEM using N435, TL-IM and RM-IM as biocatalysts.[448] A product containing a total of *n*-3 PUFAs of 13.92–17.12 wt% in the S_n-2 position under optimal conditions could be obtained using N435 and Lipozyme TL-IM.

In another research effort, castor oil was reacted with caffeic acid to produce castor oil-based caffeoyl structured lipids with a conversion and yield of monoglycerides bearing caffeic acid of near 95%.[449]

Menhaden oil and capric acid were used to produce structured lipids using two different lipases as catalysts.[442] N435 gave a yield of 28.63 mol%, while Lipozyme RM-IM gave a yield of only 9.81 mol% of incorporation of capric acid.

In another case, oleic acid and corn oil were reacted in a bubble column reactor system using N435 as a catalyst, to produce highly unsaturated glycerides; the final product contained 46.67 wt% of monoglycerides and 35.56 wt% of diglycerides.[450] This treatment decreased the oil crystallization rate significantly.

The synthesis of conjugated linoleic acid partial glycerides was performed using different lipase commercial preparations. N435 offered much better results than Lipozyme RM-IM or Lipozyme TL-IM.[451] Results could be improved by using other immobilization techniques, and the lipase from *R. miehei* immobilized on another hydrophobic support became more active and selective than N435.

Citronellic acid was used in an acidolysis process of egg-yolk phosphatidylcholine using five commercially available immobilized lipases as biocatalysts.[67] The best results were achieved using N435, with 33% yield of a phospholipid fraction enriched with citronellic acid in the S_n-1 position (39% incorporation in this fraction).

As in other cases, N435 is not always the recommended catalyst. For example, Lipozyme TL-IM, Lipozyme RM-IM and N435 were compared in the synthesis of caprylic or capric acids/long chain fatty acids/caprylic or capric acid triglycerides *via* batch acidolysis in solvent-free medium.[452] As an oil substrate, grapeseed oil (rich in linoleic acid) was utilized. The best results were obtained using capric acid and Lipozyme RM-IM.

3.3. Energy: biodiesel production

Similar to all lipases, N435 has been intensively used in biodiesel production.[12,72–74,145–147] Here, we have collected some of the most representative examples since 2016.

For example, biodiesel production from *Ceiba pentandra* oil using N435 was optimized to reach a 78.0% yield *via* stepwise addition of 9-fold methanol excess, although a decrease to less than 70% was observed after only 3 cycles.[453] In another research study, ethanolysis of triglycerides in a solvent-free reaction medium catalyzed by N435 as a biocatalyst was studied, trying to understand the acyl migration that results in 100% yield.[454] The paper shows that long-chain fatty acids with unsaturation have limitations in their access to the active site of the lipase. Triolein ethanolysis was performed in a fixed-bed reactor operated in circulating batch mode using N435.[455] Triolein was also methanolyzed by N435 using dimethyl ether (DME) as the reaction medium in a batch reactor and a continuous pipe reactor.[456] In another example, oil obtained from the seeds of *Manilkara zapota* (L.) was transesterified with methanol, comparing different lipases as biocatalysts, with the best results achieved using N435.[457] Novozym-435 gave 96% biodiesel yield after 12 h, but the activity decreased progressively (after 6 cycles, the yield was only 72%). However, the activity can be recovered by incubating it in soybean oil, 2-butanol or *tert*-butanol. In another research effort, N435 was also employed to analyze the advantages of a micro packed-bed reactor based on a two-parallel-plate configuration to produce biodiesel.[458]

In another investigation, *Eruca sativa* oil was used as a substrate in a comparison between N435 and *Aspergillus niger* lipase, obtaining a much higher yield using N435 (98.3% *versus* 56.4%).[459] N435 was also used to produce biodiesel from degummed crude palm oil and optimized by the response surface methodology.[460] Ethanolysis of babassu oil catalyzed by N435 gave yields over 98% in a fluidized bed reactor.[461] N435 gave promising values of 85 and 76% of biodiesel from waste cooking oil and *M. circinelloides* oil, respectively.[462] Fish oil was transformed into biodiesel and enriched in polyunsaturated fatty acids by methanolysis catalyzed by NS 81006 followed by hydrolysis by N435.[463]

Different lipases were compared in biodiesel production using sunflower oil and methanol.[464] Although N435, Lipozyme TL-IM and Lipozyme 62350 showed similar reaction rates, N435 was more stable and gave the highest yields. Biodiesel from crude *Citrullus colocynthis* oil and methanol was produced in *tert*-butanol with a yield of 97.8% using N435.[465]

No standard oils have been used for biodiesel production using N435. For example, black soldier fly larvae fat and methanol were used to produce biodiesel, comparing different lipases, and N435 showed the highest activity.[466] Using the response surface methodology, the process was optimized, giving a biodiesel yield of over 96%. N435 could be reused 20 times, decreasing the biodiesel yield to 92.5%. Using this oil, methyl acetate was proposed to obtain biodiesel *via* interesterification catalyzed by N435 with a yield of almost 97%.[467] This avoided methanol enzyme inactivation and the biocatalyst could be used 20 cycles while maintaining the activity. In order to avoid methanol lipase inactivation, in another study N435 was utilized as a successful model to produce biodiesel from soybean oil by drip-feeding of methanol, obtaining a yield as high as 98.75%.[468] Spent coffee oil was also used to produce biodiesel, comparing many different biodiesels, with Novozym-435 offering better results (around 96%).[469]

In another case, microalgal oil extracted from *Nannochloropsis gaditana* was used, comparing N435 and *Rhizopus oryzae* in *tert*-butanol medium.[470] The highest reaction rate was obtained using the least polar lipid content. The same group using the same algae and N435 showed direct transesterification (in the presence of the biomass) in *tert*-butanol.[471] A biodiesel yield of more than 99% was achieved and the product was recovered by hexane extraction. However, the enzyme activity rapidly decreased. Another microalgae oil, extracted from *Aurantiochytrium* sp., KRS101, was also used in biodiesel production catalyzed by N435, taking advantage of the high concentration of free fatty acids to produce a biodiesel free of glycerol with 89.5% conversion under optimal conditions.[472] Glycerol was undetectable in the biodiesel.

In a very interesting work, waste cooking oil and dimethyl carbonate (DMC) were used as reactants and N435 as a catalyst to simultaneously produce biodiesel and glycerol carbonate as a very interesting by-product.[473,474] The enzyme maintained 88% of its activity after six reaction cycles.

Not only has a suitable biodiesel been produced using N435, but also some biodiesel additives. For example, an *n*-butyl oleate ester using N435 in a stirred basket reactor was produced with a yield of 98%, as a biodiesel additive.[475]

As N435 is regarded as a highly effective biocatalyst in biodiesel production, N435 is used to show the advantages of new preparations. Sometimes the new preparations seem to offer better possibilities. For example, a polyacrylonitrile (PAN) hollow membrane was activated with nitrile-click chemistry and treated with sodium alginate and $CaCl_2$. The immobilized enzyme was 2.5 fold more active than N435.[476]

3.3.1. Synthesis of biodiesel in non-conventional systems. This biocatalyst has been used to assay different heating or stirring systems. Biodiesel was produced in a solvent-free system under ultrasonication using ethanol, soybean oil or macauba fruit oil as a substrate, reaching a yield of 88% for soybean oil and 75.2% for macauba oil.[477] Biodiesel was produced in continuous mode using an ultrasound bath, comparing N435 and Lipozyme TL-IM, and it was shown that Novozym-425 gave higher yields.[478] In another case, transesterification of *Jatropha* oil (*Jatropha curcas L.*) with ethanol in a solvent-free system under microwave irradiation and comparing 7 lipases was reported.[479] Biodiesel yields of over 93% were obtained and reaction rates were similar to that obtained using N435.

Ionic liquids have been also used to produce biodiesel using N435.[480] The paper established some rules on the effect of ionic liquids on enzyme activity. In another case, N435 was used with zwitter-type ionic liquids as a cocatalyst to improve the reaction rate using sunflower oil, obtaining a 64% biodiesel yield.[481] Using the same system with a slurry of whole-cell *Chlorella zofingiensis* in water as a substrate, the biodiesel yield reached up to 16%. The causes of this decrease are discussed in the paper.

3.3.2. Oil deacidification. In many cases, N435 has been used as a first step in biodiesel chemical production by reducing the amount of free fatty acids, which is not compatible with traditional alkaline catalysis. Rapeseed oil rich in free fatty acids was treated with N435 reduced the acid content from the initial 15% to only 0.5% F. F.A in 1 h.[482] The authors developed a system where 15 m^3 of oil could be deacidified by just 1 kg of biocatalyst. The same effort to reduce acidity was performed on poultry fat, showing that N435 permitted a higher deacidification than 3 other lipases.[483] At least a 57% decrease of free fatty acid content was achieved. In another instance, macauba (*Acrocomia aculeata*) oil has 35–43% acidity that was decreased using N435 in a esterification reaction to almost 1%, and at the same time some transesterification is performed (55% of esters may be found in the final product).[484] The biocatalyst was used up to 150 times. In another research study, deacidification of high-acid rice bran oil was achieved by enzymatic amidation between the free fatty acids and ethanolamine. The acid value was decreased from 21.5 to 1.6 mg g^{-1} after 4 h of reaction, more rapid than that using glycerin and esterification.[485] The final oil product was rich in fatty acid ethanolamides (11.9 wt%) which are bioactive lipids and can be separated from biodiesel.

3.3.3. N435 in the combi-lipase concept. The concept of combi-lipases has received increasing interest, and it is based on the fact that oils are really very heterogeneous substrates; therefore the use of an individual lipase may hardly be "optimal" for all likely components of oils. The idea has been shown in biodiesel and oil hydrolysis.[43,47,48] During the time that we focused on the revisions, some examples of combi-lipases including N435 may be found. Waste oil and fresh soybean oil were transformed into biodiesel using an ultrasound system with a combi-lipase as a biocatalyst (including N435 and Lipozyme TL IM, and Lipozyme RM IM).[44] The optimal mixture of lipases depended on the oil; for soybean oil the combi-lipase composition was 50% CALB, 22.5% TLL, and 27.5% RML, while for used frying oil, it was 40% TLL, 35% CALB, and 25% RML. The paper showed that ultrasound provided a soybean oil biodiesel yield of 90% and 70% when

employing waste oil. Simultaneously the continuous synthesis of biodiesel from waste oil using combi-lipases in a packed-bed continuous reactor was also performed using the same lipases.[45] Biodiesel was also produced from a palm oil free fatty acid distillate and methanol in a packed bed reactor *via* direct esterification using *t*-butanol, mixing Lipozyme TL-IM and N435.[486] The optimum combi-lipase was 5% N435 and 95% Lipozyme TL-IM, and this achieved a 96% biodiesel yield. In another paper, soybean oils with different acid values (8.5, 50, and 90) were used to produce biodiesel using ethanol, comparing different lipases (Lipozyme TL-IM, Lipozyme RM-IM and N435).[487] The ester content could be doubled by mixing Lipozyme TL-IM and N435.

In another example, but where the enzymes are not mixed, a very acidic and heterogeneous (having monoglycerides, diglycerides and triglycerides) oil from rice bran oil soapstock in a solvent-free system has been transformed into biodiesel, showing that the successive use of N435, Lipozyme TL-IM or Lipozyme RM-IM and Lipozyme TL-IM resulted in similar or higher levels of yield of the individual lipases (around 92%).[488]

4. Problems of N435

Thus far, N435 has a strong history of success in diverse applications, and after many years it has remained among the most used biocatalysts at least by academic researchers.[132–152] However, this seemingly "golden" biocatalyst has some serious problems that in many instances are ignored.

First, there is a general problem that is common to all biocatalysts prepared using interfacial activation of lipases *versus* supports bearing a hydrophobic surface: the enzyme may be released from the support at high temperature, in the presence of organic co-solvents, detergents, *etc.*[210] And in many instances, the substrates/products of the lipases are detergent-like molecules (*e.g.*, fatty acids, partial glycerides, and phospholipids)[211] (Fig. 8).

The other problems are a direct consequence of some negative specific features of the utilized support, the Lewatit support. And some of them are so serious that there should be thoughtful consideration before selecting N435 as an industrial catalyst.

The first problem is that even though the surface of Lewatit is hydrophobic enough to permit the immobilization of CALB *via* interfacial activation, it is relatively hydrophilic. As such, it can retain hydrophilic by-products (*e.g.*, water in esterifications and glycerin in biodiesel production), producing an apparent enzyme inactivation due to the formation of glycerin or water layers inside the biocatalyst.[236]

The second problem is related to the fragility of the particles of the support under stirring. This may produce problems with the filters of the system.

However, the most important problem is something reported many years ago but did not have the relevance that this issue deserves: the support may be dissolved in some media (that is, the crosslinking may not be efficient enough).[489,490] This implies that not only may the enzyme be released from the support, but also the polymeric components of the support could be incorporated to the reaction media, contaminating the product.

Now, we will go in more detail into each of these problems, suggesting some solutions for each of them.

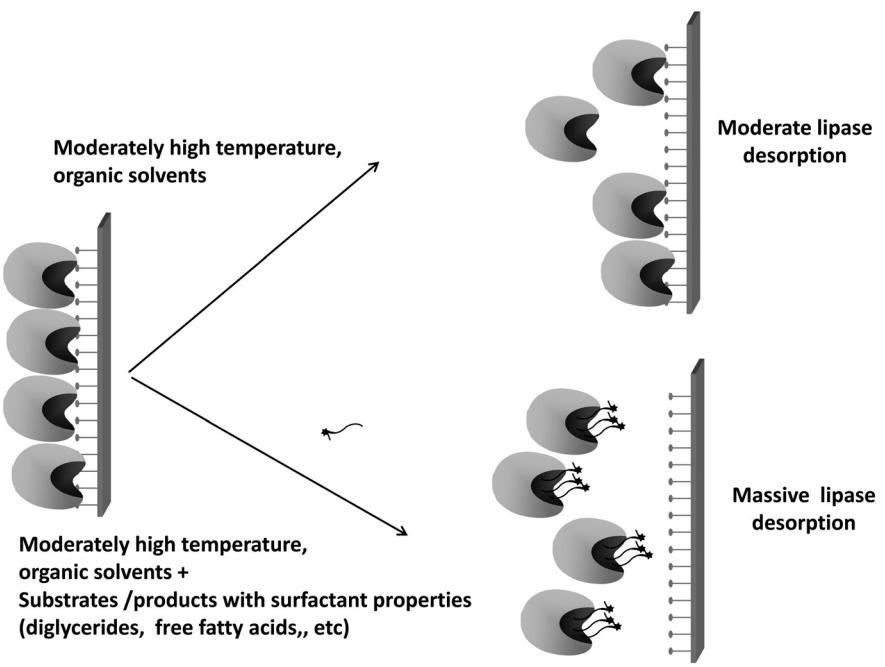

Fig. 8 Lipase release from the support is favored in the presence of detergent-like substrates or products.

4.1. Enzyme leaching

Since 2002, there have been some papers alerting us to the risks of enzyme "leaching".[131,249,491,492] This obviously affects the studies on the operational stability and reuses of N435, as well as the implementation of N435 on a large scale. The reasons for this enzyme leakage are general to all lipases immobilized using interfacial activation *versus* hydrophobic surfaces, not specific to N435 (Fig. 8). Although the enzyme–support interaction is very strong[122] due to the very large size of the hydrophobic pocket of the lipase, a high temperature may weaken this interaction and result in the release of the enzyme to the medium.[210] Usually, the enzyme immobilized by this strategy is much more stable than the free enzyme and even multipoint covalently immobilized lipases,[193,493] and it is not clear if the released enzyme molecules are previously inactivated or become inactivated after the enzyme release, but the fact is that it is possible to observe the decrease of the enzyme on the support and the appearance of the enzyme in the supernatant (Fig. 8).[210]

In a similar way, organic co-solvents may weaken the enzyme–support hydrophobic interactions and facilitate enzyme release. As such, while in thermal inactivation the lipases immobilized *via* interfacial activation tend to be more stable than the covalently immobilized enzymes, in the presence of organic co-solvents the situation is reversed and the covalently immobilized enzyme becomes more stable.[193,493] The problem is accentuated if the reaction involves detergent-like compounds.[211] These compounds need not necessarily be as long as free fatty acids or di- or monoglycerides. It has been shown that even dibutyrin or diacetin may greatly facilitate the lipase release from hydrophobic supports.[212,213]

N435 has all these problems. However, the U. K. Food Standards Agency did not find any detectable contamination from N435 on simulated food (containing isooctane and 95% (v/v) ethanol) and diverse acrylic materials.[494]

However, this enzyme release under certain conditions can be considered a proved fact. The incubation of N435 in dimethyl sulfoxide resulted in the quantitative release of the enzyme.[495] Using N435, Chen *et al.*[249] have reported that enzyme leaching from N435 can become a serious problem for the application of this biocatalyst in the production of pharmaceutical compounds, and that enzyme leaching becomes a serious problem for enzyme reuse.[253] Using N435 in the production of polymers, the enzyme leakage was again reported to be a real problem.[496] Enzyme release from N435 was also reported in the solvent-free esterification of polyglycerol-3 and related compounds, due to their surfactant features.[491] Enzyme release even in its first use has been reported in oil chemistry[497] and polymer production.[383]

Enzyme release from N435 in organic solvents and ionic liquids has been also reported to be a real problem, as "active" traces of the active material were released from the catalyst and hindered the control of the reaction (because some reactions continued after eliminating N435)[498,499] Thus, this enzyme release has been in fact considered as one of the main problems for the industrial implementation of N435.[500]

4.2. Support solubility in organic media

The solubility of a support in any reaction media may become a serious problem. The pioneering investigations regarding the possibility of solubility of Lewatit from N435 in some media started from some of the coauthors of this review, who observed that the operational stability of the biocatalyst in the enantiospecific esterification of ibuprofen in ethanol was much lower than expected.[501] The research pointed that the support became solubilized in the presence of some solvents such as ethanol.[489,490] They detected that the mass of the polymer in the biocatalyst decreased and that polymethacrylate and divinylbenzene, components of Lewatit, could be found dissolved in the medium. The problems persisted even in aqueous/ethanol mixtures. Later, this effect was also found using other alcohols.[502,503]

Table 1 shows the main conclusions of these studies. In fact, it is not possible to fully discard that part of the protein decrease may be due to the dissolution of the support in the reaction medium.

This is a very serious problem, as the polymer can go to the medium, be incorporated to the product, and produce serious problems in all the reactor operation and the recovery of the products. However, this serious problem has scarce impact in the literature.

The adsorption of alcohols on Novozym-435 was analyzed in some detail by programed thermic desorption.[503] The study revealed a very strong physical adsorption, but the existence of dimethyl ether and propylene suggested the possibility of chemisorption, which can produce methoxy and propoxy species that may be dehydrated on acid active sites, inactivating the enzyme.

The modification of the support was also studied using environmental scanning electron microscopy; the exposure of N435 to alcohols decreased the fractal value and increased the minimal cell size, showing the internal modification of the biocatalyst porous structure.[503,504] Later, the same methodologies were used to explain the operational stability of N435 in biodiesel production, showing that at 65 °C some polymers from the support could be found in the product (mainly if water was continuously eliminated from the medium). In fact, the size of the particles of the biocatalyst increased when incubated in biodiesel for 2 minutes (from an average diameter of 539 μm to 626 μm).

4.3. Support mechanical fragility

Another problem of Lewatit is its mechanical fragility under stirring.[33,505] This fragility does not seem to be very relevant

Table 1 Alterations of Novozym® 435 by incubation in methanol, ethanol or 1-/2-propanol

Alcohol	Global mass loss (%)	Protein loss (%)	% alcohol adsorbed
Methanol	11.6	1.93	2.12
Ethanol	16.6	1.27	3.81
1-propanol	5.9	0.57	4.04
2-propanol	1.2	0.79	3.68

at the laboratory scale, where the immobilized enzyme may be recovered in operational stability studies using centrifugation and the reaction may be performed in a beaker, but becomes a great problem at the industrial level.

At the academic level, this fragility may have confusing results, for example if the reaction has reduced activity due to a very high activity that results in diffusion limitations of the substrate. When the catalyst starts to break down, the size of the particles will decrease and the diffusion limitation problems will decrease. As such, apparent "hyperactivation" or a higher stability of the biocatalyst may be found by this artifact.[32]

At the industrial level, this may be a critical point, as the production of fine powders can block the filters of the reactor and result in the necessity to discard the biocatalyst even if it is fully active. Fig. 9 shows the fine powder production under stirring using N435.

Therefore, this may be a very important negative feature when considering the use of N435 in mechanically stirred batch reactors, although it may be solved using other stirred reactor configurations which are less aggressive with the mechanical structure of the beads (*e.g.*, vortex reactors).[506–508]

4.4. Retention of hydrophilic compounds

Many uses of lipases are in anhydrous media (*e.g.*, hydrophobic solvents) and during reaction some hydrophilic compounds may be released. This can lead to the accumulation of these compounds in the biocatalyst,[509] which will be more hydrophilic than the reaction medium in many instances, mainly after being coated with the very hydrophilic molecules of lipase.[510]

For example, in esterification, water is a side-product of the reaction.[511] In many instances, water is adsorbed on molecular sieves to shift the equilibrium to the direction of the synthesis (this is a thermodynamically controlled synthesis).[512,513]

However, if the water formation is more rapid than the diffusion, and the support pores or matrix is more hydrophilic than the reaction medium (imagine that the medium is octane), water can be accumulated inside the biocatalyst forming an aqueous phase.[152] This way, acids can also be accumulated in this environment, exposing the enzyme to very low pH values and promoting enzyme inactivation by these drastic conditions.[151] Moreover, water accumulation may make the thermodynamics of the process unfavorable in the enzyme proximity, reducing the enzyme activity.

In a transesterification reaction to produce biodiesel, glycerin may be the problematic side-product that can form a glycerin phase.[514,515] If this occurs, this will hinder the access of the hydrophobic substrate to the enzyme, and it can also capture water as it is a hygroscopic material. Moreover, glycerin, as a likely nucleophile in the deacylation of the acyl enzyme, may compete with methanol, or ethanol.

As such, Lewatit, even having a moderate hydrophilicity, causes some problems on the use of N435 in esterification/biodiesel production, studied in detail only in some specific cases.[236] Some solutions to this problem will be presented later.

In other cases, a perhydrolysis is desired,[83,516] and hydrogen peroxide is able to impair the enzyme activity by diverse reasons.[517–519] Although N435 is very stable even in 1 M hydrogen peroxide,[520] the use of a more hydrophobic support was found to be favorable to force a partition of this deleterious compound.[521]

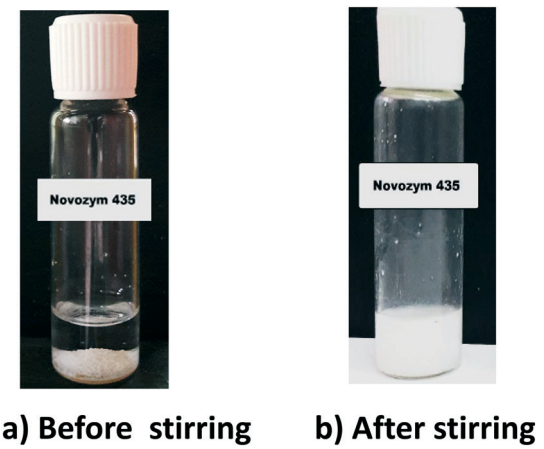

Fig. 9 Images of N435 (a) before and (b) after 6 h under magnetic stirring at 30 °C and 700 rpm in an esterification reaction in hexane.

5. Some solutions to N435 problems

5.1. Enzyme leakage prevention

The leakage of enzymes adsorbed by interfacial activation is a general problem of immobilized lipases (Fig. 9).[211–213] The most obvious solution is to cross-link the enzymes to prevent enzyme desorption. This has been tried in a covalent way using glutaraldehyde[495,522] or aldehyde dextran (Fig. 10).[218,523] A simpler solution is by physically cross-linking the proteins using ionic polymers like polyethylenimine or dextran sulfate (Fig. 10).[214,215,524,525] A combination of polyethylenimine coating and glutaraldehyde cross-linking has also proved to be efficient.[216] Now, to release the enzyme from the support, the cross-linked proteins must be simultaneously released from the support, which is much more difficult.

These strategies have been also utilized in the case of N435. In fact, the treatment of the biocatalyst with polyethylenimine or glutaraldehyde has an unexpected effect: in biodiesel production using N435 with camelina oil, a deposit can be found in the N435 preparation which cannot be found after modification.[526–528] Intermolecular glutaraldehyde cross-linking is not efficient enough to fully prevent enzyme release from the support.[491,529]

The use of heterofunctional supports, adding some ions or chemically reactive groups, is another strategy that proved to be efficient with some hydrophobic supports.[210,219–222,225–227,241,530] However, this has not been tried using N435, as the enzyme is already in the support and modifying the support may be problematic.

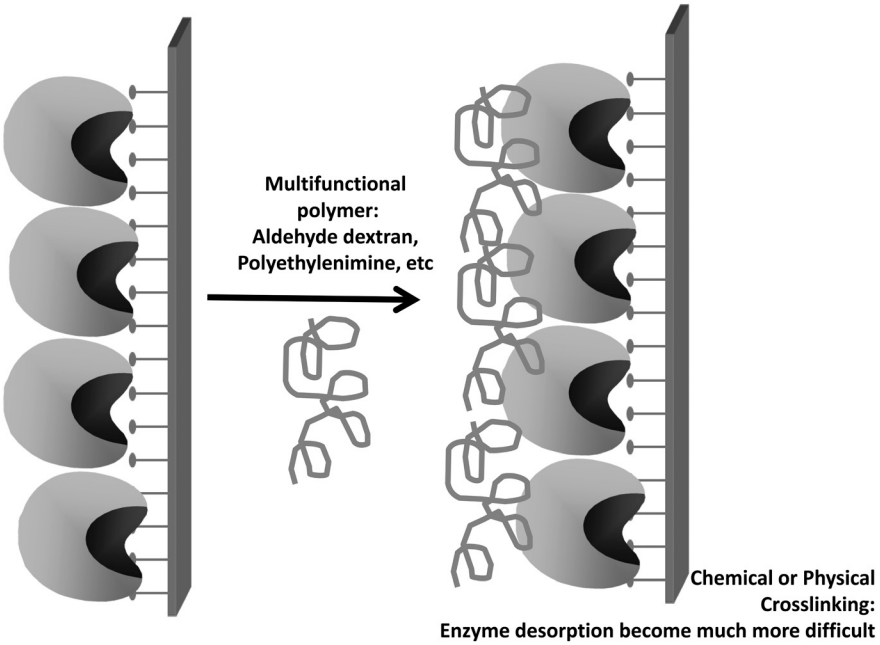

Fig. 10 Prevention of enzyme leakage *via* chemical or physical crosslinking.

5.2. Biocatalyst mechanical fragility

The biocatalyst fragility (Fig. 9) is mainly relevant on the industrial scale. To avoid the support breakage, together with using stirring methods which are less aggressive towards the support physical integrity, one of the strategies is the coating of the biocatalyst with some rigid cover that may avoid the Lewatit breakage. For example, N435 was covered with silicone and this allowed support destruction to be almost fully avoided under vigorous stirring (Fig. 11).[531] Furthermore, this treatment also reduces enzyme leaching from the support, if the biocatalyst is fully closed in a silicone matrix.[531] The biocatalyst maintained more than 90% of its activity after silicone coating.[532] In another instance, the silicone-coated biocatalyst was used in a complex chemo-enzymatic epoxidation reaction in a three-phase system.[533] The biocatalyst kept 50% of the activity after 5 days in 5 mM hydrogen peroxide. In another research study, the syntheses of poly(ethylene glycol) 400-coconut fatty acid monoester, myristyl myristate and propylene oxide copolymer-oleic acid, and ethylene oxide diester were studied with N435 and N435 treated with silicone.[534] The turnover numbers increased by a factor of up to 50.[534] The treatment did not alter the specificity of the enzyme; this way the enantiospecific acylation of racemic

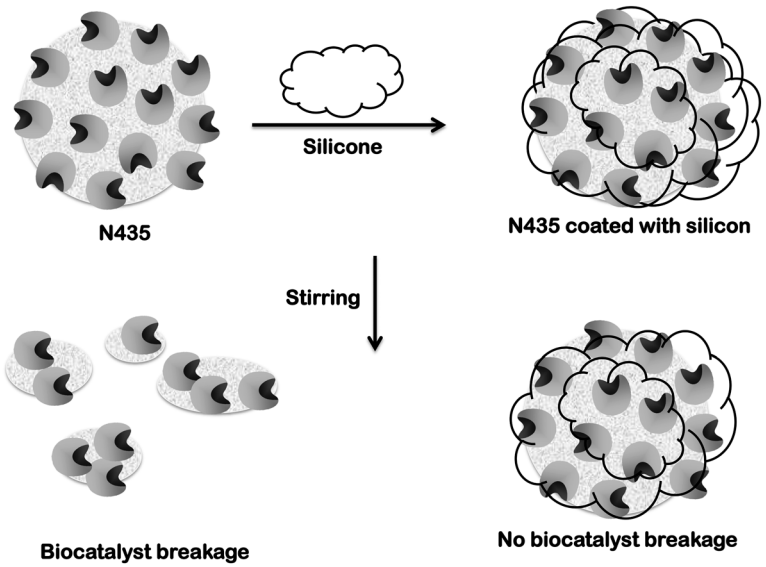

Fig. 11 Coating of N435 with silicone to prevent support breakage.

1-phenylethanol with vinyl butyrate retains the excellent resolution.[534] Later the coating was performed in a fluidized reactor and the Pd catalyst necessary for silicone polymerization was removed.[535]

It should be expected that similar protection effects may be found using other materials used to trap some weak biocatalysts, like the crosslinked enzyme aggregates trapped in silicates,[536–538] in sol–gel[539] or in LentiKats (polymers of polyvinyl alcohol).[540]

5.3. Hydrophilic compound adsorption and support dissolution

These points can have a more complex solution. Water or glycerin accumulation on Lewatit may be partially reduced using ultrasound and molecular sieves.[44,152,541–550] However, the moderate hydrophilicity of the support may complicate the understanding of the results and reuses of the biocatalyst.

The dissolution in organic solvents should be related to an inadequate crosslinking in the polymerization step. This could be (at least partially) solved after some modification of the biocatalyst which can prevent the release of polymer fragments, perhaps in a similar way to the prevention of the enzyme release. However, there are no studies in this regard.

Thus, the solution to these problems may be quite complex when keeping Lewatit as a support. The alternative is to change the immobilization matrix, using some hydrophobic ones that can be more resistant to solvent dissolution and that may be more hydrophobic to prevent water accumulation.[150,236,551,552]

However, N435 has some special features,[252] perhaps due to the existence of some acid groups in the matrix that make this a peculiar biocatalyst. It must also be considered that even if the immobilization follows an interfacial adsorption mechanism, CALB properties may be altered by changing the support hydrophobicity, internal morphology, *etc.*[185,191,259,553] We can assume the idea that a hydrophobic support may be convenient for CALB immobilization, purification and stabilization[209] A hydrophobic support that is intended to be an alternative for Lewatit should show some critical features. First, the support must not be dissolved in any media (that is, a suitable crosslinking must be performed) and it must be hydrophobic enough to reduce the adsorption of hydrophilic compounds (and perhaps allow a stronger enzyme adsorption), without affecting CALB stability. Exhibiting a high mechanical resistance will also be a desirable feature. Finally, it should keep or improve the high enzyme loading, stability and versatility that N435 has shown even with these significant problems. Moreover, it should have an adequate particle size and particle size distribution, a competitive price and also, offer some solutions towards disposal (even if immobilization is reversible, support breakage may be promoted that the reuse may no longer be convenient, mainly if the enzyme is very stable and can be used for months). If the support is compatible with some heterofunctionality that permits reduction in enzyme leaching, this will be an advantage to be considered.

Although there are many hydrophobic supports in the market,[191,234,247] a systematic study of all these properties has not been performed (perhaps because academically it is not very interesting). Until that moment, the successful history of N435 will very likely continue.

6. Conclusions

This review shows that N435, despite being a very successful biocatalyst with applications in many different areas, has some very relevant problems that in some cases may be avoided using relatively simple techniques (*e.g.*, enzyme leaching and mechanical fragility may be solved by encapsulation of the biocatalyst in silicone), while others may be much more intricate to work around, like the dissolution of the support in certain media. As N435 is mainly used in chemistry, and in these cases the final product is usually fully purified and/or crystalized, perhaps this is not a key problem at the industrial level, except that it may interfere in product purification. However this may be a critical problem in food modification, where the contaminant will be incorporated to the food. Whatever the reason, even with this problem, which was already described many years ago, N435 remains as one of the most used biocatalysts. The best solution may be to change the current support to another one with fewer problems, or to improve the crosslinking step of Lewatit (this may both reduce support dissolution and increase its mechanical resistance). However, it seems that for some reason, Lewatit VP OC 1600 and CALB are a dynamic duo that may be difficult to alter, even when nowadays there are many new alternative hydrophobic supports that have been reported to improve CALB loading, activity and stability. Price and simplicity may be among the points that make replacing Lewatit VP OC 1600 by some more suitable supports difficult. Whatever the case, it seems evident that the success of N435 will carry on at least into the near future.

Conflicts of interest

There are not conflicts to declare.

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