LECITASE ULTRA: A PHOSPHOLIPASE WITH GREAT POTENTIAL IN BIOCATALYSIS

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Abstract

Lecitase Ultra is a chimera produced by the fusion of the genes of the lipase from *Thermomyces lanuginosus* and the phospholipase A1 from *Fusarium oxysporum*. The enzyme was first designed for the enzymatic degumming of oils, as that problem was not fully resolved before. It is commercialized only as an enzyme solution by Novo Nordisk A/S. This review shows the main uses of this promising enzyme. Starting from the original degumming use, the enzyme has found applications in many other food modification applications, like production of structured phospholipids (e.g., derivatives of phosphatidylcholine), tuning the properties of flour, etc. Moreover, the enzyme has been used in fine chemistry (resolution of racemic mixtures), in the production of aromas and fragrances, polymers modification, etc. Some papers show the use of the enzyme in biodiesel production. Moreover, we present the different technologies applied to obtain a suitable immobilized biocatalyst, remarking the immobilization via interfacial activation and how heterofunctional acyl supports may solve some of the limitations. Immobilized enzyme physical and chemical modifications have also been presented. Finally, Lecitase Ultra has been one of the model enzymes in a new strategy to coimmobilize lipases and other less stable enzymes.

Key words: Phospholipase, oil degumming, structured lipids, enzyme immobilization, interfacial activation, enzyme stabilization, enzyme modulation.

1.- Introduction

Enzymes are the biocatalysts that perform all metabolic reactions and in vivo biotransformations. They have been submitted to a continuous natural selection throughout an unyielding evolution process to reach the apex as (almost) ideal catalysts for their physiological functions, exhibiting a perfect selectivity, specificity and activity under physiological conditions. These very interesting catalytic features raised the interest of researchers worldwide to use enzymes as industrial biocatalysts [1–3]. This interest has been increased with the new environmental constraints that strive to make chemical processes as "green" as possible [4–7]. Enzymes can be very interesting in this sense, as the conditions are mild (ideally the phase will be aqueous, atmospheric pressure and near room temperature) and the selectivity and specificity may save purification steps and increase the atomic economy of the process [8–13]. However, enzymes did not undergo evolution to come out as industrial catalysts, and some properties, convenient as biological catalysts are inadequate for their industrial application. For example, stability under operational conditions may not be enough in many instances. For example when used versus non-physiological substrates activity, selectivity, or specificity may be far from perfect [14]. That means that enzymes need to be enhanced in many instances to overcome these limitations as industrial catalysts.

Nowadays, there are many tools that can permit the improvement of enzyme properties. The development of metagenomics tools [15–20] opens up the opportunity of exploring almost the full biodiversity in the search of enzymes with the suitable properties (e.g., enzymes from extreme environments may have improved stabilities). Directed evolution permits to mimic the work made by Nature, but focusing on the enzyme feature(s) that is/are most relevant for the industrial application and accelerating this evolution to

months/years [21–25]. Site-directed genetic modifications of the enzymes may be better designed thanks to the improvements in modeling, crystallography, etc. In fact, nowadays it is relatively simple to produce chimeric proteins mixing the gens of two different proteins to get the desired properties of each of the involved proteins [26–28]. Chemical modification of the enzymes may be also used to improve enzyme stability or to modulate their catalytic properties [29,30].

Another limitation of industrial enzymes is their usual water solubility, which can complicate their separation from the reaction medium and reuse. Enzyme immobilization was developed to solve this limitation, producing heterogeneous biocatalysts. However, nowadays the purpose of immobilization goes beyond this goal: immobilization may improve enzyme stability, activity (mainly under harsh conditions), selectivity or specificity, decrease inhibition or increase resistance to deleterious chemicals; even purification of the enzymes can be achieved if an adequate immobilization protocol is designed [31–41].

That way, the incorporation of enzymatic biocatalysis to industrial production is becoming accelerated in the last years and may be expected to become even more relevant in the medium term.

1.1. Lipases in biocatalysis

Among the most used enzymes, lipases stand out [42,43]. The natural function of lipases is the hydrolysis of triglycerides to free fatty acids and glycerol [44]; however, in vitro lipases have been used in a wide variety of reactions, some related to oil modifications (esterifications [45–51], transesterifications [52–56], acidolysis [57–62], interesterifcations

[63–66]), some far from these substrates (resolution of racemic mixtures, regio or enantio selective reactions) and some have even exhibited a diversity of the so-called promiscuous activities far from their natural function [67–75].

Lipases are interfacial enzymes, able to hydrolyze the drops of substrate (oils are not water soluble) [76]. This is performed because the enzyme is able to become adsorbed on the surface of these drops following the so-called interfacial activation mechanism (Figure 1). Most lipases present their active center isolated from the medium by a polypeptide chain called lid. The internal face of the lid is hydrophobic and interacts with the hydrophobic surroundings of the active center [77–84]. This "closed" form is in equilibrium with an "open" form of the lipase that is the active one, where the lid is shifted and exposes the active center. However, the large hydrophobic pocket is unstable under these conditions and the enzyme is mainly in the closed form in saline aqueous homogenous media [77–84]. In the presence of a hydrophobic surface, like drops of oil, this lipase form may be adsorbed and stabilized, and for this reason they can operate at the interface of the oil drops [77–84]. This tendency to become adsorbed on hydrophobic surfaces is not limited to oil drops, and a lipase molecule may be adsorbed on any hydrophobic surface, including hydrophobic immobilization supports [85–87], hydrophobic proteins [88] or the open form of other lipases [89–91]. In fact, this strategy has become a very popular lipase immobilization protocol [92] (Figure 1).

The popularity of lipases has been promoted by their unusual properties. They are among the enzymes with a wider specificity, accepting many different substrates. This may be caused by the physiological necessity of hydrolyzing tri, di and monoglycerides of very different fatty acids. Moreover, lipases do not require any cofactor. Furthermore, some of

these enzymes are very stable under a wide range of conditions and reaction media. In fact they have been the pioneer enzymes in neomedia such as ionic liquids, supercritical fluids, etc [93–106]. They are largely widespread in biodiversity, thus Nature offers lipases with very different properties, which has resulted in many lipases being commercially available at very large scale.

1.2.- Phospholipases

Phospholipases are a diverse class of hydrolases widespread in nature present in all animals, plants and microorganisms and carry out diverse biological functions such as membrane maintenance and remodeling, regulation of cellular mechanisms and signal transduction, lipid mediators production, and digestion in humans [107]. These enzymes hydrolyze the ester bonds of the phospholipidic components of cell membranes to give simpler lipid products, such as free fatty acids, lyso-phospholipids, di-acylglycerols, choline phosphate and phosphatidates [108]. Phospholipids, being the substrate of phospholipases, consist of a glycerol-3-phosphate esterified at its *sn*-1 and *sn*-2 positions with fatty acids, whereas its phosphoryl group can be esterified with head groups such as choline, serine, ethanolamine, or inositol. Phospholipases are classified according to the position they hydrolyze on the phospholipid backbone in the following types: A1, A2, B, C and D (Figure 2). Phospholipase A1 (PLA1; EC 3.1.1.32) and phospholipase A2 (PLA2; EC 3.1.1.4) cleave the acyl ester bonds at *sn*-1 and *sn*-2 positions of phospholipids, respectively, to produce a free fatty acid and 2-acyl lyso-phospholipid or 1-acyl lyso-phospholipid, respectively, therefore they are acyl hydrolases (EC 3.1.1) [109]. There are phospholipases able to hydrolyze both of the fatty acids esterified at the *sn*-1 or *sn*-2 position of the phospholipid and they are named phospholipases B (PLB; EC 3.1.1.5) [110]. Phospholipase C (PLC; EC

3.1.4.11) cleaves before the glycerophosphate bond, releasing diacylglycerol and a phosphate-containing head group, whereas phospholipase D (PLD; EC 3.1.4.4) cleaves after the terminal phosphodiesteric bond releasing phosphatidic acid, an important intermediate involved in signal transduction, together with the head group associated at the sn-3 position of phospholipid (Figure 2). Phospholipases C and D are phosphodiesterases (EC 3.1.4.1) [107].

Apart from the crucial biological importance of phospholipases, there is a lot of interest in these enzymes from a pharmaceutical and biotechnological perspective. On the one hand, phospholipases are of great medical and scientific interest because, for example, those from snake venom induce significant pharmacological effects such as inhibition of platelet aggregation, hypotension and antitumor activity [111]. On the other hand, the use of phospholipases in industrial applications is increasing due to the development of molecular techniques for the production of recombinant heterologous proteins and the production of new redesigned enzymes with improved catalytic properties, including chemical modification as a tool to improve their stability [112,113].

Currently, phospholipases are commonly used to produce functional foods like phospholipid derivatives (*e.g.* phosphatidylserine) [114], to reduce the cholesterol content of food, and to refine certain vegetable oils, particularly in terms of oil degumming [115]. Another application is in wheat-based food systems by the production of emulsifier-like molecules such as lyso-phospholipids and monoglycerides by the action of phospholipases on the phospholipids already present in the ingredients (wheat flour, margarine and eggs) [116]. During the industrial processing of starch to produce glucose syrup, a phospholipase is used for removing unwanted lipid contaminants such as lyso-phosphatidylcholine and avoiding clogging of filters [108]. In dairy industry, phospholipases have been introduced as

a potential processing aid in cheese making, and a significant increase of cheese yield has been obtained; in this case, phospholipases release lyso-phospholipids having better emulsifying properties and favoring fat retention in the final product [117]. Phospholipases also have impact on human nutrition through the development of novel foods or ingredients with enhanced bioactivities and health effects [118].

1.3.- Lecitase Ultra

Lecitase Ultra is an example of the commercial success of a chimeric enzyme obtained via a genetic pathway. Novo Nordisk A/S commercialized the phospholipase A1 from *Fusarium oxysporum*, as Lecitase® Novo for oil degumming. This enzyme attacked the phosphatides contained in the oil phase, and was able to fully hydrolyze them to lysophosphatides, that migrated to the aqueous phase. The result was adequate, but the enzyme stability was not satisfactory for some applications. To solve this problem, the company fused the genes of the lipase from *Thermomyces lanuginosus* and the phospholipase A1 from *Fusarium oxysporum*, producing a chimeric enzyme that they named Lecitase Ultra. This way, they combined the stability of the lipase to the A1 phospholipase activity. The enzyme was first reported in 1998 [119], and since them its use has been growing in different areas, far from the initial oil degumming application. The enzyme structure has not been described to date, and only in 2016 its sequence and the amino acid residues involved in the catalytic activity were described [120]. As expected, the results concluded that most of the Lecitase Ultra sequence fits with one or the other parent enzymes. Specifically, sequence 1-284 corresponds to the lipase from *T. lanuginosus* and sequence 285-339, corresponds to the

phospholipase from *F. oxysporum*. The authors also identified the classical "Ser-His-Asp" triplet responsible for enzyme activity [120].

Using the search term "Lecitase Ultra" in "all areas" of Scopus, 280 entries may be found (access data 21 January 2019), being the first one dated in 2005 and reaching over 30 entries/year in the last 3 years. The uses of this enzyme have never been subject of a review paper, and due to its clearly growing interest, we have felt that it may be interesting to offer a review paper on this commercially available enzyme.

Curiously, this enzyme is supplied only as free enzyme, while most of the lipases may be acquired in free or immobilized forms (e.g., lipase B from *Candida antarctica* is supplied as Novozym 435, Lipase from *Thermomyces lanuginosus* as Lipozyme TL IM or from *Rhizomucor mieh*ei like Lipozyme RM IM).

2. Applications of the free Lecitase Ultra.

In this point, we will show the direct use of the commercial enzyme applications (that is, as a free enzyme), leaving for the next section the efforts to immobilize the enzyme and the uses of these immobilized biocatalysts.

2.1. Use of Lecitase Ultra in food technology

The enzyme was initially designed for food applications; therefore it is not strange that this has been the main application of Lecitase Ultra in its relatively short life. The uses in this section are restricted to those using the commercial preparation, that is, the free enzyme.

2.1.1. Oil degumming

Oil degumming, that is, elimination of phosphatides from oil, was traditionally performed by chemical processes, although the water degumming process may be described as a physical process since it does not involve any chemical reaction [121]. Degummed oil improves physical refining, reduces water consumption, the production of wastewater and the energy requirement, moreover, it may increase the yield of refined oil [121]. The employment of enzymes to remove gums from edible fats and oils started in 1992, using Lurgi's EnzyMax® process, that involved a porcine phospholipase A2 [122]. The results were unsatisfactory, and new enzymes were analyzed. Thus, Lecitase Ultra was initially designed to catalyze the degumming of oils and is very used in vegetable oils degumming with very good results [123].

The specificity of Lecitase Ultra versus different kinds of phospholipids was studied using monolayer technology [124]. The pressure of the monolayer film greatly affects the activity of the enzyme. No activity was found for sphingomyelin, while for all other phosphpolipids assayed the specificity was determined (L-α-phosphatidylethanolamine > cardiolipin L > 1,2-diacyl-*sn*-glycero-3-phospho-L-serine > L-α-phosphatidylinositol > 1,2-dioleoyl-*sn*-glycero-3-phosphocholine) [124].

Lecitase Ultra was applied in degumming of several vegetable oils; the study suggested that over 40 ºC the lipase activity decreased, making predominant the phospholipase activity [125]. In another study, Lecitase Ultra was used in the degumming of different crude rice bran oil, reducing the phosphorous content almost by 50 fold after 2 h of reaction in the oil phase [126]. In another research, the effect of the enzymatic degumming catalyzed by LysoMax® Oil and Lecitase® Ultra on the crude sunflower oil properties was

analyzed [127]. Results showed a drastic reduction of phospholipids, but also calcium and magnesium content was reduced, the oxidative stability index was decreased. In another example, soybean and rapeseed oils were degummed by Lecitase Ultra, and a high degumming can be achieved in a short time but the full phospholipids hydrolysis requires longer times [128]. This paper shows that Lecitase Ultra® hydrolyzed 80% of phosphatidyl ethanolamine after 60 min while only 40% of phosphatidyl inositol was consumed. Crude sunflower lecithin was treated with Lecitase® Ultra the enzyme hydrolyzed also non-polar lipids and in the used conditions acyl migration permitted full hydrolysis (aqueous system, T = 50 °C, pH = 5) [129]. Controlling the hydrolysis process and the presence of lysophospholipids and/or phospholipids, the emulsifying/stabilizing features of the oil could be modulated.

2.1.2. Modification of choline derivatives

This is perhaps one of the most specific and interesting uses of Lecitase Ultra, and was accomplished by diverse reactions.

l-α-glycerylphosphorylcholine was produced by Lecitase Ultra catalyzed hydrolysis of soy phosphatidylcholine [130]. The reaction medium was a biphasic water/hexane system, after optimization a purity of 99.3 g/100 was achieved (after extracting the products with diethyl ether and using a chromatographic step with a silica column). Phosphatidylcholine of soy lecithin was used in the production of L-α-glycerylphosphorylcholine in aqueous medium using Lecitase Ultra as catalyst (94.5 % yield) [131]. The hydrolysis of phosphatidylcholine by Lecitase Ultra in aqueous systems was studied in another paper, determining the thermodynamic constants (activation energy was 5.96 kJ/mol) and kinetic constants [132]. Soy phosphatidylcholine was hydrolyzed in hexane

to produce lysophosphatidylcholine, after optimizing (with a yield of 83.7 mol%) [133]. The product had a content of unsaturated fatty acid that was higher than that of the initial substrate, it was mainly due to linoleic acid.

In another research, soybean phosphatidylcholine ethanolysis catalyzed by Lecitase Ultra was used to produce lysophosphatidylcholine with a conversion ratio of 98.3% after enzyme optimization had been described [134].

2.1.3. Diglycerides production

Another use of Lecitase ultra, quite far from the degumming, and more similar to uses of a conventional lipase [135,136], is in the production of diglycerides by diverse strategies, including esterification, alcoholysis or partial hydrolysis.

For example, palm oil was hydrolyzed using four commercial lipases (Lipase A, Lipase AY, Lipozyme RM IM and Lipozyme TL IM) and Lecitase Ultra [137]. Lecitase Ultra and Lipozyme RM IM were the most selective enzymes towards hydrolysis of saturated fatty acids, Lecitase Ultra have the highest yields in free fatty acids (94.5%) [137]. Soybean oil was partially hydrolyzed by Lecitase Ultra with the objective of producing diacylglycerols, with the help of molecular distillation after hydrolysis reaction [138]. Molecular distillation promoted acyl migration in the diglycerides. The hydrolysis of soybean oil catalyzed by Lecitase Ultra in a solvent free system was also performed [139]. Diacylglycerol-enriched palm olein and soybean oil were obtained by a partial hydrolysis catalyzed by Lecitase Ultra in aqueous/oil suspension or solvent free media [140–142] Later, after product purification by molecular distillation, the oxidative stabilities of the product were analyzed [143].

In another study, palm oil was submitted to glycerolysis in a solvent-free system to produce diacylglycerol [144]. After optimization, the final content of diacylglycerides was 59.5% in the lipid layer was achieved.

Diacylglycerol-enriched oil synthesis was catalyzed by Lecitase Ultra using via esterification of free fatty acids and glycerol (a yield of 42.7 and a purity of 83.1% were obtained) [145]. In another paper, oleic acid and glycerol were esterified in a reaction catalyzed by Lecitase Ultra in solvent free medium [146]. An esterification efficiency higher than 80% and a production of almost 55% of diacylglycerides was found in the lipid layer

2.1.4.-Modification of flour in bread making

Surfactants may form amylose-lipid inclusion complexes that reduced bread crumb firming. Considering that free fatty acids or lysolipids are detergent-like molecules, the treatment of the wheat endogenous lipids with enzymes may have some positive effects. Model gluten-starch were produced in laboratory (resembling flour), and it was found that the blends adsorbed more oil when treated with Lecitase Ultra, showing that the features of this model may be tuned by this treatment [147]. Treatment of wheat flour with Lecitase Ultra had low or even a negative impact on gluten agglomeration and yield, while Lipolase treatment permitted an improvement on the glutted agglomeration [148]. Lipopan F, Lecitase Ultra, and Lipolase treatment of the whet permitted to reduce stiffness intensification, crumb firmness and the decrease in resilience [149]. Lecitase Ultra was compared to Lipopan F in their ability to alter the wheat lipids and how the lipid modification alter the gluten network strength and direct gas cell stabilization, while keeping the dough extensibility unaltered [150]. Amylose-lipid inclusion complexes reduce the access of water molecules to starch granules by forming a lipophilic shield, which increased the rigidity of the starch granule

[151]. The high content of phospholipids contained in wheat germ oil was reduced using Lecitase Ultra and Lysomax, being Lecitase Ultra the most effective (over 85% phospholipid removal) [152]. The wheat flour lipid composition during bread making was altered employing Lipopan F and Lecitase Ultra to increase the loaf volume [153]. This was related to the decrease of phospholipids and galactolipids and the increase in free fatty acids and lysolipids.

2.1.5. Other uses of Lecitase Ultra in food.

Lecitase has many other uses. For example, the acidity of mammalian fats was reduced to check its carbon and water footprint using as biocatalysts Novozym 435, Lipozyme IM TL and Lecitase Ultra for mammalian fat [154]. Unfortunately results were uneconomical with the three biocatalysts.

In another case, by combining the hydrolysis catalyzed by Lecitase® Ultra and reversed-phase liquid chromatography with electrospray ionization and sequential mass spectrometry, the regiochemistry of sulfoquinovosylmonoglycerides and sulfoquinovosyldiglycerides in the lipid contained in spinach (*Spinacia oleracea*) and parsley (*Petroselinum crispum*) was investigated and the main components determined [155]. Another application was in the extraction of proteins from some wastes. The use of Palatase 20000 L and Lecitase Ultra was found to be the best treatment for the extraction of proteins from olive pulp and stone, respectively [156].

Foaming properties of whey protein concentrated solutions were tuned by hydrolysis using Lecitase 10L, Lecitase Ultra and a lyso-phospholipase A2 from *Aspergillus niger* [157], all biocatalysts permitted to alter this properties.

2.2. Use of Lecitase Ultra in chemistry

Lipases have found many applications in chemical areas, thanks to their broad specificity in many instances coupled to a high enantio and/or region specificity and selectivity [10,158,159]. Lecitase Ultra has been employed in most applications described for lipases, although with a lower intensity perhaps due to its name as phospholipase.

For example, one of the most popular uses of lipases is in the production of low chain esters (flavors and fragrances), because when produced using biocatalysis may be labeled as natural products [160]. For example, Lecitase Ultra has been employed in a direct esterification reaction in the production of methyl benzoate and methyl butanoate in a continuous flow microreactor (with conversions of 54 and 41 %, respectively) [161].

Resolution of racemic mixtures (exploiting enzyme enantiospecificity) is another very popular use of lipases [162]. Using Lecitase Ultra, the kinetic hydrolytic resolution of different derivatives of (E)-4-phenylbut-3-en-3-ol (acetate or propionate) (4b) catalyzed by Lecitase Ultra yielded (+)-(R)-alcohol and unreacted (−)-(S)-ester (using the propionate ester the enantiospecificity was higher, enantiomeric excesses ranging from 93–99 % [163]. Esters of L amino acids with aliphatic residues are hydrolyzed by Lecitase Ultra with high enantiospecificity while if the side chain is an aromatic ring or α-hydroxy acids, the enantiospecificity greatly decreased [145].

Polymer modification and synthesis is another interesting use of lipases [164,165], and Lecitase Ultra has been also employed for this goal. For example, using ultrasounds, Lecitase Ultra was used in the synthesis of poly-4-hydroxybutyrate-co-6- hydroxyhexanoate [166], but the reaction rates were lower than using other biocatalysts although the results were better than using the lipase from *Candida rugosa*. The effect of the organic solvent in

343 the modification of poly-3-hydroxyalkanoates by glucose via esterification catalyzed by
344 Lecitase Ultra was studied [167]. Using dimethyl sulfoxide and chloroform the results
345 reached maximum polymer modification (just under 40%). In another paper, polylactic acid
346 polymers have been synthetized using Lecitase Ultra and Lipozyme TL IM , producing low
347 molecular weight polylactic acid polymers [168]. The polymers were slightly larger using
348 Lecitase Ultra.

349

350 **2.3.- Lecitase Ultra in production of biodiesel**

351 Biodiesel production is one of the most interesting uses of lipases [169,170].
352 However, in many instances this is performed using immobilized enzymes [171,172]. We
353 have been able to find just two reports using free Lecitase Ultra in biodiesel production, and
354 they are not directly related to biodiesel production, but in the previous refining to make a
355 specific oil usable to this goal.

356 The first example is in the use of the oil from *Jatropha curcas* seeds to produce
357 biodiesel [173,174]. However, the presence of a high content of phospholipids in the oil from
358 *Jatropha curcas* seeds complicates its use in biodiesel; a previous refining process is thus
359 required. LysoMax and Lecitase Ultra were successfully used for degumming of this oil,
360 being more effective Lecitase Ultra, and permitting the use of the final product in the
361 biodiesel production [175].

362 In the second example, Lecitase Ultra was combined to lipase NS81006 to produce
363 biodiesel from low quality oil, because these oils contain a great percentage of phospholipids
364 [176]. The process was a two-step method. First, the phospholipids were hydrolyzed by

Lecitase Ultra; later the lipase was used to produce the methyl esters. This permitted to increase the yield from just over 76 % to more than 96 % [176].

3. Preparation of immobilized biocatalysts of Lecitase Ultra

As Lecitase Ultra is only supplied as an enzyme solution, the enzyme has been subject of many attempts to prepare a suitable immobilized biocatalysts. As stated previously, a properly immobilized enzyme derivative, together with having a simpler separation from the reaction media, may have many properties improved when compared to the free counterpart, like stability, activity, selectivity, etc. [31–41]. In fact, even when the supplier recommends the use of the enzyme as a free enzyme, immobilization may have significant positive effects on its performance, as it is the case of Eversa lipase [177]. Thus, many research papers pursue the preparation of immobilized biocatalysts from Lecitase Ultra.

3.1. Lecitase immobilization

The enzyme has been immobilized on many different supports. For example, it has been immobilized in several instances via ion exchange. In a first example, Lecitase Ultra has been immobilized on aminated bacterial cellulose beads (via ion exchange), that had been coupled to superparamagnetic molecules to have a biocatalyst with response towards a magnet [178]. Immobilization yield of the enzyme was 70%, without altering their properties. In another example, Lecitase Ultra was immobilized on the commercial support epoxy-DILBEAD-VWR [179]. The support was previously modified with polyethylenimine and

386 crosslinked with glutaraldehyde, and the enzyme was immobilized via ion exchange. The
387 support can be reused after enzyme inactivation by enzyme release and new loading of fresh
388 enzyme. However, the authors did not investigate in deep if all enzyme molecules were
389 desorbed [179]. Lecitase Ultra immobilized on polyethylenimine/glutaraldehyde DILBEAD-
390 VWR was used in the resolution of methyl trans-(±)-3-(4-methoxyphenyl) glycidate [179].
391 The reaction was performed in xylene with excellent results (e.e. >99 %, conversion 50 %).
392 Lecitase Ultra immobilized on an anionic exchanger greatly increased its activity when
393 adding organic cosolvents or surfactants to the reaction medium (e.g., 12 folds in presence
394 of 15 % ethanol) [180]. The hydrolysis of sardine oil was also improved by these components
395 of the reaction medium, altering the selectivity by eicosipentaenic acid or docosahexaenic
396 acid.

397 The acidolysis between lecithin and capric acid was catalyzed by Lecitase Ultra
398 ionically exchanged on Amberlite XAD 7HP, and compared to the results using other lipases
399 [181]. The highest molar incorporation value of caprilic acid was obtained using Lecitase
400 Ultra (51.0 mol%). Lecitase Ultra was immobilized on Duolite A658 via anion exchange
401 and used in the hydrolysis or ethanolysis of phosphatidylcholine [182]. The study included
402 several lipases, but the immobilized Lecitase Ultra offered the best results in ethanol. This
403 biocatalyst was also used in the production of structured phosphatidylcholine via acidolysis
404 of phosphatidylcholine with conjugated linoleic acid [183]. The incorporation of CLA
405 reached 90%, but this accounted for the product, as most of the substrate was hydrolyzed
406 [183]. Later, this process was optimized by response surface methodology [184].

407 Other immobilization strategies were also assayed with Lecitase Ultra. When the
408 enzyme was encapsulated in AOT/isooctane reverse micelles; the enzyme was located close
409 to the interface [185]. Activity in esterification reactions increased after enzyme trapping.

Lecitase Ultra was also trapped on Amberlites XAD2 (hydrophobic polymer) and XAD4 (no ionic polymer) using standard stirring or ultrasound irradiation [186]. The biocatalysts were used in production of monoglycerides from acidic residue from a palm oil refining using isopropylidene glycerol as second substrate. Yields of over 50% were obtained under continuous flow conditions [186].

Lecitase Ultra was immobilized in a gelatin matrix, and this permitted a very significant increase in the enantiospecificity of the enzyme versus ethyl 2-hydroxy-4-oxo-4-phenylbutyrate (E value increases from 4.5 to 19.5 with S-enantiospecificity) [187]. The biocatalyst was also employed in the resolution of ethyl trans-(±)-3-phenyl glycidate and methyl trans-(±)-3-(4-methoxyphenyl) glycidate, yielding unreacted (2R,3S)-glycidate esters with enantiomeric excess higher than 99% at conversion of 52-55%. Later, the same group immobilized Lecitase Ultra on a macroporous gelatin organo-gel, achieving a yield of 47 % yield [188]. This biocatalyst was employed to produce trans-(2R,3S) methyl (4-methoxyphenyl)glycidate by enantiospecific hydrolysis of racemic glycidate ester The immobilized enzyme was reused several reaction batches maintaining the initial activity [188].

Another paper shows that Lecitase Ultra was immobilized in gelatin hydrogel and crosslinked with glutaraldehyde (activity recovery was more than 80%) [189]. The immobilized biocatalyst presented a clear pH-optimum of pH 7.5, while the free enzyme has a broader pH/activity curve. Optimum temperature increased 13 ºC after immobilization, and the stability increased greatly (maintained full activity under conditions where the free enzyme was fully inactivated). When used in a spinning basket bioreactor for the degumming of rice bran oil, it can be reused 6 times without significant changes in the activity (phosphorous reduction of 7-8 folds in each cycle). In another research, Lecitase Ultra

was immobilized using calcium alginate (CA), calcium alginate-chitosan (CAC), and calcium alginate-gelatin (CAG). After immobilization, the biocatalysts were treated with glutaraldehyde [190]. The best activity recovery was found using calcium alginate-gelatin. Thermal stability was increased after immobilization. The biocatalysts were used in degumming of soybean oil in a batch reactor, the reaction being slower using the immobilized enzyme preparations (20% more time was required to reach the same results using calcium alginate-gelatin) [190].

Covalent immobilization has been also performed in many instances. Agarose containing vinylsulfone groups was used to immobilize Lecitase Ultra, with very disappointing results (the enzyme became inactivated after immobilization) [191].

3.1.1. Immobilization of Lecitase Ultra on hydrophobic supports via interfacial activation

A lipase immobilization method that has been used with many different lipases is the immobilization on hydrophobic supports via interfacial activation (Figure 1), that permits the one step immobilization, purification an stabilization of the enzyme [87]. The lipases immobilized this way have the open form stabilized versus the support surface [92]. This way, this is the immobilization protocol more utilized to immobilize lipases, and also Lecitase Ultra. Lecitase Ultra, as an interfacial enzyme, may be immobilized following this protocol. One of the most used supports to immobilize Lecitase Ultra is octyl-agarose beads, at least for academic purposes (although agarose may be applied at industrial level) [192].

The effect of the reaction medium composition on lipase stability has been found to be also very dependent on the exact immobilization preparation. For example, phosphate

457 ions seem to have a very negative effect on lipase stability, and this is more significant when
458 the enzyme is immobilized via interfacial activation [177]. The same case may be found
459 using Lecitase Ultra, enzyme stability is greatly reduced in the presence of phosphate salts,
460 although to a lower extent when compared to other immobilized enzymes [193]. On the other
461 hand, some cations (e.g., Ca^{2+}) seem to stabilize some lipases immobilized on hydrophobic
462 supports via interfacial activation [194], although this effect depends on the exact
463 immobilization support [195] and inactivation conditions [196]. However, this effect was not
464 found using Lecitase Ultra [194]. Furthermore, enzyme stability did not significantly depend
465 on Lecitase Ultra loading [197], although the effect of immobilization rate has not been
466 properly evaluated using this enzyme [198], this result suggested that enzyme immobilization
467 rate on octyl agarose was not as high as using some lipase.

468 Other hydrophobic supports have been used to immobilize Lecitase Ultra. Cellulose
469 triacetate was used to immobilize Lecitase Ultra (yield near 100%), taking advantage of the
470 higher hydrophobicity if this product is compared to cellulose [199]. That way, the enzyme
471 was immobilized via interfacial activation and the immobilized enzyme has higher
472 thermostability than the free enzyme. Lecitase Ultra was also immobilized on Sepabeads C18
473 via interfacial activation and on Duolite via anion-exchange [200]. The biocatalysts were
474 used in the catalysis of the ethanolysis of sardine oil in solvent-free medium. The enzyme
475 immobilized via interfacial activation was 43 times more rapid in production of
476 eicosapentaenoic ethyl esters than in the synthesis of docosahexaenoic ethyl ester. In another
477 research, Lecitase Ultra was immobilized via interfacial activation on different core-shell
478 supports, becoming the enzyme properties very dependent on the immobilization support
479 [201]. Other example of the Lecitase Ultra immobilization via interfacial activation on some

hydrophobic supports utilized some supports from Purolite company, like Lifetech™ ECR1030M (DVB/methacrylic polymer), Lifetech™ ECR8804M (octadecyl methacrylate), Lifetech™ ECR1061M (styrene/methacrylic polymer), Lifetech™ ECR8806M (octadecyl methacylate) and Lifetech™ ECR1090M (styrene)) [202]. Results again show that enzyme performance (activity, stability, specificity) strongly depends on the used support. These biocatalyst were assayed in the production of biodiesel employing used cooking oil; results were much worse than those obtained using the lipases from *T. lanuginosus* [203]. However, it should be considered that this immobilized *T. lanuginosus* lipase biocatalyst gave values near the alkaline catalysts [204]. Later on, the combination of Lecitase Ultra immobilized on octadecyl methacylate and a proper phosphatidylcholine drying protocol improved the previously described low conversion yields of phosphatidylcholine using Lecitase Ultra immobilized on Duolite A658 [184], giving conversion yields over 95% (by preventing the hydrolysis) and reducing the reaction time from 24 h to 2 h (submitted manuscript).

Lecitase-Ultra was also immobilized in styrene-divinylbenzene beads and utilized in the esterification with different alcohols and acids under ultrasound conditions [205]. Ultrasounds permitted to increase the enzyme activity by a factor over 2. The best results were obtained using of caprylic and myristic acids and ethanol.

Another useful support to immobilize lipases is hydrophobic styrene-divinylbenzene matrix. It has been used to immobilize Lecitase Ultra, and permits higher loadings and immobilization rate than octyl-agarose [206].

Lecitase Ultra was also immobilized on hydrophobic polystyrene supports via interfacial activation, improving the results achieved using other supports [207]. This biocatalyst was later used in the glycerolysis of soybean oil ion solvent free system or using

503 organic solvents as solvent [208]. Higher yields were obtained using solvent-free systems
504 (53.7 wt.% if diglycerides in the lipid phase could be found).

505 Even though this strategy is very advantageous, it has a problem: the enzyme may
506 be released from the supports when using high temperature, organic solvents [209], or
507 detergent-like substrates/products [210]. Thus, some effort has been performed to overcome
508 this problem.

509

510 **3.1.2. Use of heterofunctional acyl supports to prevent enzyme release.**

511 One possibility is to modify the support, in a way that the first immobilization cause
512 remains the interfacial activation of the lipase on the hydrophobic support surface, but where
513 it is later possible to establish other enzyme-support interactions that prevent enzyme release.
514 Thus, Lecitase Ultra has been immobilized on glyoxyl-octyl agarose beads. This support
515 permits a first immobilization via interfacial activation (at neutral pH value) followed by
516 some covalent bond that prevents enzyme release (after incubation at alkaline pH value)
517 [209]. This improved the enzyme stability in thermal and organic solvent inactivation.
518 Lecitase Ultra immobilized on octyl and octyl-glyoxyl agarose beads were used in the
519 alcoholysis of tributirin with methanol, ethanol or isopropanol [211]. The phospholipase was
520 unable to produce isopropyl butyrate, while it could produce the other esters. However, the
521 paper shows how the combined use of high concentrations of alcohol and dibutyrin produced
522 the release of the enzyme from the octyl support, making the use of the covalent preparation
523 necessary [211].

Thus, this immobilization strategy solved the problem of undesired enzyme release. However, it turns immobilization into an irreversible process. Thus, the support cannot be reused after enzyme inactivation.

To solve this problem, ionic-acyl supports were designed. Octyl-glutamic [212], and amino-octyl [213] heterofunctional agarose beads were used to reinforce the immobilization of Lecitase Ultra and prevent enzyme desorption, while maintaining the reversibility if the enzyme immobilization. The immobilization on these supports reduces enzyme leakage and greatly alters the enzyme performance (stability, activity, specificity, etc).

3.1.3. Modulation of Lecitase Ultra properties via immobilization on different supports

It has been reported that lipase catalytic properties may be dramatically modulated via immobilization on different supports or using different immobilization protocols [35]. Lecitase Ultra has been submitted to these strategies, where the researchers do not pay too much attention to the immobilization process but on how the immobilization may affect the enzyme properties.

Thus, Lecitase Ultra was immobilized in diverse supports following very different immobilization mechanisms (octyl-agarose beads, cyanogen bromide agarose beads, polyethylenimine coated agarose beads and glyoxyl agarose beads) and assayed in the hydrolysis of (±)-2-O-butanoyl-2-phenylacetic acid and (±)-methyl mandelate, showing a high alteration on the enzyme properties [214]. For example, the covalent preparation yielded an E value of 26, (the S isomer) while the enzyme immobilized on octyl agarose produced mainly the R-mandelic acid (E value higher than 100). Similar biocatalysts were employed

in the enantioselective hydrolysis of the prochiral dimethyl 3-phenylglutarate [215]. Again, activity and accumulation of monoester were greatly depended on the immobilization protocol. The enzyme covalently immobilized on cyanogen bromide agarose beads was the most active and enantioselective biocatalyst, producing the (S)-methyl-3-phenylglutarate with a yield of 80 % and an ee exceeding 99 % [215]. The same immobilized Lecitase Ultra biocatalysts were used in the regioselective deprotection of 1,2,3,4,6-penta-O-acetyl-β-d-galactopyranose, 2-acetamido-2-deoxy-1,3,4,6-tetra-O-acetyl-β-d-glucopyranose , 1,2,3,4,6-penta-O-acetyl-α-d-mannopyranose and 2,3,4,6-tetra-O-acetyl-β-d-galacto pyranosyl-(1 → 4)-1,2,3,6-tetra-O-acetyl-β-d-glucopyranoside [216]. Enzyme specificity and regio-selectivity were tuned by the immobilization protocol. Different hydrophobic supports (butyl- and octyl-agarose and hexyl- and butyl-Ttoyopearl) were used in Lecitase Ultra immobilization [86]. Although the immobilization mechanism was the same (interfacial activation), the different morphology, hydrophobicity, etc.

These results indicate that Lecitase Ultra properties can be modulated as deeply as that of any other lipase using different immobilization protocols.

3.2. Physical or chemical modification of immobilized Lecitase Ultra to modulate enzyme properties

Chemical and physical modification of immobilized enzymes may also be a tool to tune enzyme properties, like activity, specificity or stability [29,30,217]. There are some examples of immobilized Lecitase Ultra modification that promote very relevant alteration on its performance as catalyst.

For example, Lecitase Ultra covalently attached on cyanogen bromide agarose beads and interfacially activated versus octyl-agarose beads were submitted to diverse modifications (with 2,4,6-trinitrobenzensulfonic acid, glutaraldehyde or amination) [218]. Results showed than the effects of the chemical modifications on the enzyme features significantly depended on the immobilization strategy used, in some instances the activity increased while in others it decreased. Glutaraldehyde modification or amination modification of immobilized Lecitase Ultra increased the enzyme stability of both biocatalysts at pH 7 and 9 (around a 10-fold), while only the aminated biocatalyst increased the enzyme stability at pH 5 by glutaraldehyde treatment.

In another research, the same biocatalysts (Lecitase Ultra covalently immobilized on cyanogen bromide agarose and on octyl agarose biocatalysts) were coated with polyethylenimine or dextran sulfate via ion exchange [219]. The cationic polymer increased enzyme activity (e.g., by 30 folds using methyl phenyl acetate as substrate), while the anionic polymer usually reduced enzyme activity. Both polymers permitted to increase the enzyme stability in some conditions, mainly in organic solvents [219]. Later, the covalently immobilized enzyme was incubated in some detergents that permitted to increase the enzyme activity by inducing the Lecitase Ultra open form, and in that moment were incubated with polyethylenimine, trying to freeze the open form of the Lecitase Ultra induced by the detergents [220] (Figure 3). After detergent elimination, a significantly enhanced activity (even by 50 times) was observed. The increase in the irreversible inhibition rate of the enzyme by irreversible inhibitors suggested that the open form of the enzyme had been stabilized.

3.3. Coimmobilization of Lecitase and other enzymes

The interest on enzyme coimmobilization is growing every day [221–223], as this gives some advantages in cascade reactions by eliminating the lag time required when using independently immobilized enzymes. In some instances, this may become critical, affecting not only the reaction rate but the final products yields (e.g., when the intermediate product is unstable). However, together with other points, coimmobilization generates two significant problems. The first one is that one enzyme is inactivated; the other enzymes must be also discarded, even if they remain fully active. The second one is the necessity of using the same support to immobilize all enzymes. The support that offers the best improvement of the properties of one enzyme may not be the same that offers the best performance for the other ones [36]. Recently, a strategy that may permit to avoid some of these problems has been proposed, and Lecitase Ultra was involved as one of the utilized enzymes.

The strategy is to coimmobilize lipases and other enzymes (with lower stability and hard to stabilize via immobilization), enabling the reuse of the immobilized lipase after the inactivation of the other enzyme. This was initially described using a galactosidase and the lipase B from *Candida Antarctica* [224]. The lipase was immobilized via interfacial activation on octyl agarose, coated with polyethylenimine and then the galactosidase was immobilized on the lipase-polyethylenimine molecule [224]. This permitted to reuse the immobilized lipase after incubation of the immobilized co-biocatalyst at high ionic strength and desorption of the inactivated galactosidase [225], that was much less stable than the lipase, for several cycles. However, this has a problem: the PEI was desorbed along the galactosidase, making it necessary to re-coat the enzyme with the polymer [224]. To solve this problem, a more sophisticated protocol was proposed, using Lecitase Ultra as one of the model enzymes. The idea was to obtain lipase-polyethylenimine composite resistant at high

ion strength. To do this, the lipase-polymer bonds need to be covalent, using glutaraldehyde and glyoxyl-octyl agarose beads [226]. As a first study, the effect of the modification on immobilized Lecitase Ultra was investigated. Thus, Lecitase Ultra immobilized on octyl agarose was modified with PEI, then with glutaraldehyde, and their properties were analyzed, finding a small effect on enzyme activity but a clear stabilization [227] (Figure 4). The stabilization was more significant when modifying highly loaded preparations and using polyethylenimine. This paper shows by SDS-PAGE the promotion of multiple intermolecular polyethylenimine enzyme molecules cross-linking, confirming the hypothesis used to explain the stabilization of lipase preparations adsorbed on hydrophobic supports after modification with polyethylenimine. Now, the galactosidase can be immobilized via ion exchange on the PEI layer and may be desorbed without losing polyethylenimine from the biocatalyst and immobilized Lecitase Ultra may be re-used by several cycles without necessity of any additional modification [226].

4. Conclusions

Although Lecitase Ultra was initially designed for oil degumming and this has been among the main applications, the good properties of the enzyme (activity, specificity, stability) have greatly opened the range of likely applications. Lecitase Ultra may be handled as a standard lipase, and like them, can recognize a great diversity of substrates, very far from phospholipids. Thus, it should be considered in any screening of enzymes to catalyze the resolution of racemic esters, alcohols or carboxylic acids. One of the most promising applications is in the preparation of structured phospholipids, mainly in the preparation of different derivatives of lysophosphatidylcholine. Its immobilization on any hydrophobic support via interfacial activation makes it relatively simple to prepare home-made

immobilized biocatalyst. This can avoid the lack of an immobilized Lecitase Ultra immobilized preparation, very likely one of the main problems that is refraining the general use of enzyme. It has also been showed how immobilization may greatly impact enzyme activity, specificity or selectivity. Thus, it is expected that the applications of this enzyme, both at academic and industrial scale, will continue the growth observed in the last years and may become one of the most utilized enzymes.

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Figure Legends

Figure 1. Conformational equilibrium and interfacial activation of lipases.

Figure 2. Generic phospholipid structure and cleavage site of the different phospholipases enzymes. Phospholipase A1 (PLA1) and phospholipase A2 (PLA2) cleave the acyl ester bonds at *sn*-1 and *sn*-2, respectively. Phospholipase B (PLB) can be deacylated at both *sn*-1 and *sn*-2 positions. Phospholipase C (PLC) catalyzes the hydrolysis of the ester bond from the *sn*-3 (3). Phospholipase D (PLD) removes the head group. R1 and R2 correspond to nonpolar fatty acids. The black arrows for phospholipases (PLA1, PLA2, PLB, PLC, and PLD) indicate their site of hydrolysis. The structure was drawn using the molecular graphics program JSmol.

Figure 3. Bio-imprinting of the open form of Lecitase Ultra induced by detergents after coating with PEI.

Figure 4- Co-immobilization of β-galactosidase and Lecitase Ultra. Lecitase Ultra was immobilized on octyl agarose via interfacial activation, coated with PEI and modified with glutaraldehyde to have covalent bonds between enzyme and support. After reduction of the composite, galactosidase was immobilized via ion exchange. PEI will remain immobilized on the support at any ionic strength.