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Review

Lipase from *Thermomyces lanuginosus*: Uses and prospects as an industrial biocatalyst

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ABSTRACT

The lipase from *Thermomyces laguginosus* (formerly *Humicola laguginosa*) (TLL) is a basophilic and noticeably thermostable enzyme, commercially available in both soluble and immobilized form. Although initially oriented toward the food industry, the enzyme has found applications in many different industrial areas, from biodiesel production to fine chemicals (mainly in enantio and regioselective or specific processes). This review intends to show some of the most relevant aspects of the use of this interesting enzyme. After checking the enzyme features, some of the most efficient methods of TLL immobilization will be commented. Finally, the main uses of the enzyme will be revised, with special emphasis in the modification of fats and oils, production of biodiesel, resolution of racemic mixtures, enantioselective hydrolysis of prochiral esters and regioselective process involving sugar preparations. In many instances, TLL has been compared to other lipases, the advantages or disadvantages of the enzyme will be discussed.

Contents

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1. Introduction

The development of a sustainable industrial chemistry of very specific and complex compounds is one of the main goals of modern chemistry [1–5]. In this context, the use of enzymes as industrial catalysts is a very promising alternative to conventional industrial chemistry: enzymes are very specific, selective and they display a very high activity under very mild experimental conditions (low pressure and temperature, aqueous medium, etc.) [6–12].

Among the most used enzymes, lipases have gained a clear predominance. In opposition to most enzymes, lipases exhibit a wide specificity, recognizing very different substrates. This permits to use a determined lipase as a catalyst for very different reactions, and makes that lipases may be used in pharmaceuticals and drugs production [13–16], in energy (biodiesel) [17–22] or food [23–26] manufacture, etc.

Lipases have a common property: in homogenous medium, they have their active center secluded from the medium by a polypeptide chain called lid [27–30]. In some occasions, as in the case of the lipase B from *Candida antarctica*, the lid is very small [31]. In other cases, this lid is quite large or even, as in the case of the lipase from *Bacillus thermocatenulatus*, a double lid exists [32]. In the presence of any hydrophobic interface [33] (a drop of oil [34,35], but also a hydrophobic support [36–43], a hydrophobic protein [44] or even other "open" lipase molecule [45–49]), the lid moves to permit the interaction between its hydrophobic face and the hydrophobic residues that usually surround the lipase active center with this hydrophobic surface. This way, the lipase becomes adsorbed on this hydrophobic surface (open form), and the active center is exposed to the reaction medium [27–32]. This mechanism of action is usually called "interfacial activation" of the lipases, and these drastic conformational changes should be considered in the use of these enzymes as biocatalysts. Lipases immobilized inside porous supports ("protected" from interactions with external interfaces) may reduce their activity. Moreover, due to the flexibility of the active center of lipases, they are highly sensitive to any change in the experimental conditions, the immobilization strategy employed, etc, making it possible to greatly alter their properties [50–53].

In this review, we will focus on the lipase from *Thermomyces lanuginosus* (TLL) (previously *Humicola lanuginosa*). TLL is the enzyme responsible for the lipolytic activity of Lipolase®, a commercial soluble lipase preparation supplied by Novozymes, and is also available in an immobilized form (Lipozyme TL IM®). Lipolase® is produced by a genetically modified strain of *Aspergillus oryzae* [54,55]. The support used in the TLL immobilization to produce Lipozyme TL IM is a silicate and the immobilization is via ionic adsorption [56,57].

1.1. Some properties of TLL

TLL is a single chain protein consisting of 269 amino acids. Its molecular weight is 31,700 g/mol and its isoelectric point is 4.4 [58]. TLL structure has been solved at 3.25 Å [59] and more recently at 1.8 Å [60]. It is roughly spherical in shape, with a size of 35 Å × 45 Å × 50 Å, and contains a central eight-stranded, predominantly parallel beta-sheet structure with five interconnecting alpha-helices (Fig. 1 shows the open and closed structures of this enzyme). The lid is an alpha-helical mobile surface loop consisting of amino acids 86–93 that covers the active site [60]. According to X-ray crystallographic studies, this lid is also highly mobile in the crystals. The active site includes the typical Ser-His-Asp catalytic triad. One of the four Trp residues of TLL, Trp89, is located in the lid. Holmquist et al. [61] have demonstrated the importance of this residue for efficient hydrolysis by showing that site-directed mutagenesis at Trp89 decreases the activity of TLL. In their study on the interfacial activation of TLL, Berg et al. [62] suggested that the activation is controlled through the cationic residues around the lid. Studies of the S146A mutant of TLL indicated that the reactive Ser also contributes to the stability of TLL [63]. TLL is quite stable, it maintains activity at 55–60 °C [64], with the maximum of activity at pH around 9 [65].

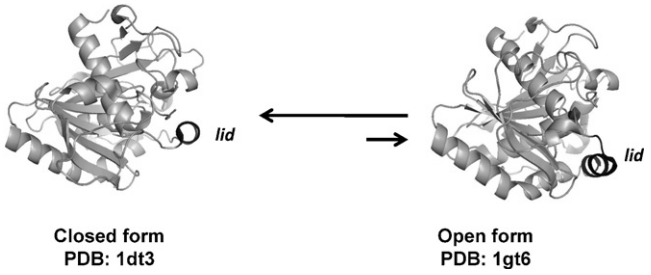

Fig. 1. Structure of open and closed forms of TLL. The 3D structure was obtained from the Protein Data Bank (PDB) using Pymol vs. 0.99.

Lipases, in general, tend to form bimolecular aggregates, confronting their open active centers [45]. TLL is one of the lipases with a stronger tendency to form these bimolecular aggregates [45], so strongly that even other immobilized lipases may be used to purify or even immobilize TLL [46,49]. This fact needs to be considered when evaluating TLL properties, because the monomer and the dimer present different activity, stability and selectivity features [45].

Some additives have effects on the TLL features. For example, betaine increased the thermal stability of TLL and protected this enzyme from unfolding by guanidine hydrochloride [66]. The effects of betaine on the structural dynamics of TLL have been explained by the increased surface tension of water caused by this solute, not by direct interactions with the enzyme [66].

The effect of detergents in TLL properties deserves some comments. On the one hand, they are able to break the enzyme-enzyme interactions, producing the monomeric form of the enzyme that has been described to be more active and less stable [45]. On the other hand, detergents can also stabilize the open form of the lipase (e.g., Jutilia et al studied the interaction of TLL with pentaoxyethylene octyl ether [67]). Both facts can explain the increased TLL activity in the presence of moderate concentrations of detergents (especially CTAB) [68]. On the other side, detergents can be inhibitors of the lipases and also produce their inactivation [69]. Moreover, detergents have been reported to modulate the specificity of TLL [70]. Thus, detergents seem to be a tool that, if properly used, may improve the management and features of TLL.

2. Preparation of industrial biocatalysts of TLL

Most enzymes require their previous immobilization to be used as industrial biocatalysts, and the immobilization of TLL has been the object of intense studies [71–73] although there is a commercially available immobilized preparation of TLL (Lipozyme TL IM®) [56,57]. The main objective of the immobilization is the reuse of the biocatalysts, and that means that the immobilized enzyme needs to be stable enough as to be reutilized for several reaction cycles [74]. In the case of TLL, the enzyme is already quite stable, and Lipozyme TL IM® has been used in many reactions (see below). At this point, we will comment some of the most successful immobilization protocols used with this enzyme. In Section 3.2, some further immobilization protocols specially focused on the biodiesel production will be commented.

Even though TLL is a quite stable enzyme, any further improvement in its stability may be interesting to further enlarge its range of applications. Stabilization of enzymes by immobilization may be achieved if an intense multipoint covalent attachment is produced

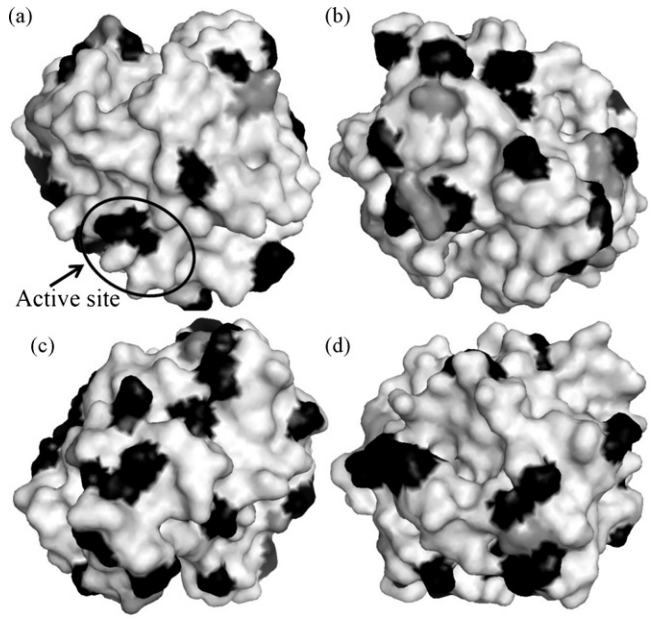

Fig. 2. Distribution of Lys, Asp, and Glu residues on four faces of the surface of TLL. Lys are shown in grey; Asp and Glu are shown in black. (a) Front face showing the active site or external face of the lid; (b) 90° rotation of the first face; (c) 180° rotation of the first face; (d) 270° rotation of the first face. The 3D structure was obtained from the Protein Data Bank (PDB) using Pymol vs. 0.99. The pdb code for closed TLL is 1DTB.

between the enzyme and the support, that way the overall rigidity of the enzyme structure increases and the enzyme becomes more stable against any inactivating reagent [53,75–80].

However, in the case of TLL, this intense multipoint covalent attachment may be difficult to achieve. The most adequate methods for multipoint covalent attachment involve mainly primary amino groups of the enzyme (e.g., glyoxyl [81,82], epoxy [83,84] or glutaraldehyde [85–87] supports), and TLL has only seven Lys groups [60]. For this reason, the chemical amination of the enzyme has been proposed to improve the prospects of getting an intense multipoint covalent attachment during immobilization via multipoint attachment on glyoxyl-agarose beads [65]. By modifying the exposed carboxylic groups with ethylenediamine after activation with carbodiimide [88,89], the number of primary amino groups could be increased by a 5-fold factor [60]. Fig. 2 shows that this aminated enzyme has far more possibilities of giving an intense multipoint attachment than the unmodified enzyme. In fact, the immobilization of the aminated enzyme permitted to get a very high stabilization factor (by a factor of several hundreds) while maintaining good activity values (70%) [65].

Other strategy that has been quite successful in the immobilization of TLL (and many other lipases) is the use of the peculiar catalytic mechanism of the lipases [27–33] to adsorb the enzyme on different hydrophobic supports via interfacial activation [36–41,90–93] (Fig. 3). This way, the immobilized enzyme will

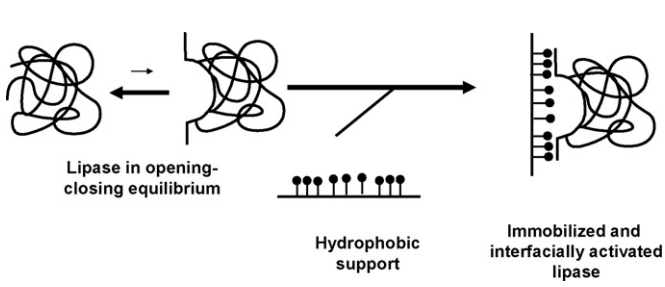

Fig. 3. Immobilization of lipases *via* interfacial activation on hydrophobic supports.

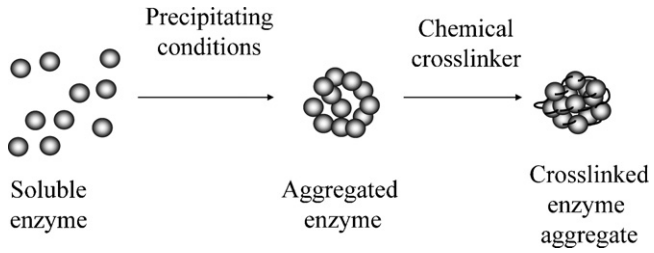

Fig. 4. Immobilization of enzymes *via* CLEA technology.

stabilize its open conformation [36,37], with properties that may depend on the nature of the support [94]. The exact orientation and mobility of TLL molecules immobilized on hydrophobic surfaces has been the object of many papers, which confirm the implication of the open form of the lipase in the immobilization [95–99]. Due to the high hydrophobicity around its active center, these preparations exhibit a lower activity versus hydrophilic substrates, while exhibiting a very high hyperactivation versus hydrophobic substrates. This enables, for example, the partial hydrolysis of polyesters [100]. Several hydrophobic supports have been used to immobilize TLL with good results in general: Accurel EP100 [93], agarose beads activated with different alkyl groups [36,37,91] or Sepabeads coated with octadecyl groups [101].

Moreover, we can mention the use of the relatively new technology of crosslinked enzyme aggregates (CLEAs) production using TLL. This immobilization technique has as main advantage that it is not necessary to use any support, it consists in the precipitation of the enzyme and the crosslinking of the precipitated enzymes with some chemical crosslinker (usually glutaraldehyde) [102–105] (Fig. 4). The aggregation and crosslinking conditions may play a relevant role in the final properties of the immobilized TLL. Thus, precipitation of TLL with $(NH_4)_2SO_4$ in the presence of SDS, followed by crosslinking with glutaraldehyde, afforded CLEAs with three times the hydrolytic activity of the native enzymes. Preparations with up to ten folds higher activity in organic medium were similarly prepared [106]. Later, Gupta et al. reported that efficient crosslinking was observed when ammonium sulfate was used as precipitant along with a 2-fold increase in activity in presence of SDS [107]. The TEM and SEM microphotographs of the CLEAs formed revealed that the enzyme aggregates are larger in size as compared to the free lipase due to the crosslinking of enzyme aggregates with glutaraldehyde. The TLL-CLEA showed more than 90% residual activity after 10 cycles of repeated use in the hydrolysis of olive oil [107].

Polymers that may be soluble in one condition and insoluble in other conditions (smart polymers) [108–111] have been also used to immobilize TLL. It was conjugated onto two water-soluble polymers, Eudragit S100 and carboxymethyl cellulose (CMC) [111]. TLL attached to Eudragit (E-TLL) exhibited reversible solubility in water, and was insoluble below pH 5.0 and soluble above pH 5.5. CMC-lipase could be reversibly precipitated from aqueous solutions using a combination of 7% (w/w) polyethylene glycol-4000 and 50 mM Ca^{2+}. E-lipase fully hydrolyzed 20% (w/v) olive oil in isooctane. The enzyme preparation was stable under stirring conditions, and could be reused multiple times without loss of enzyme activity. The nature of the polymer used for enzyme conjugation had a significant effect on the activity of the lipase used. E-lipase showed increased specificity for water insoluble substrates, while CMC-TLL showed increased specificity for water-soluble substrates. Thus, E-TLL was found more than four times more active than free lipase for hydrolysis of olive oil. CMC-lipase on the other hand, catalyzed more than four times more rapidly the hydrolysis of water-soluble p-nitrophenyl acetate, compared to soluble enzyme.

The advances in nanoscience have made that nanoparticles may be used to immobilize proteins, and TLL has been one of the targets [112,113]. Immobilization on nanoparticles offers some advantages, mainly the decrease of diffusion problems, and the possibility of using the enzymes over solid substrates [114]. However, the enzyme will be exposed on the surface of the support; therefore inactivation due to autoproteolysis, interaction with gas bubbles or drops of insoluble solvent may occur [115,116]. These problems may be prevented if the enzyme is coated with a hydrophilic polymer, which will avoid direct interaction between the immobilized enzyme and any other macromolecule or surface [117].

Finally, we will comment on the possibilities of reactivating some immobilized preparations of TLL partially inactivated by heat or the interaction with organic co-solvents. If this reactivation may be performed, the operational life of the TLL preparations should be enlarged as effectively as if the biocatalysts were more stable. It has been shown that the incubation under mild conditions (absence of organic solvents, 25 °C) of partially inactivated TLL one-point covalently immobilized preparations (especially when the inactivation was caused by exposition to organic solvents) may be enough to regain a high percentage of the lost activity [118]. Using the aminated-immobilized-stabilized TLL preparation described above [65], the percentage of recovered activity became almost 100%, if the activity was determined in the presence of CTAB [119]. This suggested that the main drawback in the reactivation of TLL was to regain the opening-closing mechanism of action of the enzyme, and that the multipoint covalent immobilization could not only prevent enzyme inactivation, but also facilitates enzyme reactivation. In a further step, the immobilized-stabilized TLL surface was modified with highly hydrophilic polymers [120]. The hypothesis was that the groups attached to the polymer could hardly change the position, during unfolding–refolding, due to steric hindrances. Moreover, the high hydrophilicity of the polymer could generate a certain "partition" of the residues of the protein, helping the hydrophilic groups to remain in the enzyme surface, and the hydrophobic groups to stay in the enzyme core. This strategy permitted to reactivate the immobilized–stabilized enzyme inactivated by organic solvents to 100% even in the absence of CTAB [120]. The incubation of inactivated enzymes in guanidine, used to destroy incorrect but stable enzyme structures [121,122], did not permit in general to significantly improve the reactivation of TLL. This could be caused by the fact that even the free enzyme did not fully unfold when incubated in concentrated guanidine (very likely due to their high stability) [123]. This partial unfolding could be also the reason for some results in experiments of folding–unfolding of soluble TLL in urea, where the "reactivated" TLL molecules showed different properties compared to the "native" TLL molecule [124].

3. Uses of TLL as industrial biocatalyst

The high activity and stability of TLL have permitted to use the enzyme in most of the available reaction media. From biphasic water–organic–solvent media [125] to solvent-free reactions [126], there are many processes catalyzed by this enzyme.

For example, TLL has been also used in the popular ionic liquids. Ionic liquids are organic salts, which are liquids at ambient temperatures [127–129]. Ionic liquids are composed of ions, and present a high polarity. Their unique properties such as nonvolatility, nonflammability, and excellent chemical and thermal stability have made them an environmentally attractive alternative to conventional organic solvents. Ionic liquids have low melting points (<100 °C) and remain as liquids within a broad temperature window (<300 °C) [127–129]. TLL was assayed, together with other lipases, in nine different ionic liquids as catalyst the synthesis of propyl butyrate from propanol and butyric acid. Activities were clearly dependent on the nature of the ions, the results being improving as the alkyl chain length of the imidazolium cation increased [130]. Regioselective acylation of sugars was also accomplished in a two-phase system containing ionic liquids. In this example [131], selective TLL-catalyzed synthesis of glucose fatty acid esters in two-phase systems consisting of an ionic liquid 1-butyl-3-methyl imidazolium tetrafluoroborate or 1-butyl-3-methyl imidazolium hexafluorophosphate ([BMIM][PF_6]) and *t*-butanol as organic solvent was performed, TLL permitted to reach yields higher than 30% (although no optimization was performed for this enzyme) [131].

TLL has been also used in water-in-ionic liquid microemulsions, a new microheterogeneous media [132]. Water-in-ionic liquid (w/IL) microemulsions formulated with non-ionic surfactants (Tween 20 or Triton X-100) in ([BMIM] [PF_6]), were used as media for lipase-catalyzed esterification reactions. The catalytic behavior of the enzymes depends strongly on the surfactant concentration and the water content. TLL lipase retained 90% of activity after ten reaction cycles in w/IL microemulsions formulated with Tween 20 [132].

There are also some examples of using TLL in supercritical fluids (scF) [133,134]. The supercritical state for a substance can be achieved above its critical temperature and critical pressure. The solvating power and the density of a scF are a function of temperature and pressure. Therefore, the solubility of a compound and the density of the scF are correlated. For example, downstream processing is possible by alteration of pressure and/or temperature. The densities of scF are comparable to liquids, while the viscosities and diffusion coefficients are comparable to gases. Thus, scF combine solvation powers of liquids with mass transfer properties of gases. Examples of the use of TLL in this media are diverse; e.g., in hydrolytic reactions [135], in the synthesis of cocoa butter [136], flavors [137], fine chemicals [138] or different ester of fat acids [139,140].

3.1. Modification of oils and fats

One of the main uses of lipases is in the modification and production of new oils and fats, to produce healthier foods [23–26]. The regiospecificity is one of the major advantages of using lipase technology in the oleochemical industry to produce high added value products, such as cocoa butter equivalents, human milk fat substitutes, and other specific-structured lipids [141]. Therefore, positional specificity of lipases has the priority and will be the target property to be exploited for commercial and industrial developments, because no chemical method presents such specificity. In this regard, the high stability of TLL may be an advantage. One of the main applications of TLL has been in this particular field.

3.1.1. Hydrolysis of oils and fats

TLL has been used in the hydrolysis of different oils and fats to produce free fatty acids. This enzyme is specific for the positions 1 and 3, therefore the hydrolysis of the ester in position 2 should mainly follow acyl migration to position 1 (Fig. 5). The kinetics of the hydrolysis catalyzed by Lipozyme TL IM has been studied in the hydrolysis of palm olein in an aqueous-organic phase [142]. The results fitted a ping-pong bi-bi model with substrate inhibition by water. The critical water content before inhibition occurs was found to be 3.6% (v/v). The rate of formation of fatty acids is limited by the formation of glycerol. No inhibition by palm olein was observed up to a content of 874.76 g/l. The hydrolysis of emulsified tributyrin by TLL has been also studied [143]. The results of the paper suggested that the hydrolysis of tributyrin by TLL proceeds through two separate stages which partially overlap. In the first one, the main reaction is the hydrolysis of one of the outer ester groups (sn-1 or 3) of the tributyrin molecule. The second stage starts when the

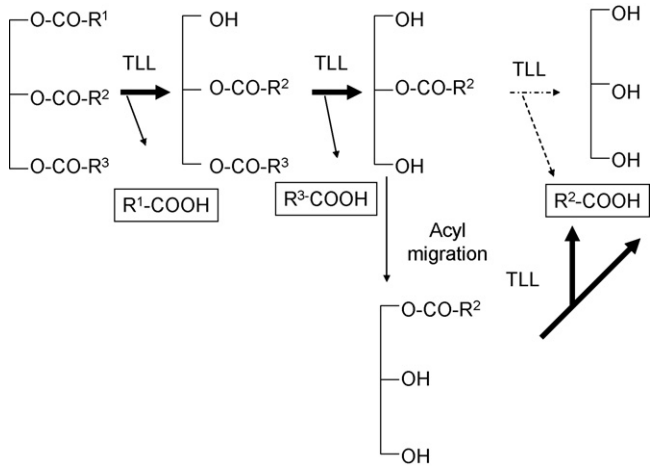

Fig. 5. Hydrolysis of triglycerides catalyzed by TLL.

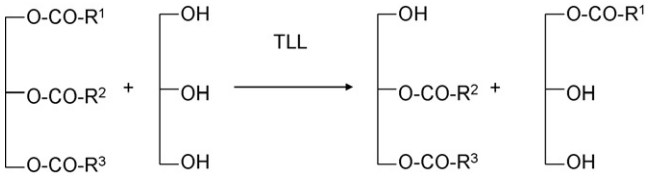

Fig. 6. Glycerolysis of triglycerides catalyzed by TLL.

interface becomes saturated with diglyceride, and involves mainly the hydrolysis of the remaining outer ester bond [143]. In fact, the hydrolysis of the successive products of the hydrolysis of triglycerides has been also analyzed in many papers. For example, the hydrolysis of 1-monoolein or 1,2-diolein monomolecular films, in absence or presence of β-cyclodextrin by TLL has been studied by Ivanova et al. [144,145]. The presence of β-cyclodextrin accelerated considerably the hydrolytic process reaction rates, by increasing the concentration of the available substrate.

Special interest has been focused on those papers that compare TLL to other lipases. It should be noted that comparisons are complex; usually fixed conditions are employed, and moreover, in many instances there is not a unique "best" lipase for all the substrates studied, or the parameters analyzed.

TLL has been compared with other lipases with different structures as catalysts of the hydrolysis of sunflower oil, soybean lecithin and their mixtures at 60 °C in a biphasic mixture heptane-buffer pH 7.0 [146]. Lipozyme TL IM® evidenced the highest conversion to fatty acids when sunflower oil (85.3% conversion to fatty acids) and its mixture with lecithin (100% conversion to fatty acids) were tested, while the free lipase from *Rhizomucor miehei* evidenced the highest activity with lecithin as substrate (35.6% conversion to fatty acids). In the case of Lipozyme TL IM® and the artificial mixture sunflower oil-lecithin, the product distribution was: 14.91% mono-glycerides; 15.46% diglycerides and 69.63% free fatty acids [146].

One of the most interesting applications of lipases is in the hydrolysis of fish oils to produced poly-unsaturated fatty acids. In a recent study, the fatty acid specificity of five lipases (including TLL) towards eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) was evaluated in the hydrolysis of fish oil, squid oil and a model system containing methyl esters of EPA, DHA and palmitic acid [147] Results suggested that both EPA and DHA were more easily hydrolyzed from a glyceride than from a methyl ester for all lipases. In the oils, DHA was initially discriminated the most by TLL. However, after longer reaction times the enrichment of DHA in the glyceride fraction of the oils was greatest for the lipase from *Candida rugosa*. In another paper, the ability of TLL lipases to mediate the hydrolysis of the soybean oil to yield concentrates of essential fatty acids was compared to that of the lipases from *Candida rugosa* and porcine pancreatic lipase [148]. In terms of free fatty acids, microbial lipases were more effective to promote the enzymatic hydrolysis of the soybean oil (over 70%) than the porcine pancreatic lipase (24%). In spite of this, porcine pancreatic lipase showed the most satisfactory specificity towards both essential fatty acids.

Other interesting point is the hydrolysis of semisolids fats. The enzymatic fat-splitting catalyzed by TLL was studied using a reactor that alternated layers of enzyme support material and separator screens [149]. Melted edible tallow at 51 °C was pumped through the immobilized enzyme layers and swept from the downstream separator screens by buffer recycled from a continuous oil/water separator. The activity per square centimeter of 10-layer reactors was nearly as much as that of single-layer reactors at the same enzyme loading and oil feed rate [149].

3.1.2. Alcoholysis or transesterifications of oils and fats

In some cases, instead of hydrolyzing the oil, the alcoholysis of oils is intended, obtaining an ester (Fig. 6). This may be denominated alcoholysis or transesterification.

There are many examples where the alcohol is glycerin. For example, sunflower oil was used as the starting material to produce mono-glycerides containing poly-unsaturated fatty acids via enzymatic glycerolysis [150]. Lipozyme RM IM, Lipozyme TL IM and Novozym 435, were employed in a batch reaction system, being Novozym 435 the biocatalysts that showed the best properties in this glycerolysis.

In other transesterification examples, considering that saturated fatty acids enhanced the oxidative stability of phospholipids, palmitic and stearic acid-rich phosphatidylcholine oils have been prepared using lipase-catalyzed transesterification from phosphatidylcholine isolated from egg and soybean lecithins [151]. Novozym 435 and Lipozyme TL IM were compared. Palmitic acid could be incorporated up to 58.6 and 57.1% using Lipozyme TL IM and 56 and 61% using Novozym 435 in egg and soybean phosphatidylcholine from an initial content of 37.4 and 16.8%, respectively. Similarly, stearic acid incorporation was up to 44.7 and 46.3% using Lipozyme TL IM and 37.2 and 55.8% using Novozym 435 in egg and soybean phosphatidylcholine from an initial content of 8.6 and 2.1%, respectively. That is, changing the substrate, the optimal biocatalyst was different [151].

Once again, it must be highlighted the papers where TLL is compared to other lipases. For example, the fatty acid specificity of TLL, *Rhizomucor miehei*, *Pseudomonas cepacia* or *Pseudomonas fluorescens* lipases towards EPA and DHA was evaluated when performing ethanolysis of squid oil [150]. During the first part of ethanolysis, no DHA ethyl esters were detected. Using TLL, some conversion of EPA located in a triacylglycerol to ethyl ester could be observed. This pattern was not found for the lipase from *Rhizomucor miehei*. TLL showed the lowest specificity towards DHA and the highest DHA recovery during DHA enrichment in the acylglycerol fraction. It was thus used to catalyze the ethanolysis of squid oil on a larger scale. The product contained 34 mol% DHA and 17 mol% EPA, compared with 19 mol% DHA and 12 mol% EPA in the original squid oil [152].

Lipases from *Pseudomonas cepacia* and TLL were immobilized in a phyllosilicate sol–gel matrix and studied for their ability to catalyze the alcoholysis of fats and oils to simple alkyl esters [153]. At 50 °C and after 48 h of reaction, immobilized TLL gave the highest alkyl ester yields (70–100%) from fats and oils regardless of chain length or degree of unsaturation of the acyl groups in the triacylglycerols. Both immobilized lipases catalyzed ester forma-

tion (80–90%) from greases containing a range of free fatty acids (2.6–36%) [153].

Another example is the enzymatic transesterification of a commercial preparation of medium-chain triacylglycerols with fully hydrogenated soybean oil [154]. Three different immobilized lipase preparations were tested: Lipozyme TL IM, lipase PS (*Burkholderia cepacia*), and Chirazyme L2 (*Candida antarctica*). The rates of disappearance of the triacylglycerols originally present in both the medium-chain triacylglycerols and the fully hydrogenated soybean oil were the fastest when Lipozyme TL IM was used; the slowest rates were observed for lipase PS. Although the relative compositions of the newly formed triglycerides at equilibrium depended on the particular lipase used and the initial weight ratio of the substrates, the product families containing 2 stearic residues and a residue of either capric acid or caprylic acid were the most abundant product species [154].

Recently, monoacylglycerol synthesis by enzymatic glycerolysis of soybean oil catalyzed by Lipozyme TL IM in *tert*-butanol/isopropanol systems was studied [155]. The yield of monoacylglycerol was 72.0% where the triacylglycerol content was reduced to only 1.0%. Fatty acid ester formation from the solvents was very low in the final product (1.3%) [155].

3.1.3. Esterification of fatty acids

Direct esterification of fatty acids and alcohols is not the most used strategy, although some examples may be found in the literature. Here, free acids are used and directly attached to alcohols (Fig. 7). Only some examples of this strategy may be found using TLL.

Co-products of vegetable oil refining such as a mixed deodorizer distillate resulting from the refining of various vegetable oils, a crude distillate resulting from the physical refining of coconut oil and commercial mixtures of distilled sunflower and coconut fatty acids were used as starting materials for the enzymatic preparation of diglycerides, by lipase-catalyzed esterification/transesterification using a mixed deodorizer distillate and glycerol as starting materials [156]. The study compared TLL to lipases from *Rhizomucor miehei* and *Candida antarctica B*. While *C. antarctica B* gave moderate good results, TLL was less active as esterification biocatalysts [156].

Other esterifications have been performed in less conventional media. Supercritical carbon dioxide has been studied as a medium for esterification of camel hump fat and tristearin in producing cocoa butter analog using Lipozyme TL IM as a biocatalyst. Pressure, temperature, tristearin/camel hump fat ratio, water content, and incubation time were the key parameters in the reactions optimization [136].

The nonthermal effect of microwave irradiation on the 1,3-specificity in the enzymatic esterification of glycerol and caprylic acid in the presence or absence of the solvent was studied [157]. Comparison on the 1,3-specificity was made between reactions driven by low power consecutive microwave irradiation and conventional heating. For the four assayed lipases (TLL included), the microwave irradiation did not change but weaken the lipases 1,3-specificity regardless the form of the lipase employed in the solvent-free medium [157].

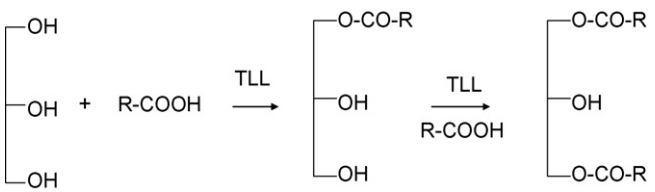

Fig. 7. General scheme of esterifications of fat acids and glycerol catalyzed by TLL.

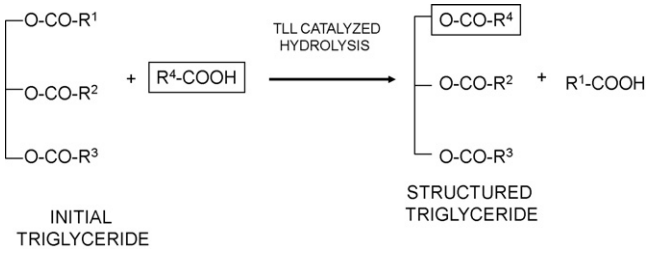

Fig. 8. General scheme of acidolysis catalyzed by TLL.

3.1.4. Acidolysis and interesterification of oils

The acidolysis (using free fatty acids) (Fig. 8) or interesterification (using an ester of the fatty acid) (Fig. 9) are the most intensively studied strategies using TLL. These terms have been considered synonymous in many instances, and even confused with transesterification, a completely different reaction (see below), making it difficult to keep track of the literature. Both strategies follow a similar mechanism.

In the case of glycerides, if one is to substitute a new fatty acid into the glycerol moiety, the ester bond between the native fatty acid residue (the original substituent group) and the glycerol moiety must first be hydrolyzed. This reaction liberates the native fatty acid and produces a lower (less substituted) glyceride containing at least one hydroxyl group. The hydrolysis step is followed by the formation of a new ester bond by reaction of the newly created hydroxyl group with the incoming replacement fatty acid [158]. If this acyl-enzyme derived from a free fatty acid, this will be termed acidolysis (Fig. 8), if this came from an ester (other glyceride, a simpler ester), it is termed interesterification (Fig. 9).

3.1.4.1. Modification of soybean oil. Soybean oil is one of the most modified oils using this strategy [159]. For example, a structured lipid was produced using lipase-catalyzed acidolysis of soybean oil and caprylic acid using Lipozyme TL IM as biocatalysts [160]. Acidolysis reactions were carried out in free-solvent system. Under optimal conditions, a lipid containing 27.01% C8:0, 6.71% C16:0, 3.48% C18:0, 19.46% C18:1, 40.49% C18:2 and 2.85% C18:3 was obtained. The C8:0 incorporation decreased by 30% when the immobilized lipase was used by 10 cycles [160].

Lipases from *Candida antarctica*, *Rhizomucor miehei*, *Pseudomonas* sp., and TLL were compared in the acidolysis of fully hydrogenated soybean oil with conjugated linoleic acid. Reaction was carried out in a batch reactor at 75 °C [161]. The lipase from *Rhizomucor miehei* produced the fastest reaction rates, and the greatest extent of incorporation of conjugated linoleic acid residues in acylglycerols was achieved in 12 h. Lipases from *C. antarctica* and TLL produced slower initial rates, and maximum extents of incorporation of conjugated linoleic acid residues were achieved in 24 h [161].

Structured phospholipids were produced by lipase-catalyzed acidolysis between soybean phospholipids and free fatty acids

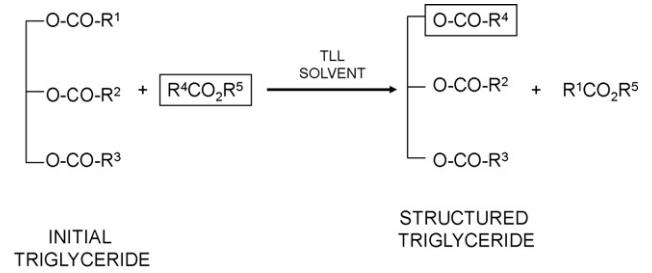

Fig. 9. General scheme of interesterifications catalyzed by TLL.

in a solvent-free system [57]. Three commercially available immobilized lipases were compared, and Lipozyme TL IM was finally selected. Incorporation of different fatty acids, including caprylic acid, conjugated linoleic acid (CLA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), into phospholipids was achieved, although with different reaction rates. The nature of the phospholipids also affected the incorporation rates of caprylic acid catalyzed by Lipozyme TL IM (phosphatidylcholine > phosphatidylethanolamine > phosphatidic acid > phosphatidylinositol). After optimization, 39% incorporation of caprylic acid could be obtained [57]. Later, the possibilities of producing structured phospholipids between soybean phospholipids and caprylic acid by lipase-catalyzed acidolysis were examined in continuous packed-bed enzyme reactors [162]. Acidolysis reactions were catalyzed in both a solvent system and a solvent-free system by Lipozyme TL IM. The water content was crucial for the acidolysis reaction in packed-bed reactors. If no water was added to the substrate during reactions under the solvent-free system, very low incorporation of caprylic acid was observed. In both solvent and solvent-free systems, acyl incorporation was favored by a high substrate ratio between acyl donor and phospholipids, a longer residence time, and a higher reaction temperature. Under certain conditions, the incorporation of around 30% caprylic acid can be obtained in continuous operation with hexane as the solvent [162].

3.1.4.2. Production of structured lipids resembling human milk fat. Structured lipids resembling human milk fat are one of the targets of many papers using TLL and these reactions. For example, structured lipids resembling human milk fat and containing γ-linolenic acid were synthesized by an enzymatic interesterification between tripalmitin, hazelnut oil and γ-linolenic acid in *n*-hexane [163]. Several commercially immobilized 1,3-specific lipases, Lipozyme RM IM and Lipozyme TL IM, were used as the biocatalysts. Human milk fat substitutes containing GLA that can be included in infant formulas were successfully produced using both Lipozyme RM IM and Lipozyme TL IM enzymes. The effect of the two enzymes on the incorporation of GLA and oleic acid were found to be similar. In another example, palm oil, palm kernel oil, olive oil, sunflower oil, and marine oil blend, was subjected to interesterification catalyzed by Lipozyme TL IM in order to obtain a product that contains similar triacylglycerol structure to that of human milk fat. The predominant TAGs of the reaction product contained mainly 1,3-diunsaturated-2-saturated structure, like human milk fat [164]. Lipozyme TL IM was used in a solvent-free batch and microaqueous system for enzymatic interesterification of anhydrous milkfat with linseed oil in binary blends and with rapeseed oil in one ternary blend [168]. The change in triacylglycerol profiles in binary blends of anhydrous milkfat/linseed oil showed that quasi-equilibrium conditions were reached after 4–6 h of reaction. Free fatty acid contents was <1%. The decrease in solid fat content and in dropping point temperature obtained with increasing content of linseed oil and interesterification resulted in good plastic properties for some of the products originating. The ternary blend composed of anhydrous milkfat/rapeseed oil/linseed oil 70/20/10 gave satisfactory rheological and improved oxidative properties, fulfilling the requirements for a marketable spread and, moreover, offering increased potential health benefits due to the enriched content in poly-unsaturated fatty acid residues [165].

3.1.4.3. Preparation of glycerides adequate to formulate margarines. The preparation of glycerides suitable to produce healthier margarines is another of the applications of TLL in these reactions [166–169]. For example; structured lipids for formulating *trans*-free margarines were synthesized by lipase-catalyzed interesterification of the blends of canola oil, palm stearin and palm kernel oil [170]. Some of the structured lipids had atherogenic indices similar to or lower than those of the commercial *trans* and similar to the *trans*-free margarine fats. Products from the blends with palm kernel oil contained a wide range of fatty acids (C6-C20) and had more β' than β polymorphs. The products obtained with all the assayed oils were suitable for formulating trans-free margarines with low atherogenicity and desirable textural properties. Another interesting example is the interesterification of a blend of palm stearin and coconut oil catalyzed by Lipozyme TL IM. This was studied for the production of margarine fats in a 1- or 300-kg pilot-scale batch-stirred-tank reactor [171]. Lipozyme TL IM was stable in the 1-kg scale reactor at least for 11 batches and the 300-kg pilot-scale reactor at least for nine batches. Due to the regiospecificity of the lipase, enzymatically interesterified products had different fatty acid distribution at sn-2 position from the chemically randomized products, implying the potential nutritional benefits of the new technology [171].

Six commercially available immobilized lipases were evaluated in batch experiments for the interesterification of butterfat blended with rapeseed oil (70/30, w/w) [175]. Lipozyme TL IM and Lipozyme RM IM, were chosen for further studies in a continuous packed-bed reactor, where Lipozyme TL IM gave the fastest reaction [172].

3.1.4.4. Modification of other oils. There are many other examples of the use of TLL with other oils and acids. In many papers there is an interesting comparison between TLL and other lipases, with a variety of results depending on the conditions and substrates. For example, structured phospholipids were synthesized with the functional lipid conjugated linoleic acid using 4 different lipases and a phospholipase A_2 [173]. Only Lipozyme RM IM and Lipozyme TL IM were effective in the incorporation of conjugated linoleic acid into phospholipids. The maximum incorporation achieved by the latter enzyme was 16% with soy phospholipids in 72 h. Structured lipids were also synthesized by acidolysis of perilla oil with caprylic acid using two lipases, Lipozyme RM IM and Lipozyme TL IM [174]. The results showed that the caprylic acid incorporation increased in parallel with solvent content to 49.0% with Lipozyme RM IM and to 63.8% with Lipozyme TL IM. After 24 h incubation in *n*-hexane, caprylic acids were incorporated to 48.5 mol% with Lipozyme RM IM and to 51.4 mol% with Lipozyme TL IM, respectively, whereas linolenic acid content was reduced from 61.4 to 31.5 mol% with Lipozyme RM IM and to 28.4 mol% with Lipozyme TL IM, respectively. Lipozyme TL IM showed a higher acyl migration rate than Lipozyme RM IM when acidolysis was performed in the reaction system containing *n*-hexane as a solvent, whereas the difference in acyl migration between the two lipases in the solvent-free system was negligible [174]. In other case, 5 lipases were evaluated in the enzymatic acidolysis of lard with caprylic acid. Lipolase TL IM permitted the highest incorporation of caprylic acid into lard [175]. After optimization, caprylic acid incorporation was up to 34.2 mol%. Lipozyme TL IM-catalyzed acidolysis was also examined for the production of structured phospholipids in a hexane system [176]. Under optimal conditions, an incorporation of caprylic acid up to 46% and recovery of phosphatidylcholine up to 60% can be obtained. Lipozyme TL IM, Lipozyme RM IM and Novozym 435 were employed as biocatalysts in the interesterification of extra virgin olive oil and fully hydrogenated palm oil in a batch reactor operating at 75 °C [177]. In this case, equilibrium was approached in the shortest time with Novozym 435 (80% conversion in 4 h). The lipase-catalyzed interesterification of virgin olive oil and fully hydrogenated palm oil was studied in a batch reactor operating at 75 °C comparing Lipolase TL IM, Lipolase RM IM and Novozym 435 [178]. Equilibrium was reached faster with Lipozyme TL IM than with the other two lipases. Lipozyme TL IM and Novozym 435 were very stable with residual activities of 90 and 100%, respectively, after 15 batch reaction cycles [178].

In other cases, the reaction was directly optimized using only TLL preparations. Lipase-catalyzed interesterification between fish oil and medium-chain triglycerides was investigated in a packed-bed reactor with Lipozyme TL IM [179]. The lipase was stable in a 2-week continuous operation without adjustment of water content or activity of the column and the substrate mixture. TLL was also employed to catalyze the interesterification reaction between corn oil and tristearin at 45 °C in a solvent-free system [180]. Addition of molecular sieves to the reaction medium reduced the percentage of hydrolysis from 15 to 7 [180]. In other study, the production of structured lipids by inserting capric acid (C8:0) into tripalmitin catalyzed by Lipozyme TL IM was studied. The product, containing 44.9 mol% capric acid, produced under optimal conditions may be considered a reduced calorie fat [181]. Solvent-free acidolysis of tristearin with conjugated linoleic acid has been carried out in a packed-bed reactor catalyzed by Lipozyme TL IM [182]. Elevated temperatures (75 °C) were utilized to eliminate solid substrates. Conjugated linoleic acid was preferentially incorporated at the sn-1,3 positions of the glycerol backbone, although 10% of the sn-2 positions were occupied by conjugated linoleic acid residues. At a pseudo space time of 0.6 h, 38% of the initial conjugated linoleic acid was incorporated in acylglycerols; the associated extent of hydrolysis was 8.3% [182]. Lipozyme TL IM-catalyzed interesterification of refined, bleached, deodorized palm olein was performed in a pilot continuous packed-bed reactor operating at 65 °C [183]. After interesterification and fractionation, the olein fractions contained higher unsaturation content ranging from 64.7% to 67.7% compared to the starting material (58.3%), while the saturation content was reduced from 41.7% to the range of 32.3% to 35.3%. In another example, interesterification of a mixture of olive oil and fully hydrogenated canola oil was carried out in a batch reactor using Lipozyme TL IM [184]. The use of a protocol involving a steep decrease in temperature significantly decreased catalyst deactivation, while presenting similar results in terms of interesterification yields. Lipase-mediated interesterification of sesame oil and fully hydrogenated soybean oil was studied at 70 °C in both a batch reactor and a continuous-flow packed-bed reactor with Lipozyme TL IM as the biocatalyst [185]. Primary differences between the performances of the two reactors were the maximum level of net hydrolysis (ca. 3 and 10 wt% lower acylglycerols at equilibrium for the continuous-flow packed-bed reactor and batch reactor, respectively), the time or space time required to approach quasi-equilibrium conditions, and less migration of acyl groups in the continuous-flow packed-bed reactor trials. The predominant product families formed by interesterification were LLS, PSO, PSL, SSL, and SSO (L = linoleic; S = stearic; P = palmitic; O = oleic).

The triglyceride (fatty acid) selectivity of Lipozyme TL IM was investigated in the interesterification between two mono-acid glycerides in *n*-hexane [186]. None of the methods used showed any significant differences between the performances of the lipase, indicating that Lipozyme TL IM is nonselective toward fatty acids or triglycerides in the system used.

The effect of high pressure in the interesterification kinetics of fat blends, in solvent-free medium, catalyzed by Lipozyme TL IM has been evaluated [187]. Reaction media were ternary blends of palm stearin, palm kernel oil and a concentrate of triacylglycerols rich in ω-3 poly-unsaturated fatty acids. Reactions were carried out at 60 °C, at 0.1, 50, 100 and 150 MPa. The biocatalyst presented interesterification activity at least up to 150 MPa. High pressures seem to affect lipase selectivity towards lauric acid. Batch operational stability tests showed an indirect correlation between pressure and enzyme stability.

Enzyme stability used to be one of the critical parameters in the optimization of the reactions. In fact, some papers focus on the operational stability of Lipozyme TL IM during reaction. For example, this was studied in the interesterification of two fat blends, in solvent-free media, in a continuous packed-bed reactor [188]. Blend A was a mixture of palm stearin, palm kernel oil and sunflower oil and blend B was formed by palm stearin, palm kernel oil and a concentrate of triacylglycerols rich in n-3 PUFA. The bioreactor operated continuously at 70 °C, for 580 h (blend A) and 390 h (blend B), at a residence time of 15 min. The inactivation profile of the biocatalyst could be well described by the first-order deactivation model: Half-lives of 135 h and 77 h were estimated when fat blends A and B, respectively, were used. Higher levels of PUFA in blend B, which are rather prone to oxidation, may explain the lower lipase stability when this mixture was used. The free fatty acid content of the interesterified blends decreased to about 1% during the first day of operation, remaining constant thereafter [188]. Thus, not only the reactions conditions may determine the stability of the enzyme biocatalysts, but also the properties of the substrate may be critical.

In an interesting paper, enzymatic interesterification of palm stearin with coconut oil was conducted by applying a dual lipase system in comparison with individual lipase-catalyzed reactions [189]. The results indicated that a synergistic effect occurred for many lipase combinations, but largely depending on the lipase species mixed and their ratios. The combination of Lipozyme TL IM and RM IM was found to generate a positive synergistic action at all test mixing ratios. Only equivalent amount mixtures of Lipozyme TL IM with Novozym 435 or Lipozyme RM IM with Novozym 435 produced a significant synergistic effect as well as the enhanced degree of interesterification. The interesterification catalyzed by Lipozyme TL IM mixed with thermally inactivated immobilized lipase preparations indicated that the carrier property may play an important role in affecting the interaction of two mixed lipases and the subsequent reactions. A dual enzyme system, consisting of immobilized lipases and a non-immobilized one (Lipase AK), in most cases apparently endows the free lipase with a considerably enhanced activity. 70% Lipase AK mixed with 30% immobilized lipase (Lipozyme TL IM, RM IM and Novozym 435) can achieve an increase in activity greater than 100% over the theoretical value when the reaction proceeds for 2 h. The co-immobilization action of the carrier of the immobilized lipases towards the free lipase was proposed as being one of the reasons leading to the synergistic effect and this has been experimentally verified by a reaction catalyzed by a Lipase AK-inactivated preparation. No apparently synergistic effect of the combinations of Lipozyme TL IM and RM IM was observed when the dual enzyme systems applied to the continuous reaction performed in a packed-bed reactor [189].

In another interesting example, interesterification of fat blends rich in n-3 poly-unsaturated fatty acids (n-3 PUFA); catalyzed by Lipozyme TL IM was carried out batch-wise [190]. Mixtures of palm stearin, palm kernel oil and a commercial concentrate of triacylglycerols rich in n-3 PUFA were used as substrates. The solid fat content values of the blend at 10 °C, 20 °C varied between 18 and 48 and at 35 °C between 6 and 24. The authors affirmed that although these values fulfill the technological requirements for the production of margarines, the accumulation up to 8.3% free fatty acids is a problem that needs to be solved before implementation of the process [190].

A blend of rapeseed and butter oil was interesterified using Lipozyme TL IM as catalyst [191]. A few of the triacylglycerols detected were typical combinations of fatty acids originating from rapeseed oil, such as α-linolenic acid, and short-chain fatty acids from butter oil. Due to the regioselectivity of the lipase, the transesterification reaction involved mainly fatty acids in the sn-1 and sn-3 positions. However, significant changes in the fatty acid composition in the sn-2 position were detected after 6 h [191].

Lipozyme TL IM was also employed to catalyze the continuous interesterification of sesame oil and fully hydrogenated soybean oil in a packed-bed reactor operating at 70 °C [192]. The predominant

triglycerides in the quasi-equilibrium products obtained from the mixture initially containing 90% (w/w) sesame oil and 10% fully hydrogenated soybean oil were LOL (26.22%) and OLO (21.92%) [L = linoleic; O = oleic; P = palmitic]). For interesterification of 80% sesame oil and 20% fully hydrogenated soybean oil, the major product species were OOP (21.27%), LOL (17.46%), and OLO (13.93%). OOP (24.38%) was the major product for reaction of 70% sesame oil with 30% fully hydrogenated soybean oil. Appropriate choices of reaction conditions and initial ratios of sesame oil to fully hydrogenated soybean oil lead to TAG with melting profiles and solid fat contents similar to those of a variety of commercial products [158].

3.1.4.5. Chemoenzymatic routes. In some cases, chemoenzymatic routes have been proposed to reach the desired products. In one study, two important isomers of conjugated linoleic acid, i.e. c9,t11 and t10,c12, were produced up to 73% of total fatty acids, employing alkali isomerization of safflower oil. Interesterification (acidolysis) of purified conjugated linoleic acid with canola oil was then conducted by TLL. The conjugated linoleic acid content incorporated into the triacylglycerols (TG) was 26.6 mol% after 48 h of reaction time [193]. Adhikari et al. have proposed other chemoenzymatic route to produce trans-free hard fat stock from fractionated rice bran oil, fully hydrogenated soybean oil, and conjugated linoleic acid [194]. Rice bran oil was fractionated into 2 phases, solid and liquid, using acetone at -18 °C. Then, trans-free hard fat was synthesized from trans-free substrate of solid rice bran oil and fully hydrogenated soybean oil at different molar ratios (solid rice bran oil: fully hydrogenated soybean oil; 1:1, 1:1.5, 1:2, and 1:3) with Lipozyme TL IM [194]. Conjugated linoleic acid (12.2–14.2%) was found on the triacylglycerol backbone of the interesterified products along with stearic (37.6–49%), palmitic (15–17.9%), and oleic acids (13.3–19.2%). The interesterified product contained higher levels of saturated fatty acids (62.6–70.1%) at sn-2 position [194].

Villeneuve et al. have proposed another interesting chemoenzymatic process for the production of structured triacylglycerols containing conjugated linoleic acids at sn-2 position and lauric acid at external ones [24]. First, castor bean oil was chemically dehydrated and isomerised to obtain a new modified oil with very high proportion of conjugated linoleic acids (>95%). Then, this new oil was used for enzymatic transesterification allowing the grafting of lauric acid at external positions of the TAG backbone by using 1,3 regioselective enzymes. Lipozyme TL IM and lipase from *Carica papaya* latex allowed good reaction yields. With alkyl esters TLL provided a final incorporation of 58.9% after 72 h corresponding to 88.4% transesterification yield. Concerning *C. papaya* lipase, incorporation of lauroyl residues was lower than Lipozyme TL IM. This lipase exhibited higher performance with lauric acid accounting for 44.7% lauroyl incorporation at the end of reaction for a 67.1% transesterification yield. The effect of the substrates mole ratio was also evaluated. It was observed that a 1:3 TAG/acyl donor mole ratio was the most efficient for both lipases. Finally, fatty acids regiodistribution of the newly formed structured lipids was determined. With Lipozyme TL IM, the proportion of lauric acid incorporated at the sn-2 position did not exceed 5.4% after 72 h while with *C. papaya* lipase a more pronounced incorporation of lauroyl residues at the central position (8.8%) was observed [24].

Thus, TLL is an enzyme with very good prospects to be used in this kind of reactions.

3.2. Production of biodiesel

Environmental problems associated with the petroleum industry and the foreseen lack of supply in the long term has converted biodiesel in an alternative to diesel, since it is made entirely from vegetable oil or animal fats, thus being renewable and biodegradable [195–197]. Biodiesel is produced by transforming triglycerides

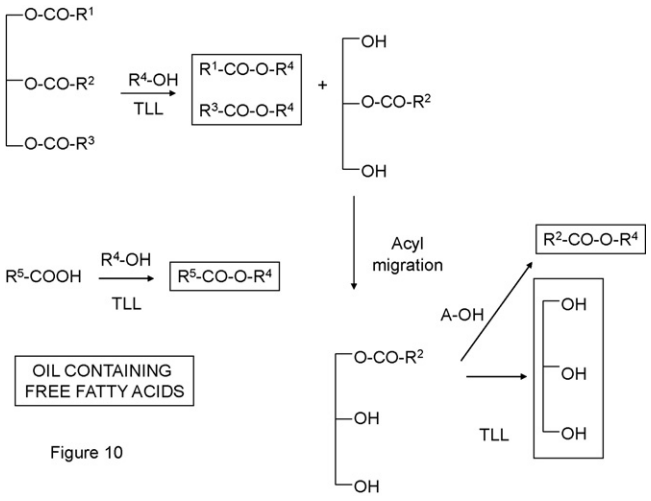

Fig. 10. General scheme of production of biodiesel catalyzed by TLL.

into fatty acid alkyl esters, in the presence of an alcohol, such as methanol or ethanol, in most cases using an acid or alkali as catalyst, with glycerol as a byproduct [198]. Alternatively, the enzymatic reaction with lipases can be used [17–22]. The separation and purification of enzymatically produced biodiesel is simplified due to the absence of soap and other by-products. To turn the biocatalytic route economically competitive, the development of stable and active biocatalysts is very important in order to improve conversion yields in the shortest possible time and to allow the reuse of enzyme for as much batches as possible. The reaction is similar to the presented in Section 3.1.2 of this review, only the objective is different, now the target is the esters, not structured lipids (Fig. 10).

Lipases can carry out the transesterification of a wide variety of oil feedstocks in the presence of acidic impurities, such as free fatty acids frequently present in oil samples, even using recycled oils [199–202]. Thus, this has been one of the uses of TLL. One of the problems that should be solved to use this lipase is to overcome its sn-1,3 regiospecificity, because yields need to be near to 100% to make the enzymatic process competitive to the chemical one. That way, acyl migration from sn-2 position to sn-1,3 position should occur during the reaction. In fact, this migration spontaneously occurs in many instances. Some papers deal with the acyl migration in reactions catalyzed by TLL and its cause, trying to accelerate this. For example, in the transesterification of soybean oil for biodiesel production catalyzed by TLL, Du et al. obtained a 90% yield; this suggested that an acyl migration was occurring during the reaction [203]. Different factors which may influence the acyl migration were explored further and it has been found that the silica used to immobilize TLL contributes significantly to the promotion of acyl migration in the transesterification process. The final biodiesel yield was only 66% when 4% lipozyme TL used, while about 90% biodiesel yield could be achieved when combining 6% silica gel with 4% lipozyme TL IM. Temperature [204] and solvent [205] were the most significant parameters to control the acyl migration. For example, transesterification and alcoholysis reactions catalyzed by immobilized TLL in hexane gave fatty acid esters that did not reflect the expected 1,3-specificity of the enzymes, due to competing acyl migrations in the partial glyceride products. However, using diethyl ether as solvent, strict 1,3-specific reactions were obtained [205]. Triethylamine, an acyl migration enhancer, could efficiently improve the yield of the methanolysis of corn oil, giving an improvement in the yield from 85% to 92.0% [206].

One of the critical problems in the production of biodiesel is the negative effect of the alcohol in the TLL stability. Lipozyme TL IM was inactivated when more than 1.5 molar equivalent of methanol

was added to the oil mixture. This makes it convenient to perform successive additions of alcohol in order to maintain an active lipase [207]. To fully convert the oil to its corresponding methyl esters, the conversion of soybean oil to biodiesel in a solvent-free medium was performed successfully by a three-step addition of 1 molar equivalent of methanol and under the optimized conditions, the maximum methyl ester (ME) yield was 98% after 12 h reaction. However, this stability-requirement seems to depend on many factors, even on the reactor design. Using also soybean oil, Du et al. found that in non-continuous batch operation, the optimal oil/alcohol ratio and temperature were 1:4 at 40–50 °C; however, during the continuous batch operation, the optimal oil/alcohol ratio and temperature were 1:1 and 30 °C [208].

Another problem for the reutilization of the biocatalyst is the presence of glycerin in the pores of the support. This may be eliminated by washing with different solvents. The washing of the immobilized enzyme with *n*-hexane, water, ethanol, and propanol after each synthetic batch, gave the best results using hexane, around 80% of the enzyme activity still remains after seven cycles of synthesis [209]. In another example, 95% of enzymic activity remained alter 10 synthetic batches when isopropanol was adopted to remove glycerol [208].

Again, the comparison of TLL with other lipases may be of special interest. Results are not clear, but they leave clear that TLL is a very good option, and that the optimal catalyst strongly depends on the substrates and experimental conditions.

For example, the enzymatic alcoholysis of crude palm oil with methanol and ethanol was investigated using Lipozyme RM IM and Lipozyme TL IM in a solvent-free system [210] Lipozyme TL IM showed the best catalytic performance. In another example, using *n*-hexane as organic solvent, TLL was compared with different immobilized lipases [211]. Highest conversion (97%) was observed with TLL after 24 h. In contrast, this lipase was almost inactive in a solvent-free reaction medium using methanol or 2-propanol as alcohol substrates.

The esterification of soybean oil deodorizer distillate with ethanol catalyzed by Lipozyme RM IM, Lipozyme TL IM, and Novozym 435, showed that the last offered the most rapid reaction [212]. In fact, literature suggests that Lipozyme TL IM and Novozym 435 are the two best commercially immobilized preparations for this reaction. The comparison between both enzymes has been subject of some papers. Both enzymes were used in the enzymatic syntheses of biodiesel from sunflower, borage, olive and soybean oils [213]. Loss of lipase activity induced by the nucleophile was greater with methanol than with ethanol, and was greater for Lipozyme TL IM than for Novozym 435. However, alcoholysis of the vegetable oils is faster with Lipozyme TL IM than with Novozym 435, although final yields were higher using Novozym. In an interesting paper, Rodrigues et al. performed a deeper analysis of comparison between TLL and CALB commercial preparations [214]. Biodiesel synthesis by alcoholysis of three vegetable oils (soybean, sunflower and rice bran) catalyzed by Novozym 435 and Lipozyme TL IM were performed. Both enzymes displayed similar reaction kinetics with all three oils and no significant differences were observed. However, each lipase displayed the highest alcoholysis activity with a different alcohol. Novozym 435 presented higher activity in methanolysis, at a 5:1 methanol:oil molar ratio; Lipozyme TL IM presented higher activity in ethanolysis, at a 7:1 ethanol:oil molar ratio. The optimal temperature was in the range of 30–35 °C for both lipases. When washing the biocatalyst with *n*-hexane, approximately 90% of the enzyme activity remained after seven synthesis cycles.

In some cases, immobilized TLL preparations have been specially designed to be used in these reactions. Considering the strong influence that the immobilization may have in the lipase properties [50–53] and the stabilization that may be achieved if it is properly designed [53,75–80], these improved preparations have permitted to enhance the kinetic and yield of the production of biodiesel.

TLL has been immobilized within hydrophilic polyurethane foams using polyglutaraldehyde to produce biodiesel with canola oil and methanol [215]. Maximum methyl esters yield was 90%. The immobilized lipase proved to be stable and lost little activity when was subjected to repeated uses (activity was maintained after 10 batches if washed with *tert*-butanol). Later, after further optimization, the maximum biodiesel yield was 97% at 50 °C in 24 h reaction. The immobilized enzyme retained its activity during the 10 repeated batch reactions [216].

Biodiesel was produced by TLL immobilized onto a novel microporous polymeric matrix containing aldehyde groups [201]. TLL was covalently attached onto the support with 80%, 85%, and 89% immobilization efficiencies using bead, powder, and monolithic forms, respectively. Immobilized enzymes were successfully used for the production of biodiesel using sunflower, soybean, and waste cooking oils. It was shown that immobilized enzymes retained their activities during 10 repeated batch reactions at 25 °C, each lasting 24 h.

TLL was also immobilized in silicone-based matrix (static emulsion) [202]. This preparation was used in the solvent-free esterification of free fatty acids from by-products of biodiesel fabrication and 2-ethyl-1-hexanol. The immobilized TLL activity was higher by a factor of 4.9–9.4 than the activity of the native enzyme. In biodiesel by-products, the immobilized lipase catalyzed the esterification of free fat acids as well as the transesterification of residual fatty acid methyl esters to the desired ester oils. A conversion of 90% in esterification and 35% in transesterification gave a total yield of 60%. The inactivation coefficients during repeated use in a stirred-tank reactor with intermittent pressure reduction were exceptionally low. Favorable properties of immobilization matrix in terms of stability and immobilization yield were observed [202].

Finally, a similar strategy to the described in [65] was followed (see Section 2 of this review), to produce a very stable TLL biocatalysts, but changing agarose by Lewatic, a support able to stand in anhydrous conditions [84,217]. Thus, aminated TLL was immobilized on glyoxyl-Lewatic and used in the enzymatic transesterification reaction of ethanol and soybean oil [218]. The reaction was carried out as a 7.5:1 ethanol:soybean oil molar ratio, 4% of water and 30 °C. In the presence of *n*-hexane, the transesterification yielded 100% of conversion, while in solvent-free system the yield was 75%. The ethanolysis carried out by three stepwise addition of ethanol produced 80% of conversion, but when two-step ethanolysis was employed, 100% of yield conversion was reached in 10 h of reaction, both for solvent and solvent-free systems.

Thus, TLL may be one of the best alternatives for this reaction, although further improvement of the enzyme stability in these medium seems to be convenient.

3.3. Uses of TLL in organic chemistry

Another important area of application of lipases is in the production of different chemicals, useful for foods, pesticides, pharmaceuticals, etc. [23–26].

In some cases, TLL specificity has been enlarged via genetic techniques. For example, TLL does not present ferulic acid esterase activity, but 13 variants of TLL were constructed based on a model of *Aspergillus niger* ferulic acid esterase A [219]. Two of the variants of TLL had significant ferulic acid esterase activity (the best one having around 20% of the model enzyme).

TLL has been also utilized in the ring-opening copolymerizations of the oxiranes (e.g., glycidyl phenyl ether or diglycidyl ether of *bis*phenol-A) with some di-carboxylic acid anhydrides [220]. The enzyme presented higher activity in this reaction than Novozyme 435 or the lipase from *Candida cilindracea*.

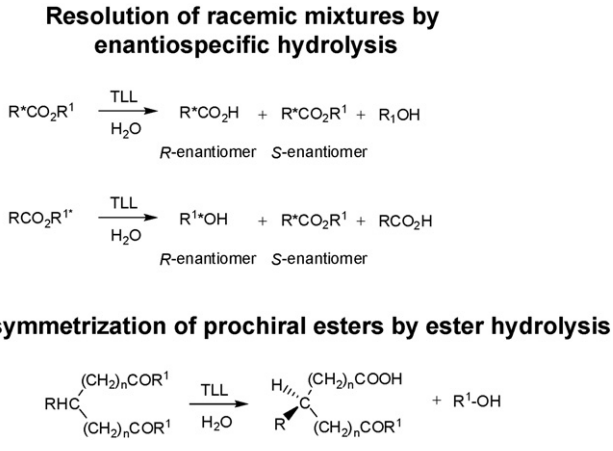

Fig. 11. General scheme of enantiospecific and enantioselective hydrolysis of esters catalyzed by TLL.

However, most of the uses of TLL in this area are related to its enantiospecificity, enantioselectivity or regioselectivity.

3.3.1. Resolution of racemic mixtures by exploiting the enantioselectivity of TLL

TLL has been used in the resolution of many racemic mixtures of acids and alcohols (Fig. 11). For example, TLL was described as the best enzyme in the kinetic resolutions of (*Z*)-4-triphenylmethoxy-2,3-epoxybutan-1-ol, a synthetically useful chiral building block, using vinyl acetate as acyl donor [221]. Enantiomeric enrichment of the optically active acetate isomer was accomplished by selective crystallization of the racemic part of the enantiomeric mixture. Enzyme catalyzed hydrolysis of the acetate also provided an optically pure epoxybutanol derivative. Best results afforded an ee over 98%. TLL was also selected as the best enzyme in the acetylation of 3-phenylthio-2-propanol with vinyl acetate. The (*R*)-enantiomer was selectively acetylated while the (*S*)-enantiomer was non-reactive [221]. In other instance, TLL-catalyzed the double enantioselective hydrolysis of (*1S,2R,3R,5S,6R*)-2-bromo-3-butanoyloxy-6-hexanoyloxybicyclo [3.2.0] heptane, allowing to obtain the corresponding (−)-bromodiol and (−)-epoxyalcohol with high optical purity [222]. Another interesting paper presents the TLL resolution of an N3-acetoxymethyl-activated dihydropyrimidone (precursor of dihydropyrimidone antihypertensive agent (*R*)-SQ 32926) [223]. In other paper on this compound, the attachment of an acetoxymethyl residue at the N3 position of the dihydropyrimidones scaffold led to an activated ester, which was selectively cleaved by TLL (enantiomer ratio > 200) to furnish, after deprotection, dihydropyrimidones (*R*)- and (*S*) on a semi-preparative scale. Treatment of the (*R*) isomer with trichloroacetyl isocyanate produced the antihypertensive agent (*R*)-SQ 32926 [224]. Using TLL, *rac*-2-carboxyethyl-3-cyano-5-methylhexanoic acid ethyl ester can be resolved to form 2-carboxyethyl-3-cyano-5-methylhexanoic acid [225]. A heat-promoted decarboxylation of this compound efficiently generates (*S*)-3-cyano-5-methylhexanoic acid ethyl ester (3), a known precursor of pregabalin (enantiomer ratio was 86). Racemic β- and γ-hydroxy sulfides were resolved by TLL-catalyzed transesterification using vinyl acetate both as acyl donor and solvent [226]. TLLl catalyzed also the ammoniolysis of phenylalanine methyl ester. Using a *T* of −20 °C, an enantiomeric ratio of 84 was obtained [227].

The enantiospecificity and activity of immobilized TLL in the hydrolysis of racemic methyl mandelate was improved via chemical amination, suggesting that chemical amination of TLL may be a way of tuning the catalytic features of the enzyme [228].

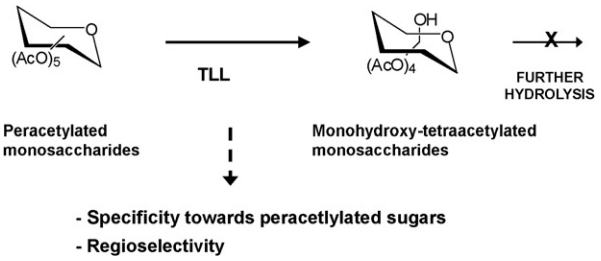

Fig. 12. General scheme of the regioselective hydrolysis of peracetylated sugars catalyzed by TLL.

3.3.2. Using the enantioselectivity of TLL hydrolyzing prochiral esters

Enantioselective hydrolysis of prochiral compounds has the advantage of producing 100% yield of the desired product (Fig. 11). Prochiral dimethyl and diethyl phenylmalonate were partially hydrolyzed to the corresponding chiral monoesters by different immobilized preparations of lipase from TLL [70]. This enzyme does not hydrolyze the monoesters and hence its hydrolysis was carried out without production of the final achiral di-carboxylic acid, quantitatively yielding the chiral monoester. Asymmetry factor with preference towards the production of the (+)-isomer could be increased from 1.5 up to 10 depending on the immobilized preparation, the type of acyl donor and the presence of co-solvents. The presence the cationic detergent CTAB promoted very significant improvements of activity (by a 40-fold factor) and enantioselectivity (from 3.5 to 20). Under these conditions a moderately enantioselective asymmetric hydrolysis, obtaining the (+)-1-(ethoxy-carbonyl)-phenylmalonic acid with an ee over 90% can be obtained [70].

3.3.3. Use off the regioselectivity of TLL

The modifications of sugars or sugar derivatives are among of the most abundant examples in the use of TLL (Fig. 12 shows a general scheme of the regioselective hydrolysis of peracetylated sugars). These reactions may be performed as regioselective acylation of free sugars or regioselective deprotection of peracetylated sugars.

3.3.3.1. Hydrolytic processes. As previously commented, lipases properties can be strongly modulated by following different immobilization protocols [50–53]. TLL is one of the examples where results are clearer. The effect of the immobilization strategy on the activity, specificity and regioselectivity of TLL in the hydrolysis of peracetylated β-monosaccharides was evaluated in a study by Palomo et al. [229]. The octyl-TLL immobilized preparation was the most efficient biocatalyst in the hydrolysis of 1,2,3,4,6-penta-*O*-acetyl-β-D-galactopyranose, producing specifically 6-hydroxy-l,2,3,4-tetra-*O*-acetyl-β-D-galactopyranose in 95% overall yield, whereas the covalently immobilized TLL preparation was 48 times slower and regioselective towards the anomeric position, producing the 1-hydroxy derivative in 70% yield. The enzyme adsorbed in a support coated with PEI was the most efficient catalyzing the hydrolysis of 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose, permitting to obtain up to 70% of the 6-hydroxy product.

These results suggest that the use of just one TLL preparation in a regioselective process may not give the best possible results, making it convenient to use as many TLL preparations as possible during the enzyme evaluation.

Lipozyme TL IM catalyzes the deacylation of 4-C-acyloxymethyl-3,5-di-*O*-acyl-1,2-*O*-(1-methylethylidene)-β-l-threo-pentofuranose to form 3,5-di-*O*-acyl-4-C-hydroxymethyl-1,2-*O*-(1-methylethylidene)-α-D-xylo-pentofuranose in a highly

selective and efficient manner [230]. The activity of TLL versus tributanoyl furanose is 2.3 times faster than the one against the triacetyl furanose derivative [231]. TLL has been also successesfully used in the deacylation of 4′-C-acyloxymethyl-2′,3′,5′-tri-*O*-acyl-β-L-threo-pentofuranosylthymine and 4-C-acyloxymethyl-3,5-di-*O*-acyl-1,2-*O*-(1-methylethylidene)-β-L-threo-pentofuranose [232].

3.3.3.2. Synthetic processes. The effect of immobilization is also a key point in this reaction. For example, TLL was immobilized on granulated silica via adsorption, on polypropylene via interfacial activation (Accurel EP100), on Celite by deposition and on Eupergit C via covalent attachment [233]. Granulated lipase converted >95% of sucrose into 6-O-lauroylsucrose in 6 h. Accurel-lipase was also very active, converting 70% of sucrose into monoester in 2 h. The residual activity of granules after five reaction cycles under the best reaction conditions was 72%; while the lipase adsorbed on Accurel retained only 15% activity after five cycles.

Highly regioselective acylation of 6-azauridine with fatty acid vinyl esters catalyzed by TLL for the preparation of its 5′-*O*-acyl derivatives has been successfully reported [234]. The effects of some crucial factors on the enzymatic palmitoylation of 6-azauridine were further examined. Under optimal conditions the reaction rate, the substrate conversion and the regioselectivity were 13.3 mM/h, 98.4% and 99.0%, respectively. Although 5′-*O*-acyl derivatives of 6-azauridine were exclusively obtained with all the tested acyl donors, the enzymatic activity varied widely with different acyl donors.

TLL was found to display better catalytic activities and excellent 5′-regioselectivities (94–>99%) in the acylation of some ribonucleosides as compared to those in the acylation of 2′-deoxynucleosides [235]. Unexpected inversion of the 3′:5′-regioselectivity was observed in the enzymatic methacryloylation, crotonylation and cinnamoylation of floxuridine (1.5:1, 2.3:1 and 4.4:1, respectively), where TLL preferentially catalyzed the acylation of 3′-hydroxyl rather than that of 5′-hydroxyl group [236]. The possible reason suggested by the authors is the presence of a remote interaction between the unsaturated bond in the acyl group and the aromatic ring of amino acid residue Trp89 in the lid of the lipase.

Lipozyme TL IM was successfully applied to the regioselective synthesis of 5′-*O*-acyl 5-fluorouridines, more powerful anti-tumor drugs than 5-fluorouridine itself [237]. The chain length of acyl donors affected the initial rate and the maximum substrate conversion of the regioselective acylation. Under optimal conditions, the maximum substrate conversion and the regioselectivity were 98.4 and >99%, respectively, after a reaction time of around 6 h.

The enzymatic transesterification of raffinose and melezitose with divinyl adipate using Lipozyme TL IM permitted to obtain polymerizable vinyladipoyl sugar esters with excellent selectivity and high yields [238]. Monoesters prepared with lipase catalysis have also been used as substrates for a second acylation step catalyzed by subtilisin. Diesters were obtained with high selectivity although the isolated yields were slightly lower than those obtained for the corresponding monoester derivatives. This two-step enzymatic approach allows regioselective control in the incorporation of the sugar inside the polymer structure.

The TLL-catalyzed synthesis of 6-*O*-lauroylsucrose and 6-*O*-palmitoylsucrose was performed by transesterification of sucrose with the corresponding vinyl esters in a medium constituted by 2-methyl-2-butanol containing a low percentage (not higher than 20%) of dimethyl sulfoxide [239]. Several lipases were able to catalyze the transesterification, but TLL adsorbed on diatomaceous earth was particularly useful. Under optimized conditions, a sucrose conversion of 70% to 6-*O*-lauroylsucrose was achieved in 24 h using 50 mg biocatalyst/mL. As a side product, a low percentage (<5% in 24 h) of the initial sucrose is converted into the diesters 6,1′-di-*O*-lauroylsucrose and 6,6′-di-*O*-lauroylsucrose. The above methodology was also extended to the synthesis of 6-*O*-palmitoylsucrose. The acylation process was even faster, with 80% conversion to monoester in 48 h using 25 mg biocatalyst/mL. The silica granulated TLL preparation has been compared to Novozym 435 in the synthesis of sugar esters by transesterification of sugars with fatty acid vinyl esters in 2-methyl-2-butanol:dimethylsulfoxide mixtures [240]. The lipase from *C. antarctica B* is particularly useful for the preparation of 6,6′-diacylsucrose, whereas TLL catalyzes selectively the synthesis of 6-*O*-acylsucrose. The granulated TLL retained more than 80% of its initial activity after 20 cycles of 6 h. Both lipases were similarly effective for the regioselective synthesis of 6′-*O*-palmitoylmaltose and 6-*O*-lauroylglucose (active as anti-tumor and antibacterial agents) [240,241]. The interest of these reactions has made that several computational conformacional studies of the catalysis of TLL with different sugars and acyl donors have been performed [242,243]. This has permitted to suggest mutations to exploit the differences towards changing the observed selectivity.

The use of several lipases (TLL among them) and proteases in the enzymatic transesterification of several tri- and tetrasaccharides with vinyl laurate suggest a final complementary picture respect to the primary hydroxyls of the oligosaccharides studied [244]. By using the sugars in their amorphous form, complete solubility is achieved in the reaction media (*tert*-butanol/pyridine mixtures for the lipases and pyridine for the protease) and high isolated yields of the corresponding monoesters are obtained. Good to excellent regioselectivity is observed for all the enzymes.

Ionic liquids have been also used in TLL acylations of sugars [131]. Selective lipase-catalyzed synthesis of glucose fatty acid esters in two-phase systems consisting of an ionic liquid 1-butyl-3-methyl imidazolium/tetrafluoroborate or 1-butyl-3-methyl imidazolium/hexafluorophosphate and *t*-butanol as organic solvent was investigated. The best enzyme was commercially available lipase B from *Candida antarctica*, but also TLL gave good conversion.

Some more complex reactions, as feruloylation of glycosides have been described [245]. *Humicola insolens* lipase, TLL and *Aspergillus niger* lipase were assayed for transesterifications of ferulic acid from its active esters to various glycosides at their primary hydroxyl group in acetonitrile or methyl isobutyl ketone as solvents. The use of TLL was optimized for these syntheses in preparative scale.

3.4. Other uses of TLL

TLL has found many other applications in some other different areas. Here we will summarize some of them.

3.4.1. Environmental applications

3.4.1.1. Degradation of polymers. The degradation of polymers is one of the most relevant environmental problems. TLL has been applied in many instances as catalyst of these reactions, in many instances comparing several lipases in the reaction.

The hydrolysis of the side chain of poly (vinyl acetate) in toluene at 60 °C catalyzed by several lipases was performed [246]. Longer side chains are hydrolyzed in the order hog-pancreas lipase > Novozyme 435 > TLL > *Candida rugosa* lipase whereas the short chains are hydrolyzed in the reverse order.

Another example is the study of the effect of various solvents on the biodegradation of poly (-caprolactone). The reaction was performed at 45 °C using two different lipases: Novozyme 435 and TLL. While the degradation rate decreased with viscosity, it increased with the polarity of the solvents. The inactivation rate was higher

using TLL than Novozyme 435 in non-aqueous solvents [247]. Both enzymes showed the maximum degradation of the polymers at an optimum value concentration of 8.7 wt.% of water in acetone.

Poly(*bis*phenol-A carbonate) was another polymer degraded by TLL. The reaction was performed in solution at various temperatures (26–70 °C) by different lipases, *Candida rugosa*, hog-pancreas, TLL and Novozyme 435 in various solvents [248]. The optimal temperatures for hog-pancreas lipase and other lipases were 50 and 60 °C, respectively. The overall degradability activity of the lipases was in the order of TLL > *Candida rugosa* > Novozyme 435 > hog-pancreas. The effect of viscosity and polarity of the solvents was the same than that shown for the degradation of poly-caprolactone [247].

In other instance, TLL and cutinases from *Thermobifida fusca* and *Fusarium solani* hydrolyzed poly(ethylene terephthalate) fabrics and films and *bis*(benzoyloxyethyl) terephthalate endo-wise [249]. Due to interfacial activation of the lipase in the presence of Triton X-100, a seven-fold increase of hydrolysis products released from 3PET was measured. In the presence of the plasticizer *N,N*-diethyl-2-phenylacetamide, increased hydrolysis rates of semi-crystalline poly(ethylene terephthalate) films and fabrics were measured both for lipase and cutinase.

The linear aromatic polyester poly(trimethylene terephthalate) was also treated with TLL and enzymes from *Penicillium citrinum*, *Thermobifida fusca* and *Fusarium solani pisi* [250]. The cutinase from *T. fusca* was found to release the highest amounts of hydrolysis products from the polymer and was able to open and hydrolyze a cyclic dimer. In contrast, TLL also showed activity on the polymer fibers and on *bis*(3-hydroxypropyl) terephthalate but was not able to hydrolyze the polymer film, mono(3-hydroxypropyl) terephthalate nor the cyclic dimers of poly(trimethylene terephthalate). Poly(D,L-lactide) [251] and poly(trimethylene carbonate) [252,253] may be also degraded by TLL.

Thus, TLL may find applications in the degradation of very different polymers that pose a real environmental problem.

3.4.1.2. Treatment of wastewaters from the meat industry. Wastewaters from the meat industry usually present high contents of oils and fats, which present low biodegradability. Enzymatic hydrolysis may contribute to increase the biodegradation of fatty wastewaters, accelerating the treatment process and decreasing the oxygen demand of the waters.

Wastewaters from a swine and bovine meat industry with high content of oil and grease were treated in batch anaerobic reactors with and without an enzymatic pretreatment [254]. Two different lipases were tested: TLL and lipase SEP. Although a high amount of enzyme maximizes hydrolysis of oil and grease, it decreases biodegradation, mainly when TLL is used, probably due to inhibition caused by excess of free acids. However, the benefits of the enzymatic prehydrolysis became evident when the wastewater was treated with only a 0.1% (w/v) of lipase. In this case, the performance of lipase SEP was better than TLL, which could be related to the lower activity of TLL in the wastewater, when compared to Lipase SEP. Later, in a paper focused on wastewater of the swine meat industry, the same authors reported that using lipase SEP, 100.1 μmol of free acid/mL could be released using 5.0% (w/v) enzyme at 45 °C, while TLL yielded 52.1 μmol of free acid/mL using the same enzyme amount, but at 37.5 °C [255].

3.4.2. Pretreatment of wool

The lipase pretreatment of wool has improved the dyeability of wool fabric with reactive dyes using TLL [256]. The treatments revealed improvement in the dyeability of wool with reactive dyes. The use of the enzyme pretreatment enables wool dyeing under mild temperature conditions with increasing dye consumption and an increase of the rate of dyeing.

3.4.3. Sensor of fat quality

Using lipase specificity, a microtiter plate method using TLL was developed to determine the quality of fats that are used in large-scale processing using lipase catalysis, following two approaches [257]. In the first approach, the fats were interesterified with *p*-nitrophenol laurate using TLL; in the second approach, pH indicators were added to the fat samples containing TLL. The results showed that the analytical method using *p*-nitrophenol as pH indicator allowed a rapid and reliable assignment of bad fats and an acceptable differentiation between fats of moderate and good quality.

3.4.4. Cleaning application

Lipase degradation of oils and grease makes the lipases to have a great interest in cleaning of any material, mixed or not with detergent, and TLL is not an exception of this application.

Thus, the effect of incorporating TLL into detergent formulas for washing fatty soils on hard surface was studied [258]. The results showed that, in the presence of lipases, soil removal was achieved by three consecutive mechanisms: (i) fundamental removal of the soil by the bath flow; (ii) emulsion of the soil in the washing medium; and (iii) enzymatic hydrolysis of the dispersed soil. The use of surfactant formulas with the lipolytic enzyme showed a positive effect of the enzyme on the detergency values registered with the fatty alcohol ethoxylate surfactants Findet® 10/15 and Findet 1214N/23, and with the anionic surfactant linear alkylbenzene sulfonate. However, the commercial surfactants Glucopon® 600, Glucopon 650, Findet 10/18, and Findet Q/21.5NF alone each presented high detergency values for fatty soils, and the effect of the incorporation of the lipase was not significant. The results could be studied later in terms of inhibition of the detergents of the TLL activity [259]. Non-ionic surfactants seem to prevent or delay enzyme penetration at the interface, thereby decreasing lipase activity. Notably, no inhibitory effect of the anionic surfactant on lipase action was found, higher conversions being achieved after 20 min of enzymatic hydrolysis in the presence of this surfactant than in its absence. Employing two different oily stains (tributyrin and triolein), it was found that the lipase by itself increases detergency significantly, preventing the subsequent re-deposition of the removed dirt.

4. Conclusions

From the results presented in this review, it is evident that TLL is a lipase that has been mainly used in oil and fat modification following very different strategies, perhaps because this was the first application of the enzyme. However, there are some examples concerning the use of the enzyme in other processes, like biodiesel manufacture, pure enantiomers or regioisomers production (mainly in sugars modifications), which suggest that the enzyme may have very good prospects of application in other areas of the chemistry. In fact, TLL is a very useful enzyme in all the processes commented in this paper, not as popular as those from yeast of the genus Candida, but in some instances with even better properties. Special interest has its high stability. Among the commented processes, TLL seems to offer some specific advantages for biodiesel production and for regioselective processes involving sugars.

The results from different research groups are in many cases heterogeneous and in some instances, contradictory. This may be due to the easy alteration of the lipase properties by any change in the biocatalysts preparation, experimental conditions o substrate nature [53].

Using soluble TLL, the fact that the enzyme tends to form bimolecular aggregates even at very low concentrations or to be adsorbed to any hydrophobic component of the crude extract must

be carefully considered. These aggregates have very different properties when compared to the monomeric form of the lipase. This makes evaluation of the enzyme very complex [45–49]. Mild immobilization protocols (ideally via just one-point and using long and inert spacer arms) [260] in the presence of detergents to break the dimers [45–49], may be one simple way to get monomeric and pseudo-native lipase structures, that may facilitate comparison between several lipases. The use of hydrophobic supports may be a good option to have interfacially activated and monomeric TLL molecules [36–38,45].

On the other hand, considering that lipases properties strongly depend on the immobilization protocol [53], and that TLL is quite sensitive to this [51], the evaluation of the enzyme as a catalyst for a determined process should include several different enzyme preparations. The comparison of results from different authors using different TLL preparations may not really reflect the properties of the TLL, but the TLL-support properties, which may be quite different. This may be extended to the comparison of TLL properties to those of other lipases.

The application of the new genetic tools, immobilization protocols [53] and chemical modification to TLL enzyme may open new opportunities for the tuning of the TLL properties, and that way increase the industrial use of this interesting enzyme.

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