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Review

Lipase from *Rhizomucor miehei* as a biocatalyst in fats and oils modification

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ABSTRACT

The lipase from *Rhizomucor miehei* (RML), formerly *Mucor miehei*, is a commercially available enzyme in both soluble and immobilized forms with very high activity and good stability under diverse experimental conditions (anhydrous organic solvents, supercritical fluids, etc.). The uses of the enzyme were initially oriented towards food industry, that way the enzyme has found a broad application in this area. This review intends to show the enzyme features and some of the most relevant aspects of the use of this interesting enzyme in oils and fats modification. The enzyme has been the first lipase whose structure was reported and its interfacial activation is very well known, making this enzyme a good template for modeling studies. Finally, the main uses of the enzyme in fatty acids, oils and fats modification will be revised (hydrolysis of glycerides, transesterification, esterification, acidolysis and interesterification). This lipase seems to be advantageous when compared to other lipases mainly in esterificaton reactions in anhydrous media, while other lipases can be preferred in transesterification reactions.

Contents

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1. Lipases as biocatalysts in the fats modification industry

The production of tailor-made foods is a demand of the twenty first century society. Concerning this, the modification of oils or fats of foods may greatly alter their nutritional properties. Some fatty acids (e.g., polyunsaturated fatty acids) [1–3] are recognized as nutraceuticals, some fatty acids allow the preparation of low-calorie foods [4], etc.

The modification of these foods with lipases presents several advantages: the specificity and selectivity of the lipases may produce oils with desired composition, and the amount of side products may be greatly decreased, facilitating the final downstream of the products [4]. A special case is when the biocatalyst becomes incorporated to the food. In these cases, enzymes may be the only alternative.

Lipases are among the most widely used enzymes in enzyme technology [5–8], because they recognize a wide variety of substrates and may catalyze many different reactions: hydrolysis or synthesis of esters bonds [9–11], alcoholysis [12], aminolysis [13–17], peroxidations [18–20], epoxidations [21–23], interesterification [24], etc. The apparent promiscuity of these enzymes make lipases useful in many different reactions, finding application in pharmaceuticals and drugs production [25–28], in the production of biodiesel [29–31] or in food modification [32–34].

The hydrolysis of fats and oils in aqueous medium (as emulsions or insoluble drops) by lipases follows a well-known mechanism: the so-called interfacial activation. In aqueous media, lipases have their active center secluded from the medium by a polypeptide chain called lid or flap [35–38]. The lid may be very small and simple, not fully isolating the active center of the enzyme in the closed form (e.g., this is the case of lipase B from *Candida antarctica* [39]) or, as in the case of the lipase from *Geobacillus thermocatenulatus*, to be a quite complex structure involving a large percentage of the amino acids of the enzyme and forming a double lid [40]. In the presence of the drop of oil [41,42], the lid moves to permit the interaction between its hydrophobic internal face and the hydrophobic residues that usually surround the lipase active center with this hydrophobic surface. Thus, the lipase becomes adsorbed on this hydrophobic surface, and the active center is exposed to the reaction medium (open form) [35–42]. Other hydrophobic surfaces may "mimic" these drops of oils, allowing the adsorption of the open form of the lipases via interfacial activation: a hydrophobic support [43–47], a hydrophobic protein [48] or even other "open" lipase molecule [49–53].

In this review, we will focus on the uses of the lipase from *Rhizomucor* (previously *Mucor*) *miehei* (RML) as catalyst in the modification of oils, fats or free fatty acids, while the uses of the enzyme in energy and organic chemical industries will be presented in another paper [54]. A review on the immobilization of the enzyme on different supports may be also found in the same paper [54].

1.1. Some properties of RML

This extracellular enzyme was first described in 1973 [55]. Few years later, a first use of the enzyme was described in food transformation. This paper showed that the mould produced an esterase able to attack a number of natural fats such as vegetable oils, beef tallow, and lard oil and a number of synthetic substrates including sorbitol esters of fatty acids [56]. In this first paper, the authors found a strong pH dependence of the substrate specificity of the lipase by the different fatty acids, at lower pH values shorter fatty acids were hydrolyzed better than at high pH values.

The native enzyme presented two different forms, differentiated only by a partial deglycosylation (the B lipase formed by partial deglycosylation of the A lipase) [57–59]. Later, the enzyme was expressed in *Aspergillus oryzae* [60]. The enzyme is currently commercially available from Novozymes in free form (Palatase 2000L) and in an immobilized form (Lipozyme RM IM). The support of the immobilized enzyme is Duolite ES 562, a weak anion-exchange resin based on phenol–formaldehyde copolymers [57,58,61]. In these first studies, it was reported that the immobilization altered the lipase specificity for different fatty acids [58].

RML was the first lipase whose structure was known (it was resolved at 1.9 Å resolution [38]) (Fig. 1). The molecular size of RML is 31,600 Da and its isoelectic point is 3.8 [62]. The structure revealed a Ser-His-Asp trypsin-like catalytic triad with an active serine buried under a short helical fragment of a long surface loop (the lid). The enzyme was described as a single polypeptide chain of 269 residues. It is folded into a singly wound β-sheet domain with predominantly parallel strands, connected by a variety of hairpins, loops and helical segments [38]. All the loops are right-handed, creating an uncommon situation in which the central sheet is asymmetric and all the connecting fragments are located on one side of the sheet. A single N-terminal α-helix provides the support for the other, distal, side of the sheet. Three disulfide bonds (residues 29–268, 40–43, 235–244) stabilize the molecule. There are four *cis*-peptide bonds, all of which precede a proline residue. In short, this enzyme consists of a six-residue beta-strand (strand 4 of the central sheet), a four-residue turn of 'type II' with serine in the epsilon conformation, and a buried α-helix packed in a parallel way against strands 4 and 5 of the central beta-pleated sheet [63]. One year later, the crystal structure (at 3 Å resolution) of a complex of RML with *n*-hexylphosphonate ethyl ester was presented. Now, the enzyme active site was exposed by the movement of the helical lid [64]. The authors proposed that the structure of the enzyme in this complex is equivalent to the activated state generated by the oil–water interface. After another year, the crystal structure of the enzyme inhibited irreversibly by diethyl *p*-nitrophenyl phosphate (E600) was solved by X-ray crystallographic methods and refined to a resolution of 2.65 Å [65]. The higher resolution allowed a detailed analysis of the changes observed in the inhibited enzyme. The movement

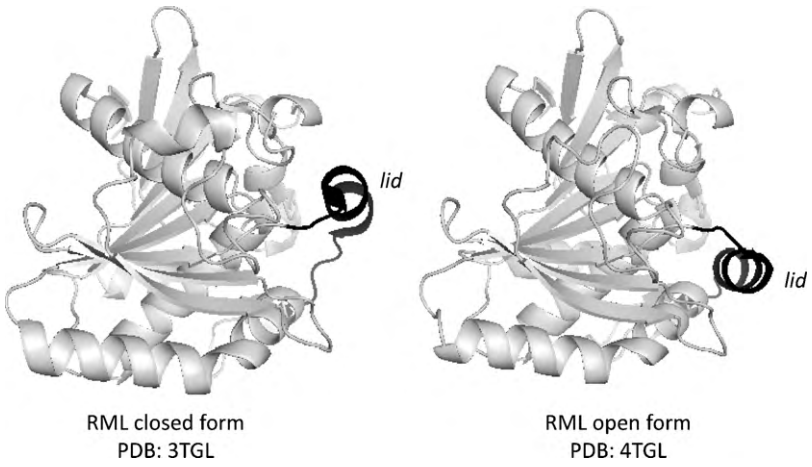

Fig. 1. Structure of open and closed forms of RML. The 3D structure was obtained from the Protein Data Bank (PDB) using Pymol version 0.99.

of a 15 amino acid long "lid" (residues 82–96) is a hinge-type rigid-body motion that transports a short α-helix (residues 85–91) by over 12 Å. There are two hinge regions (residues 83–84 and 91–95) within which pronounced transitions of secondary structure between α and β conformations are observed. As a result of this movement a hydrophobic area of 800 $Å^2$ (8% of the total molecule external surface) becomes exposed. A docking using trilaurylglycerol as template substrate led to a first model of the complex of RML/substrate [66].

Due to the large available information, RML interfacial activation is perhaps one of the most studied ones. Molecular dynamics simulations for the lid covering the active site of RML postulated that, among other interactions, Arg86 in the lid stabilized the open-lid conformation of the protein by multiple hydrogen bindings to the protein surface [67]. Experimentally, the implication of an Arg in stabilizing the active open-lid conformation of the enzyme was suggested when it was found an inhibition of RML by both the presence of structural analogs of the Arg side chain and the chemical modification of Arg residues [68]. The desolvation of Arg86 at the water–lipid interface plays a key role in the activation process, while the contribution from hydrophobic stabilization of the open form is less important [69]. Later, it was shown that electrostatic interactions of Arg86 play an important role in terms of both the intrinsic stability of the lid and its displacement, through enhancement of hinge mobility in a high dielectric medium [70]. In a further research, it was determined that Arg86 and Asp91 form the strongest electrostatic interactions with the rest of the protein [71]. Moreover, other key residues whose interactions with the lid are significantly perturbed by the change in the dielectric of the medium were identified: Asp61, Arg80, Lys109, Glu117 and the active-site residues Asp203 and Asp256, all of which lie within approximately 20 Å of the lid. In a later work, a functionally important electrostatic network was identified [72]. This network consists of residues belonging to the catalytic triad (Ser144, Asp203, His257), residues located in proximity to the active site (Tyr260), residues stabilizing the geometry of the active site (Tyr28, His143), and residues located in the lid (Asp91) or close to the first hinge (Arg80).

Later, many other studies were performed by molecular dynamics simulations using RML as model protein, e.g., explaining the interrelation of the ionic network [73], the effect of different solvents on the dynamics of RML [74], the relation between the structural changes in catalysis and RML selectivity or specificity [75,76], the effect of temperature in enzyme dynamics [77] or analysis of chain flexibility and fluctuations [78]. Thus, RML has become one of the most studied and best-known lipases [79].

These data make RML a good model to modify their properties by site-directed mutagenesis. For example, its chain length specificity was altered by substituting Phe94 in the protein groove, which is responsible for accommodating the acyl chain of the substrate [80,81].

The stability of RML has been analyzed under different conditions, e.g., different temperature and pressure [82,83]. It has been shown that, under non-denaturing pressure conditions, pressurization protects RML against thermal deactivation [84]; this may be relevant when using the enzyme in supercritical fluids (see below). At denaturing pressure conditions, polyols stabilized the enzyme [82]. Other studies focused on the properties of the lipase in organic medium. RML is a quite active and stable lipase in systems with low water activity. Comparison of the sequences of RML with the homologous lipases from *R. niveus* and *Humicola* sp. suggests that changes in charged residues in the 'hinge and lid' region of the structure may play a significant role in the low a_w tolerance of this enzyme [85].

Some additives may be relevant for the management of RML. RML is activated by some detergents, even in organic media [86]. The detergents sodium cholate, Tween 80 or Tween 20 are possible activators of the enzyme, increasing RML activity in organic media 2–2.5 times. In contrast, RML activity was inhibited in the presence of Brij 58 (other non-ionic detergent) or the cationic detergent cetyltrimethylammonium bromide (this was attributed to the inactivation of the enzyme by these detergents). Some studies trying to show the interactions of RML with detergents were performed using the amphoteric surfactant sodium *N*-(2-hydroxydodecyl)sarcosinate, whose charge may be controlled by adjusting the pH [87]. It was concluded that only when the head group is positively charged the enzyme binds with the surfactant. However, the number of surfactant molecules that bind to each enzyme was found to be dependent on the surfactant structure. It was suggested that below the critical micelle concentration of the detergent, binding occurs with negatively charged sites on the enzyme interacting with cationic surfactant head groups, with some enhanced adsorption by interaction between a hydrophobic domain adjacent to the negatively charged site of the enzyme and the tail of the cationic surfactant. Later, another study confirmed that cationic surfactants form a complex with RML over a broad pH range (even below its isoelectric point). No such interactions were found for neither anionic nor non-ionic surfactants [88]. The interaction between cationic surfactant and RML leads to a reduction of reaction rate in hydrolysis of a palm oil. The authors propose that anionic and non-ionic surfactants with bulky hydrophobic tails (to prevent the RML recognition as substrates) are the preferred

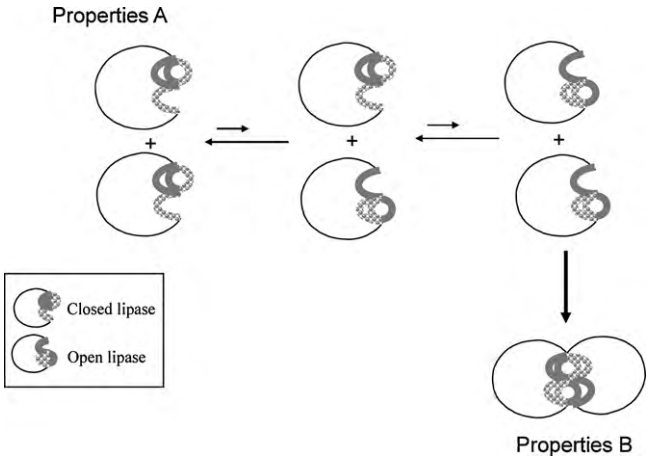

Fig. 2. Lipase dimers formation by interaction between two open molecules of RML.

surfactants for microemulsion-based reactions using RML as catalyst.

Interactions between RML and emulsion droplets were studied in oil-in-water emulsions [89]. The emulsions were stabilized by either the cationic surfactant didodecyldimethylammonium bromide, the anionic surfactant sodium *bis*(2-ethylhexyl)sulfosuccinate or the non-ionic surfactant 1-(2-ethylhexyl)-3-(2-ethylbutyl)-2-hexaethylene glycerol ether. It was shown that RML bound to droplets stabilized by the cationic surfactant, but not to those stabilized by the negatively charged or non-ionic surfactant. The lipase-catalyzed hydrolysis of *o*-nitrophenyl palmitate was studied in emulsions stabilized by the different surfactants. The highest reaction rate was observed in systems with the non-ionic surfactant and also with the cationic surfactant, while in the anionic system almost no reaction was observed. RML prefers positively charged surfaces than negatively charged surfaces to become adsorbed [89].

Considering that RML tends to form bimolecular aggregates with reduced activity (Fig. 2) and that the presence of detergents may break these dimers [49–53] (Fig. 3), this is another effect of detergents that needs to be considered when they are used in reactions catalyzed with this enzyme.

2. Use of RML in foods for oil modifications

In some cases, RML has been added directly to the foods to modify them. In these cases, the enzyme is incorporated to the foodstuff. Therefore, many studies devoted to analyze the safety in the utilization of RML in foods have been performed. Initial studies using the enzyme expressed in *R. miehei* showed no effect when it was supplied 1 g/kg body weight/day for rats or 4 g/kg body weight/day for dogs. There was no evidence of teratogenic or mutagenic potential and no effect on fertility and general reproductive performance [90]. Later, the studies used the enzyme preparation expressed in *Aspergillus*. The recombinant enzyme is also described as non-mutagenic, and with no effect if feeding rats at a concentration lower than 1600 mg/kg. At higher concentrations, there were effects upon food intake and even minor kidney damage [91]. At 40000 mg/kg diet the enzyme was considered to have exacerbated the onset of normally occurring chronic myocarditis in male Sprague–Dawley rats. Thus, the enzyme was considered adequate to be used in human foods.

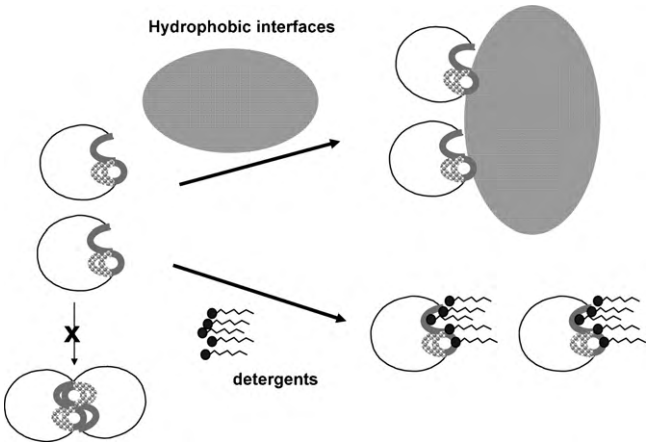

Fig. 3. Different ways of breaking RML dimers.

The first report of the use of RML as catalyst was in the hydrolysis of animal fat and vegetable oil to improve the characteristic flavor of Italian cheese when the enzyme was added to the milk [56]. In this paper, RML is classified as an esterase. The enzyme is more specific for triglycerides containing low molecular weight fatty acid at pH 5.3 than at pH 8.0.

Much later, RML and a protease were used to improve the production of highly flavored cheese-like hydrolyzates from fluid milk with a high concentration of volatile compounds [92].

In another paper, RML encapsulated in liposomes were incorporated into cheese milk [93]. Treated cheeses had higher moisture and lower protein, fat and ash contents than the control cheese. Moreover they were less firm, but more elastic and cohesive, than the control cheese. Under proper conditions, milk fat had slightly better flavor intensities than control cheese. The use of an excess of RML produced cheeses with a pronounced soapy off-flavor after 2 and 3 months of ripening.

Later, cheddar cheese proteolysis and lipolysis were accelerated using liposome-encapsulated enzymatic cocktails (RML and flavourzyme—a commercial protease) [94]. Certain enzyme treatments resulted in cheeses with more mature texture and higher flavor intensity in a shorter time compared with control cheeses.

RML has been also used in the preparation of different sausages. The enzyme significantly increases all free fatty acids analyzed, which were proportionally lower for linolenic acid and linoleic acid. The final juiciness and taste of the product were slightly better [95–97]. The simultaneous use of RML and a protease produced an increase of many free amino acids, and significant increases in palmitic, oleic and linolenic acids [98]. Despite the observed changes, no differences were found in the sensory quality compared with the control, except for a slight softening.

RML also was used to increase the formation of flavor volatiles in black tea, which contributed to its aroma [99].

3. Oil hydrolyses catalyzed by RML

The hydrolysis of oils is the natural function of lipases, for this reason some applications of RML in this reaction were reported in the literature and are reviewed here.

In some instances, the partial hydrolysis of the glycerides in the oil may allow the modification of its specific properties (Fig. 4). For example, commercially available epoxidized soybean oil has a certain percentage of saturated fatty acids, which produces branches when it is used to produce polymers. The removal of these fatty acids creates a potentially valuable degree of freedom in the processing of soybean oil for polymer synthesis applications. RML selectively hydrolyzed saturated fatty acids in soybean oil but the lipase was not selective towards the epoxidized soybean oil [100].

Hydrolysis of triglycerides is an essential reaction for the production of fatty acids or special glycerides. Although RML is a

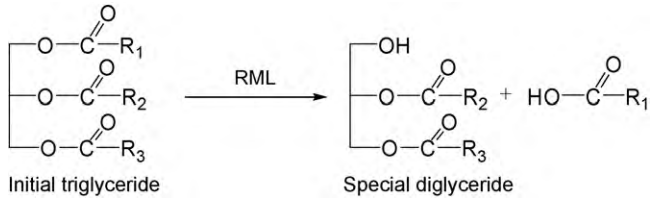

Fig. 4. General scheme of the production of special diglycerides via hydrolysis of triglycerides catalyzed by RML.

sn-1,3-specific lipase, the spontaneous acyl migration from position 2 to positions 1 or 3 allows the full hydrolysis of the triglycerides (Fig. 5). The usual method of hydrolysis of oils to fatty acids and glycerol uses high temperature and pressure [101]. With these extreme conditions, polymerization of the oil and by-product formation takes place resulting in a dark colored fatty acid. Enzymatic hydrolysis is a good alternative to overcome these disadvantages as the use of enzymes for the hydrolysis not only gives colorless pure products but also reduces the by-product formation, due to enzyme specificity. In other cases, the target of the reaction is the production of di- or monoglycerides with a special composition.

The 1,3-specificity of RML causes that the first hydrolytic product to be a chiral diglyceride. Using analytical techniques that allowed the resolution of these chiral diacylglycerols, RML has been found to preferentially hydrolyze the ester bond at position *sn*-1 versus hydrolysis in position *sn*-3 [102].

These hydrolyses have been performed in a wide range of reactors and reaction media. RML immobilized by adsorption on microporous, asymmetric hollow fiber membrane reactors, was used to hydrolyze palm and olive oils [103]. Experimental results were fitted to a multi-substrate kinetic model derived from the Ping-Pong Bi-Bi mechanism.

Hydrolysis of peanut oil using RML in aqueous media has been also successfully performed [104]. RML has been used to hydrolyze triglycerides in an organic solvent system too [105]. The presence of secondary amines, i.e., diethylamine, *N*-methylbutylamine, or the tertiary amine, methylamine, greatly increased the extent of hydrolysis, very likely by facilitating the migration of the acyl moiety in position 2. In other paper, the hydrolytic activity of RML in aerosol-OT-stabilized water-in-oil microemulsions in *n*-heptane was studied [106]. The pH/activity curve gave a bell-shaped profile with a maximum activity at pH 7.5, similar to that reported for a purified RML in a tributyrin-based aqueous emulsion assays [107,108]. RML hydrolysis of canola oil in supercritical carbon dioxide has been also reported [109]. There was a drop in triglyceride conversion over a 24-h reaction time at 38 MPa and 55 °C, which may be an indication of loss of enzyme activity. On-line extraction-reaction process using supercritical CO_2 shows great potential for new process design to obtain products from agricultural commodities for use as ingredients in food and other industries [110]. In a further paper, the critical role of the water content in this reaction medium was assessed [111]. Lipozyme RM IM was also used in the hydrolysis of blackcurrant oil in this media [112]. It was found that lipozyme displayed specificity towards linolenic acids; the release of α-linolenic acid was the fastest while that of γ-linolenic acid was slower than the release of other constituent acids present in blackcurrant oil. In a further paper, it was found that the use of Lipozyme RM IM on these supercritical fluids allowed having a more active catalyst [113].

Thus Lipozyme RM IM has been used in many instances and with good properties in this reaction, using its specificity to liberate target fat acids. Its activity and stability in not conventional media is one of the advantages of the enzyme even in this hydrolytic reaction.

3.1. Production of special glycerides via hydrolysis of triglycerides

In some of the first papers on the use of this lipase, the hydrolysis of oils catalyzed by this enzyme was proposed to have a docosahexaenoic acid-enriched fraction of glycerides [114,109].

Later, RML has been used to improve the content of polyunsaturated fatty acid in the glyceride fraction [115–119]. RML released polyunsaturated fatty acid extremely slowly, resulting in their accumulation in the triglyceride fraction. Less than 2% of the total amount of polyunsaturated fatty acids was lost in the fatty acid fraction [115]. The contents of γ-linolenic acid [117], and C18 fatty acids are enriched in the acylglycerol fraction while substantially high levels of erucic acid and other very-long-chain monounsaturated fatty acids were hydrolyzed [118]. Partial hydrolysis using Lipozyme RM IM in a solvent-free system was also used to produce a diacylglycerol-enriched palm olein (a yield of 32 wt.% was obtained) [119].

The enzymatic deacylation of l,2-diacyl-*sn*-glycero-3-phosphocholines to *sn*-glycerol-3-phosphocholine catalyzed by Lipozyme RM IM in a microemulsion system has been also reported [120].

3.2. Production of special free fatty acids

The production of hydroxy fatty acids (lesquerolic and auricolic) via 1,3-specific lipolysis of *Lesquerella fendleri* oil in several reaction systems and catalyzed by RML has been reported in [121]. Of the 35% free fatty acids obtained after partial hydrolysis, 75–80% are hydroxy acids [122].

The RML-catalyzed hydrolysis of blackcurrant (*Ribes nigrum*) oil was performed in mixtures of isooctane and phosphate buffer at 30 °C, displaying specificity towards α-linolenic acid and γ-linolenic acid [123]. Later, the authors carried out this reaction in supercritical CO_2 [124]. Compared to oil, the liberated fatty acids

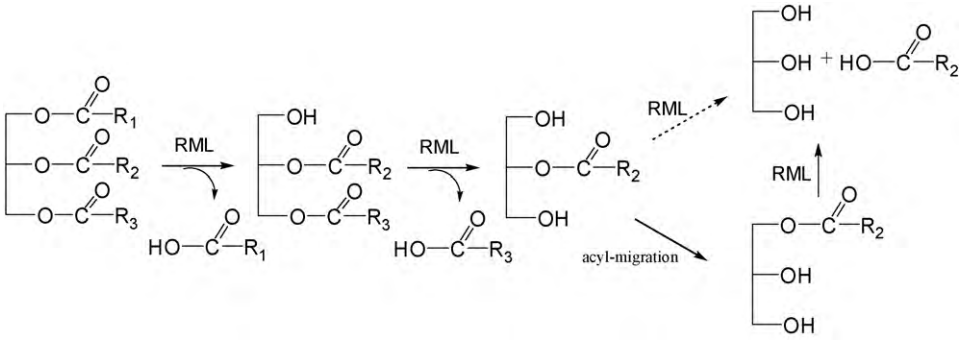

Fig. 5. General scheme of the full hydrolysis of triglycerides catalyzed by RML to produce free fatty acids.

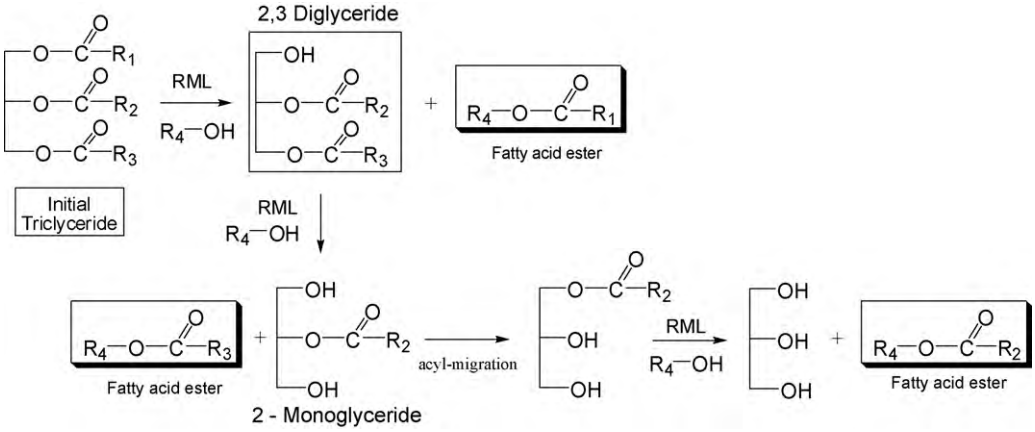

Fig. 6. General scheme of the alcoholysis or transesterification of triglycerides catalyzed by RML to produce esters of fatty acids or special glycerides.

contained more α-linolenic, palmitic and stearic acids, while di- and monoacylglycerols contained increased levels of γ-linolenic and stearidonic acids. Moreover, high erucic acid seed oil has been hydrolyzed by RML to produce erucic acid, a valuable industrial feedstock [125].

4. Transesterifications or alcoholysis of glycerides

In some cases, instead of using water as nucleophile to produce free fatty acids, an alcohol is used to produce the corresponding ester (Fig. 6). In some cases the target may be to produce partially deacylated glycerides, in other cases the target will be the produced esters.

Among the several uses of RML in transesterification the main application is to obtain 2-monoglycerides. As RML is a specific *sn*-1,3 lipase the reaction can be easily performed. Ethanol is the most common alcohol used in this reaction [126–130]. However, methanol [127,131] and glycerol [132,133] has also been tested. One of the major problems to get good results in alcoholysis is the presence of water. With the increased addition of water, the amount of water available for oil to form oil–water droplets increases, thereby increasing the available interfacial area where lipase may act, but high concentrations of water could led to hydrolysis. In absence of free water or in low concentrations, RML exhibited no ethanolysis activity, at 7–9 wt.% of water shows its best performance [126]. Reaction was carried out in the presence of solvent or in solvent-free systems, and was fully 1,3-specific in reactions when diethyl ether was used as a solvent [127]. An interesting application of production of 2-monoglycerides was reported by Turner et al. [131]. The authors firstly produced 2-monoricinolein from RML-catalyzed methanolysis of triricinolein, and after that, esterified this product using ricinoleic acid as the acyl donor to obtain, 1,2(2,3)-diricinolein.

Stereoselective ethanolysis of trioctanoylglycerol by RML was studied for the preparation of optically pure *sn*-2,3-dioctanoylglycerols [134]. The results indicated that RML was highly stereoselective toward the *sn*-1-position (enantiomeric excess >99%) The larger the amount of ethanol was, the higher the stereoselectivity became. After optimizing the reaction, optically pure *sn*-2,3-diacylglycerols were obtained at 61.1 mol% in the glyceride fraction. When fatty acids longer than decanoic acid were used, the yields and enantiomeric purities of 2,3-diacylglycerols were dramatically reduced [134].

RML-catalyzed alcoholysis of soy phospholipids with different alcohols was used to simultaneously make lysophospholipids and fatty acid esters [135]. Lysophospholipids (in 69–78% molar yield) were obtained from soy phospholipids, and the yields of esters of various alcohols nearly agreed with theoretical yields.

Some trials of separation of eicosapentaenoic acid and docosahexaenoic acid may be found. Ethyl eicosapentaenoate obtained from tuna oil was reduced and the ethyl docosahexaenoate was increased by selective alcoholysis of ethyl esters with lauryl using RML [136]. Similar process was performed by the transesterification of various fish oil triglycerides or esterification of their free fatty acids with a stoichiometric amount of ethanol by RML under anhydrous solvent-free conditions, resulting in a good separation of both acids, although the use of the free fatty acids instead of the triglycerides greatly improved the results [137]. This was because it avoided complications related to regioselectivity of RML and non-homogeneous distribution of eicosapentaenoic acid and docosahexaenoic acid into the various positions of the triglycerides. In other paper, ethanolysis of squid oil catalyzed by RML produced ethyl eicosapentaenoate and ethyl docosahexaenoate from the first stages of the reaction [138].

Synthesis of esters with long-chain alcohols, the so-called wax esters, catalyzed by RML was the objective of several researches [139–142]. RML presented a good performance in this reaction in both solvent-free medium (86.8–99.2%, yield depending on the alcohol) [139] and in the presence of *n*-hexane (approximately 95% yield) [130]. The advantage of use long-chain alcohols is that these alcohols present a better solubility in oils and fats and this way the reaction can be successfully conducted in solvent-free system.

5. Esterification of fatty acids in oils or fats

This reaction is the inverse of the "natural" reaction of lipases in nature. The reaction consists in the direct condensation of a free fatty acid and an alcohol, that may be glycerin, a mono- or diglyceride, or other alcohol (Fig. 7). One of the experimental conditions with higher impact in this reaction is the water activity, because water is one of the products of the reaction and must be removed somehow to shift the thermodynamic equilibrium towards the synthesis. Thus, water control along the reaction has been described to be one of the main problems of this reaction. It was found that the water activity could be lowered to extreme values to favor the synthesis, without any sacrifice on the productivity of the process, using RML [143]. Esterification may be used to obtain desired glycerides or to reduce the acidity of an acid oil [144] (Fig. 7). As in the case of the hydrolysis, the use of enzymes in esterification may have some advantages compared to chemical processes, as the lower production of side products and less purification steps. The enzymatic and autocatalytic esterifications of free fatty acids and monoglycerides and diglycerides in rice bran oil, palm oil and

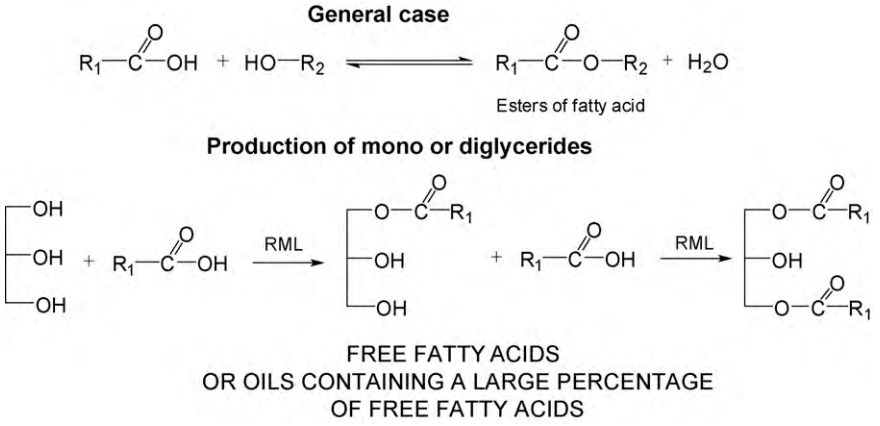

Fig. 7. General scheme of esterification of fatty acids and alcohols (e.g., glycerol) catalyzed by RML.

palm kernel oil have been compared [145]. The enzymatic process was more effective in reducing free fatty acids and produced less saponifiable matter and color in the final product. To produce edible-grade oil, a single deodorization step would be required after enzymatic esterification; whereas, alkali refining, bleaching, and deodorization would be required after autocatalytic treatment.

RML is defined in hydrolysis as *sn*-1,3-specific, but some studies are related to the selectivity of RML in esterification reactions by positions 1 and 3. In a first paper it was reported that RML has *sn*-1 stereopreference [146]. However, using a new HPLC-based method for separation of isomers of monoacylglycerol, and many different fatty acids, RML was found to have *sn*-3 preference in this reaction, with 16.6–65.5% ee [147]. The stereopreference increased as the carbon number decreased with an exception for 14:0, for which RML showed the lowest preference. In addition, higher enantiomeric purity was observed using *t*-amyl alcohol as reaction medium compared to *t*-butanol. The enzymatic esterification of *rac*-1-*O*-octadecylglycerol with oleic acid in hexane catalyzed by RML has been also reported. *X*-1(3)-*O*-octadecylmonooleoylglycerols were the only products formed with a slight stereoselectivity for the *sn*-1-position [148]. Thus, preference for 1 or 3-positions seems to be not very high and depended on many variables, giving different results from different groups.

RML specificity in esterification by different fatty acids has also been the subject of some studies. For example, RML specificity was assessed in multi-competitive esterification reaction mixtures containing a homologous series of *n*-chain even carbon number fatty acid (C4–C18) and a single alcohol cosubstrate [149–152]. Reaction was conduced in different reaction mediums as *tert*-butyl methyl ether [149,150] and hexane [151,152]. As alcoholic substrates glycerol, 1,2-propanediol, or 1,3-propanediol [149,150] and glycerol, or α or β-monoglycerides containing C4, C10, C16, or C18:1 [151,152] were tested. The ordinal patterns of fatty acids selectivities observed were; with glycerol, C8 > C12 > C10, C14 > other fatty acids, with 1,2-propanediol, and 1,3-propanediol, C8 > C12 > other fatty acids. The water activity in the reaction can alter this enzyme specificity [150]. Similar patterns of broad fatty acids RML selectivity toward C8–C18 were generally observed for esterification into specific acylglycerol pools with the different α/β-CX-monoglycerides cosubstrates. Exceptions were the enrichment of C18 in the monoglyceride pool with α-C16-monoglyceride substrate, a general suppression of C4/C6 fatty acids reactivity and a specific discrimination toward >C8 fatty acids incorporation into the triglyceride pool, both for reactions with α-C10- and α-C16-monoglycerides [152,152].

Esterifications of *n*-butanol and several unsaturated fatty acids have been the subject of several manuscripts [153–156]. A strong discrimination against unsaturated fatty acids having a *cis*-4, *cis*-6 or a *cis*-8 unsaturation as substrates for esterification was observed compared to the other fatty acids, such as myristic acid (14:0) and *n*-3 18:3 [153,155]. Other fatty acids having unusual structures, e.g., cyclopentenyl (hydnocarpic and chaulmoogric), hydroxy (ricinoleic and 12-hydroxystearic) and epoxy (*cis*- and *trans*-9,10-epoxystearic) acids have been also analyzed [153] and cyclopentenyl, hydroxy and epoxy acids were very well accepted as substrates. The esterification of *cis* or *trans*-9-octadecanoic acid (oleic and elaidic acid respectively) with *n*-butanol in *n*-hexane shows that RML-catalyzed the esterification of oleic acid 3–4 times faster than the corresponding reaction with elaidic acid [154]. When the esterification between glycerol and free fatty acids was carried out in solvent-free system [156], long-chain fatty acids were esterified at a higher rate than medium-chain ones. Furthermore, it was found that the higher the number of unsaturations, the lower the rate of synthesis and the lower the final yield.

The specificity of RML in esterification reaction between various alcohols (*n*-propanol, *n*-butanol, isoamyl alcohol, *n*-hexanol, *n*-octanol, 2-ethylhexanol, *n*-decanol, and lauryl alcohol) with lauric and oleic acid has been reported in another paper [157]. Higher reaction rates were obtained with oleic acid than with lauric acid. A bimodal distribution pattern was observed for the reaction rate as a function of alcohol chain length. Two superimposed "bells" were obtained with maxima at C4 (butanol) and C10 (decanol). Using supercritical fluids, a similar influence of the rates on the alcohol chain length was found [158]. The highest rate and maximal conversion were near to the critical point of carbon dioxide.

Next, a survey of the main esterification reactions performed with RML will be presented.

5.1. Production of oleic acid glycerides

In some cases, the target of the reaction is to fully acylated glycerin, while in other cases the target is to prepare mono- or diglycerides as healthier foods or as a first step in the production of structured lipids. RML can produce triolein because the ester enzymatically formed with the primary alcohols isomerizes, through acyl migration, to an ester on the secondary hydroxyl [159]. It was found that oleic acid reacts with monooleoylglycerols predominantly by esterification, with dioleoylglycerols to a greater extent by acidolysis, and with trioleoylglycerol almost exclusively by acidolysis at the *sn*-1,3-positions (see below for the description of acidolysis) [160].

The control of water activity during this reaction has been the subject of many papers [161–164]. Molecular sieves [161], and some batch reactors using different means of removing water, as

high temperature, vacuum, or dry air bubbling [162], showed good results. Moreover, solvent hydrophobicity also affected the glyceride composition during the lipase-catalyzed esterification [164]. Other important point in this reaction is the molar ratio of substrates. When the synthesis of triglycerides from oleic acid and glycerol was performed in stoichiometric amounts [161,162] 90% yields of triolein were obtained. However, varying the ratio of glycerol over oleic acid allows for the preferential synthesis of one of the glycerides [162].

Besides triolein, RML was used to prepare di- and monoacylglycerols from oleic acid and glycerol [165,166]. In both cases, triacylglycerol may be considered as an undesired side product of the reaction. The relation of all kinds of glycerides in the composition of the final esterification of glycerin and free fatty acids was optimized and scaled in a pilot packed bed enzyme reactor [165]. Lipozyme RM IM displayed higher selectivity toward the production of oleic acid-enriched diglyceride. An increase in solvent polarity, using mixtures of *n*-hexane and 2-methyl-2-butanol, improves drastically the selectivity toward monoglyceride formation, from 6 molar % of the total products at the thermodynamic equilibrium to 94% [166].

RML has been also used in the synthesis of acetone glycerol acyl ester from acetone glycerol and fatty acid, which may be the first step for some routes of monoglyceride production [167]. Using an excess of oleic acid and removing water, the conversion degree was almost 100%.

5.2. Esterification of oleic acid

The use of enzyme in these reactions permits to use the product as a "green" one, with the relevance that this has to use them in different industries. RML-catalyzed esterification of oleic acid and octanol (useful in cosmetic, lubricant and pharmaceutical industries) has been the subject of many studies in different systems [168–172]. The enzymatic synthesis of *n*-octyl oleate by direct RML-catalyzed esterification of oleic acid and 1-octanol was investigated in a stirred-batch reactor in solvent-free system, supercritical carbon dioxide and *n*-hexane [170], dense carbon dioxide [171] and packed bed bioreactor [172].

Esterification between oleic acid and oleyl alcohol catalyzed by Lipozyme RM IM has been also performed. The reaction was carried out in a batch-stirred tank reactor with supercritical carbon dioxide as solvent. This medium allowed higher reaction rates at supercritical conditions than in the solvent-free system [173].

The synthesis of *n*-butyl oleate has been the target of many other papers [174–177]. The reaction appeared to proceed via a Ping-Pong bi-bi mechanism with 1-butanol inhibition when a biphasic system was studied in a batch reactor [177]. RML-catalyzed esterification of oleic acid ethanol [178–183] was performed in supercritical CO_2, with similar results [179–181] to those obtained in *n*-hexane [178]. Afterward, a biphasic system was employed in this reaction using free or immobilized RML. The authors found that using Lipozyme RM IM, a higher affinity of RML by oleic acid could be found when compared to the soluble enzyme [182].

5.3. Esterification of polyunsaturated fatty acids

Many studies have been performed using conjugated linoleic acids as substrates for RML-catalyzed esterifications [184–190]. The reaction was carried out using glycerol [184,188,190], *sn*-1,3-diacylglycerols [185,186] and butanol [188]. When glycerol was used, diacylglycerols containing conjugated linoleic acid were mainly synthesized. RML incorporated the original conjugated linoleic acid as acylglycerol residues in primarily 1,3-diacylglycerol and 1-monoacylglycerol [188]. The esterification of isomers of conjugated linoleic acid with *sn*-1,3-diacylglycerols catalyzed by Lipozyme RM IM allowed to produce structured triacylglycerols using different media (hexane, isooctane and solvent free). Isooctane was the best medium, with yields near to 50% [185,186]. A blend of two RML and a lipase from *Alcaligenes* sp. was used in this reaction [190]. The content of triglycerides containing conjugated linoleic acids reached 82–83%. Using this biocatalysts mixture, the reaction rate was accelerated by 3, compared to using RML alone.

RML strongly discriminated against γ-linolenic and docosahexaenoic acids. Utilizing this property, γ-linolenic acid contained in fatty acids of evening primrose oil has been enriched seven to ninefold, and four- to fivefold in cod liver oil by selective esterification of the other fatty acids with butanol [191]. As long as the reaction is stopped before reaching equilibrium, very little of either γ-linolenic acid or docosahexaenoic acid were converted to butyl esters, which results in high yields of these acids in the non-esterified fatty acid fraction [191]. Later, it was established that RML preferred fatty acids and methyl esters with a (first) *cis* double bond in Δ9-position [192]. In another study, the substrate selectivity of RML was examined in the esterification of the conjugated linoleic acid isomers *cis*-9, *trans*-11-, *cis*-9, *cis*-11-, *trans*-9, *trans*-11 and *trans*-10, *cis*-12-octadecadienoic acid with *n*-butanol in *n*-hexane. The enzyme had a preference for the *cis*-9, *trans*-11-octadecadienoic acid [193].

RML has been used in the synthesis, esterification, and separation of *n*-3 polyunsaturated fatty acids. Synthesis of glycerides from glycerol and *n*-3 polyunsaturated fatty acids has been reported in isooctane and hexane [193]. Glycerides containing predominantly eicosapentaenoic acid and docosahexaenoic acid can be easily synthesized. More than 95% of polyunsaturated acids or their esters were converted to triacylglycerol by esterification or transesterification catalyzed by RML [194].

Fish oils generally contain high concentrations of *n*-3 polyunsaturated fatty acids, and this way, some studies were performed to esterify its fatty acids to produce oil-enriched in *n*-3 polyunsaturated fatty acids [195–197]. Using RML docosahexaenoic acid was recovered in the residual free fatty acids while eicosapentaenoic acid was recovered in the acylglycerol fraction [195,196]. It was found that different immobilization protocols can alter RML specificity [198].

Using Lipozyme RM IM, 1,2- isopropylidene glycerol and *n*-3 polyunsaturated fatty acid concentrate, yields of 80% monoglyceride were attained [199]. The resultant monoglyceride contained 43.3% eicosapentaenoic acid and 32.7% docosahexaenoic acid.

5.4. Esterification of lauric acid

Monolaurin and dilaurin were syhthesized by the reaction between lauric acid and glycerol in a foam reactor [200]. Some esters with other alcohols have been also produced, like hexyl laurate (a fruity flavor and an important emollient for cosmetic applications) in a solvent-free system with a yield over 90% [201,202]. Later, the same researchers used hexane as reaction media and a continuous packed bed reactor [203]. The use of a continuous packed bed reactor and a solvent-free reaction was the subject of a further paper [204]. Butyl laurate has been also produced using Lipozyme RM IM via direct esterification in a biphasic system [205]. The enzyme was inactivated by the butanol in the aqueous phase.

5.5. Synthesis of other glycerides

Mono-, di- and triacylglycerols are prepared by esterification between different fatty acids with alcohols, mainly glycerol. Because the *sn*-1,3-specificity of RML, diglycerides are largely produced from glycerol and fatty acids, such as a mixture of oleic and linoleic acids [206], fatty acids from palm oil deodorizer distillate [207,208], fatty acids from corn oil deodorizer distillate [209], fatty

acids from rapeseed oil [210] or palmitate [211]. In some cases triglycerides were synthesized, when free fatty acid, monoacylglycerol and diacylglycerol in high-free fatty acid rice bran oil were continuously esterified with glycerol to form triacylglycerol, using Lipozyme RM IM [212]. Mono-, di-, and tri-caprin were synthesized in isooctane from glycerol and capric acid, in an esterification reaction catalyzed by RML [213]. Later, the same authors found a non-very strict 1,3 selectivity for the enzyme in this reaction [214]. In another paper, tricaprylin was synthesized in a medium solely composed of glycerol and caprylic acid in stoichiometric amounts with air ventilation in all open batches to eliminate the produced water [215]. Apart from glycerol, 1,3-propanediol or 1,2-propanediol were used in the esterification of undecanoic acid using Lipozyme RM IM as catalyst in organic solvents [216]. As solvent apolarity increased, the extent of acylation of glycerol in the final product mixture also increased.

It is important to remark that in almost all cases the reactions conditions were different and particular for each case, and this makes difficult a comparison of the results since the enzyme presents different activities and selectivities depending of its concentration, the reaction medium, pH, temperature and other parameters, that may affect enzyme properties and acyl migrations.

Regioisomerically pure 1,3-*sn*-diacylglycerols are conveniently prepared in high yields (>80%) and in large quantities by enzymatic esterification of glycerol catalyzed by RML in the presence of *n*-hexane, diethyl ether or *t*-BuOMe [217]. In a further paper, the same authors prepared regioisomerically pure 1(3)-rac-monoacylglycerols using RML, in high yields (>75%) using free fatty acids, fatty acid alkyl esters, vinyl esters and triacylglycerols, as well as natural fats and oils [218].

The synthesis of 1-stearoyl-3(2)-oleoyl glycerol-enriched diglyceride was the subject of another publication [219]. After optimization, 51 wt.% diglyceride and 22 wt.% triglyceride were achieved.

5.6. Esterification of other fatty acids and alcohols

Free fatty acids from soybean oil deodorizer distillate were esterified with ethanol [220] and butanol [221]. In the first case [220], reaction was performed to facilitate the purification of tocopherol by distillation, and no significant losses of this compound were observed during the process.

Wax esters were produced from fatty alcohols and uncommon fatty acids by esterification catalyzed by RML [222] and from oleic acid esterification with 1-dodecanol [223].

5.7. Esterifications using microwave irradiation

Acceleration of reactions using microwaves has been observed for a wide range of organic reactions [224–226]. It should be considered that the microwave heating process is fundamentally different from the heating process used conventionally. Microwave irradiation has an effect on enzyme activity as a function of enzyme hydration [227]. These changes result from material–wave interactions leading to thermal effects (connected to dipolar and charge space polarization) and specific (purely non-thermal) effects [228].

This reaction system has been applied to the enzymatic esterification between *n*-alcohol homologues and *n*-caprylic acid catalyzed by Lipozyme RM IM [229]. *n*-Alcohol homologues used in this experiment showed substrate specificity in terms of the odd and even carbon numbers. THF expressed abnormal solvent effect. However, in the enzymatic esterification by conventional heating, the above mentioned substrate specificity and solvent effect were not observed. Microwave irradiation reduced the apparent activation energy of the enzymatic reaction.

Later, the effect of consecutive microwave irradiation on the RML-catalyzed esterification of pentanol with caprylic acid was found to increase the enzyme affinity to pentanol [230]. A pentanol inhibition under microwave irradiation was observed if the pentanol concentration was over 0.75 mol/L and caprylic acid concentration was over 0.25 mol/L, which did not happen in the conventional heating reaction.

6. Interesterification

Interesterification may be realized using a blend composed by several oils, just one oil having different fatty acids or one oil and determined esters (Fig. 8). It is one of the most widely employed strategies to produce structured lipids using RML. In the case of glycerides, if we want to introduce 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 replacing fatty acid (that needs to be also released from the ester) [24].

The Lipozyme RM IM-catalyzed interesterification of menhaden oil and ethyl ester of conjugated linoleic acid can be adjusted to a Ping-Pong Bi-Bi mechanism [231]. Both lower acylglycerols and ethanol are intermediates that are formed in the mechanism associated with hydrolysis of a triacylglycerol or the ethyl ester, respectively. However, diacylglycerols are present at low concentrations and monoacylglycerols and glycerol are absent during the reaction.

The use of enzymatic interesterification presents the usual advantages of selective and mild modification versus non-selective modification of the chemical methods: more control of the reaction products features, less side products, etc. A comparison of enzymatically and chemically interesterification of corn, soybean, and rapeseed oils was performed and the results were compared by the steryl ester content of the product [232]. Chemical interesterification, catalyzed by sodium methoxide, led to random positional distribution of fatty acids in triacylglycerols and some increase in the steryl ester content of all three oils. Enzymatic interesterification, catalyzed by Lipozyme RM IM resulted in a distinct reduction in steryl ester content, but essentially no alteration in positional distribution of fatty acids in triacylglycerols occurred [232]. In another example, a mixture of beef tallow and rapeseed oil was interesterified using the same chemical or biological catalysts [233]. The *sn*-2 and *sn*-1,3 distributions of fatty acids in the triglyceride fractions were random after chemical interesterification but when Lipozyme RM IM was used, the fatty acid composition at the *sn*-2 position remained practically unchanged, compared with the starting blend [234].

6.1. Interesterification of one oil

In some cases, a specific oil or fat is submitted to this interesterification process to obtain an improved product. For example, the interesterification of milk fat catalyzed by Lipozyme RM IM resulted in a decrease in the content of triacylglycerols group from C34 to C42, with a simultaneous increase of long-chain triacylglycerols from C44 to C52 content [235]. Butterfat was interesterified in organic media using Lipozyme RM IM [236]. The addition of 0.8–1% water shifted the RML affinity towards low molecular weight fatty acids.

In another paper, three ways have been undertaken to modify solid fat content of butter oil: (i) interesterification, (ii) adjunction

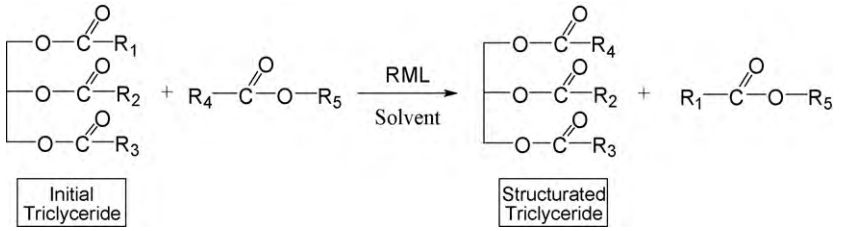

Fig. 8. General scheme of interesterification catalyzed by RML.

of high-melting point glycerides and (iii) joint effect of adjunction of high-melting glycerides and interesterification [237]. A solvent-free interesterification, carried out with RML, resulted in an increase of the solid fat content by about 114%. Adding high-melting glycerides trimyristin and tripalmitin also led to an increase of the solid fat content. The joint effect of the addition of high-melting point glycerides and interesterification was quite significant, mainly for triglycerides that included myristic and palmitic acids [237].

6.2. Interesterification of a blend of oils

It has been shown that in many instances the properties of a blend between a common oil and a healthier one may be further improved after their interesterification with RML, even though the global amount of fatty acids will remain identical. For example, blends of coconut oil with groundnut oil or with olive oil were prepared to contain balanced amounts of saturated to unsaturated fatty acids and subjected to interesterification reactions catalyzed by Lipozyme RM IM [238]. Although the blends had similar global fatty acid compositions as that of interesterified oils, the hypocholesterolemic effects of interesterified oils were significantly greater than that obtained from oils prepared by simple blending. In another example [239], blended oils comprising coconut oil and rice bran oil or sesame oil were interesterified by RML to give oils with saturated fatty acid/monounsaturated fatty acid/polyunsaturated fatty acid at a ratio of 1:1:1 and polyunsaturated/ saturated ratio of 0.8–1. Triglycerides containing one or two linoleic acids per triacylglycerol molecule were obtained by interesterification of tri-caprin and trilinolein [240].

In many cases, the objective is to improve the physical properties of the oils like its fluidity, lower melting point, etc. In others, to prepare healthier products, low-calorie structured lipids, etc.

Palm stearin is the main compost used in the interesterification reaction to change the physical properties of the oils. The reduction of the melting point of fats and oils was obtained by the interesterification of palm stearin, anhydrous milk fat, different vegetable oils and palm kernel olein, by many authors [241–249]. The properties of the final oils were very interesting. The interesterified products were suitable for use as *trans* acid-free or polyunsaturated fatty acid-rich shortening and margarine fat bases [241,245]. Moreover, a solid frying shortening similar to commercial solid frying shortenings was obtained [246,247], and the product met the melting criteria for fats used in ice cream formulation [249].

Interesterification with RML may be also used to convert mixtures of fats to plastic fats, making them more suitable for using in edible products [250]. To this goal, the transesterification of mixtures of palm stearin and sunflower oil in a solvent-free medium was performed resulting in a more fluid product. In another paper, palm stearin and palm kernel olein blends were interesterified by Lipozyme RM IM, yielding a product with physical properties similar to margarine fats [251].

The stearin fractions obtained from anhydrous milk fat was blended with sunflower or soybean oils and interesterified by RML, yielding fat products having desirable properties in making melange spread fat products with reasonable content of polyunsaturated fatty acids and almost zero *trans* fatty acid content [252].

As stated before the synthesis of structured lipids is one important application of RML. Thus, RML has been used to catalyze the interesterification of simple triglycerides to produce low-calorie structured lipids in *n*-hexane [253,254]. Interesterification of tricaprylin with peanut oil using RML (that gave the best yields among the studied enzymes, 79%) was carried out to produce structured triglycerides that contain medium- and long-chain fatty acids [255]. Cocoa butter equivalents were synthesized by catalyzed-RML interesterification [256,257].

The interesterification of palm oil, palm kernel oil and their binary blends was performed by Lipozyme RM IM [258]. Interesterified palm oil presented more medium and long-chain triacylglycerols, with MMM/OLL, MMP, OOO and PPP (M, myristic acid; O, oleic acid; L, linoleic acid; P, palmitic acid) increasing in concentration. In contrast, interesterified palm kernel oil resulted in the formation of more short and medium-chain triglycerides with LaLaO and LaMO (La, lauric acid; C, capric acid) experiencing noteworthy increases. Formation of high percentages of partial glycerides was found when using palm oil but not using palm kernel oil. The blend of these oils caused similar triglycerides compositional changes where the interesterification process focused on the medium-chain triglycerides, while hydrolysis was observed in the short and long-chain triglycerides [258].

Infant milk fat analogs were synthesized by a Lipozyme RM IM-catalyzed interesterification between tripalmitin, coconut oil, safflower oil, and soybean oil in hexane [259]. The optimal products had total and *sn*-2 positional palmitic acid levels comparable to that of human milk fat.

Another paper shows the interesterification of virgin olive oil (rich in OOO (32.36%), OPO (21.7%) and OLO (11.6%), being L: linoleic; O: oleic; P: palmitic acids), and fully hydrogenated palm oil (36.5% PSR 28.8% PPR 23.2% SPS, being S: stearic acid), performed in a batch reactor and using Lipozyme RM IM as biocatalyst [260]. The reactions produced semi-solid fats. The semi-solid fat prepared using optimal reaction conditions contained 17.20% OPO, 13.61% OOO, 11.09% POP, and 10.35% OSP.

6.3. Interesterification between oils and esters of specific fatty acids

This strategy allows controlling the fatty acid that we desired to introduce in the triglyceride and may really alter the final global composition of fatty acids in the triglycerides of the oil. Triacylglycerols of ucuhuba (*Virola surinamensis*) that contain almost exclusively (90%) lauroyl and myristoyl moieties were interesterified with methyl esters of saturated fatty acids (C13, C15, C17, C19, C20) or triacylglycerols containing C18 acyl moieties (trioleoylglycerol, sunflower oil, corn oil) with Lipozyme RM IM [261]. Transfer of acyl moieties occurred at the *sn*-1,3-positions of the triacylglycerols, and the rates of interesterification with various substances were of the order triacylglycerol > methyl ester. Rate of

interesterification of methyl esters decreased with increasing chain length.

A few solid and semi-solid fats of sal (*Shorea robusta*), kokum (*Garcinia indica*), mahua (*Madhuca latifolia*), dhupa (*Vateria indica*) and mango (*Mangifera indica*) were transformed into cocoa butter substitutes by RML-catalyzed ester interchange with methyl palmitate and/or stearate [262]. Interesterified kokum fat resembled cocoa butter in both solid fat content and peak melting temperature.

Long-chain (20–24) saturated fatty acids present in the *sn*-3 position of peanut oil play a role in atherogenesis, as well as oxidized polyunsaturated fatty acids present in peanut, sunflower, safflower, soybean, and linseed oils, while oleic acid does not exhibit these adverse effects [263]. Thus, the replacement of those fatty acids with oleic acid was performed by a batch-stirred interesterification reaction of the oil in *n*-hexane with methyl oleate using RML.

However, in most cases the objective has been to enrich the oil in polyunsaturated fatty acids. Esters of eicosapentaenoic acid and docosahexaenoic acid were incorporated into groundnut oil [264], crude melon seed (*Citrullus colocynthis L*) oil [265], trilinolein [266] and tricapryloylglycerol [267] by interesterification with RML. Eicosapentaenoic acid ethyl ester was interesterified with crude melon seed (*Citrullus colocynthis L*) oil in a reaction catalyzed by RML, with a 97% of incorporation [268]. Moreover, the level of *n*-6 fatty acid in the glycerides was decreased. In all cases, substitution was successfully performed. An interesting observation is that the amounts of *sn*-1-substituted products were greater than their *sn*-3-substituted counterparts suggesting that RML had a stereopreference towards the *sn*-1-position over the *sn*-3 position in this reaction [267] (the first step is the hydrolysis of the triglyceride, it should be expected to keep the preference obserbed in that reaction).

Lipozyme RM IM was used as biocatalysts for the interesterification of conjugated linoleic acid ethyl ester and tricaprylin in hexane [268]. The enzyme exhibited high selectivity for the *sn*-1,3 position of the triacylglycerol early in the interesterification, with small extents of incorporation of conjugated linoleic acid into the *sn*-2 position, probably due to acyl migration, at later reaction times [269]. In another example, structured lipids were successfully synthesized by interesterification of caprylic acid ethyl ester and triolein [270]. Lipozyme RM IM converted most triolein into structured lipids.

Modification of the fatty acid composition of soy lecithin, principally at its *sn*-1-position, was investigated by RML-catalyzed interesterification with the methyl ester of individual fatty acids [271]. 8.4% Capric acid was incorporated, while lauric acid and myristic acid were introduced at 14.1 and 15.7%, respectively in the *sn*-1-position. This meant an increase in the linolenic acid percentage by about 10 units.

Coconut oil has been used as a starting raw material for the production of caprylic acid and capric acid via RML-catalyzed interesterification of the oil and methyl esters of medium-chain fatty acids [272]. The production of margarine-type fats using triolein with stearic acid methyl ester in *n*-hexane was the objective of other research [273].

7. Acidolysis

This is the most used strategy to produce structured lipids using RML. The mechanism of this reaction is similar to interesterification (Fig. 9). After the hydrolysis of an ester bond between the native fatty acid residue (the original substituent group) and the glycerol moiety of the triglyceride, the native fatty acid is released and a glyceride containing at least one hydroxyl group is produced. The hydrolysis step is followed by the formation of a new ester bond by reaction of the newly created hydroxyl group with the incoming new free fatty acid [24] (Fig. 9).

However, the purity of the final triglycerides may be decreased by the existence of acyl migration. If this occurs during the acidolysis reaction, it may cause the production of by-products [274]. Using as a model the Lipozyme RM IM-catalyzed acidolysis of rapeseed oil by capric acid in solvent-free media, this phenomenon has been investigated. Water content, enzyme load, reaction time, substrate ratio and mainly reaction temperature had strong positive influences on acyl migration, moreover it was determined that interactions existed between the parameters. This migration has been the subject of many other studies [275]. A high percentage of diacylglycerols during the reaction, even though these are important intermediates in the reaction increases the risks of acyl migration [276]. Under certain conditions, up to 18% incorporation could be observed in the *sn*-2 position during the lipase-catalyzed acidolysis of soybean phosphatidylcholine with caprylic acid in solvent-free media [277].

The selectivity and specificity of RML in this reaction has been the subject of many studies. The acidolysis of tripalmitin with oleic acid mainly produced 1-oleoyl-2,3-dipalmitoyl-*sn*-glycerol, which indicated that the enzyme was more active at the *sn*-1-position of the triglyceride than at the *sn*-3 position [278]. The stereoselectivity of the enzyme strongly depends on the initial water activity (a_w) of the reaction mixture, with greater selectivity occurring at lower a_w. The *sn*-1 selectivity was essentially maintained using various solvents, or using solvent-free medium, when a_w was kept low. Shorter-chain fatty acids of the triglyceride or longer chain length of the free fatty acid increased RML stereoselectivity. By using low a_w, approximately 80% of the chiral triglyceride found in the reaction mixture was the sn-1 enantiomer, at high reaction conversion [278]. This was later confirmed using a stereospecific deuterium-labeled triacylglycerol substrate and ultra-high resolution ^{13}C nuclear magnetic resonance spectroscopy [279].

The RML preference in the incorporation of 11 fatty acids, ranging from C10:0 to C22:6, into coconut oil triglycerides during acidolysis was studied [280]. The overall preference of lipase for the incorporation of different fatty acids into coconut oil triglycerides was 18:0 > 18:2, 22:0 > 18:1, 18:3, 14:0, 20:4, 22:6 > 16:0 > 12:0 ≫ 10:0.

In another study, triolein and stearic acid were used as substrates for acidolysis reactions and eight commercially available lipases were tested for their suitability in the reaction. There was no correlation between their hydrolytic and acidolysis activities [281]. RML was the most suitable enzyme for this reaction. At zero water activity, high conversion was achieved, although acidolysis activity was lower than that at a water activity of 0.25. RML was immobilized covalently with glutaraldehyde or with six alkyl chains as spacers onto Florisil (magnesium silicate). Interesterification activities of the immobilized RML with the hydrophobic spacers were increased when compared to the free enzyme.

Many different oils have been modified by RML using this strategy. Now, we will try to review the most relevant applications.

7.1. Production of lipids enriched in polyunsaturated fatty acids

The preparation of oils enriched in polyunsaturated fatty acids (PUFA) is one of the main objectives in food technology, due to the beneficial properties of these compounds.

Thus, the preparation of triglycerides having more than 70% of *n*-3 polyunsaturated fatty acids by acidolysis of cod liver oil and free *n*-3 polyunsaturated fatty acids catalyzed by RML was one of the first uses of the enzyme in acidolysis [282].

Displacement of the equilibrium of the RML-catalyzed acidolysis between medium-chain triglyceride and long-chain polyunsatu-

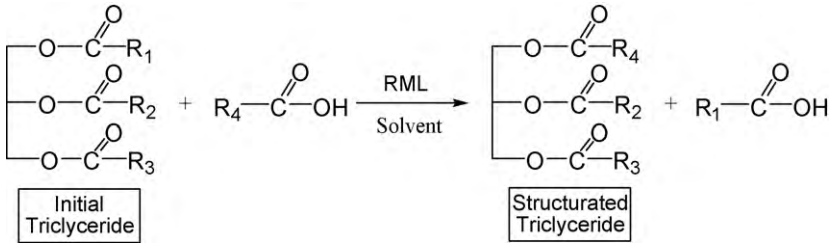

Fig. 9. General scheme of acidolysis catalyzed by TLL.

rated fatty acid was accomplished by the removal of by-products with continuous supercritical carbon dioxide extraction [283]. The incorporation of eicosapentaenoic acid to triglyceride was appreciably improved by this method and was 1.3 times higher than that of the equilibrium state that was obtained in a closed system.

Eicosapentaenoic acid (EPA) and docosahexaenoic (DHA), the most essential long-chain *n*-3 PUFAs, are recognized to have special pharmacological and physiological effects on human health acid, are the main polyunsaturated fatty acids used in oil enrichment. RML-catalyzed the incorporation of EPA and DHA, both or just one, in many cases. Enzymatic acidolysis was performed in soybean oil [284,285], borage oil [286,287], palm oil [288], and high-laurate canola oil [289]. In one case, acidolysis was used not only to improve the PUFAs content but also to reduce the content of linoleoyl moiety in soybean oil [285].

Other applications are the acidolysis of coconut oil triglycerides [290] and rice bran oil [291,292] by Lipozyme RM IM in *n*-hexane to incorporate *n*-3 or *n*-6 PUFA, Acidolysis of triolein catalyzed by RML was satisfactorily achieved using stearic, α-linolenic, γ-linolenic, arachidonic and docosapentaenoic acids [293]. Enriched *n*-6 triglyceride was used as substrate for the acidolysis reaction with *n*-3 PUFA catalyzed by lipases in supercritical carbon dioxide [294]. RML yielded the highest degree of incorporation among the used lipases. Structured triacylglycerols with caprylic acid located at positions 1 and 3 of the glycerol backbone and docosahexaenoic acid at position 2 were produced by acidolysis of tuna oil with caprylic acid catalyzed by Lipozyme RM IM in a packed bed reactor, with only moderate results [295].

7.2. Modification of lipids rich in polyunsaturated fatty acids

In other cases, PUFAs rich-oils are modified to further improve their properties or the stability of these fragile compounds. Synthesis of 1,3-dicapryloyl-2-eicosapentaenoylglycerol (CEC) catalyzed by Lipozyme RM IM was performed by acidolysis of trieicosapentaenoylglycerol (EEE) with caprylic acid or interesterification with its ethyl ester [296].

Menhaden oil is the most used oil in acidolysis catalyzed by RML [297–305]. Also, canola oil [297], single cell oil (very rich in docosahexaenoic acid) [306,307], and blubber oil [303] were submitted to RML-catalyzed acidolysis. To perform these reactions caprylic acid ($C_{8:0}$) was the main fatty acid used [297,298,306,307]. Other options with good results are capric acid ($C_{10:0}$) [300,301], γ-linolenic acid [303] and pinoleic acid (an isomer of γ-linolenic acid) [303], conjugated linoleic acid [300,301] and concentrate of *n*-3 PUFAs [304]. In most reports the content of PUFAs, mainly EPA and DHA, remained unchanged after the modification [297,299,303–305,307], and a significant proportion of the *n*-3 fatty acid residues were liberated in the process [301,302]. In one case, incorporated caprylic acid did not replace docosahexaenoic acid, but the content of eicosapentaenoic acid decreased somewhat with an increase in caprylic acid incorporation [306].

Oils containing both *n*-3 and *n*-6 fatty acids have important clinical and nutritional applications [308]. Nevertheless, acidolysis of PUFA-rich oil is important because fish oil containing capric acid may be nutritionally more beneficial than unmodified oil [299].

A deeper analysis of the results is difficult because the reactions were carried out by different biocatalyst (free or immobilized), in different conditions (solvent-free, organic solvents, supercritical CO_2) and all these factors alter RML properties.

7.3. Incorporation of conjugated linoleic acid

The studies of the very relevant isomers of conjugated linoleic acids hold a special interest. Triacylglycerols of butter oil (milk fat) were submitted to acidolysis with conjugated linoleic acid in a solvent-free medium. RML was among the best lipases for this reaction [309]. The fatty acids liberated from the butter oil were primarily short-chain fatty acids, especially butyric and caproic acids.

Conjugated linoleic acids were used in the acidolysis of the following vegetable oils: corn oil [310], fully hydrogenated soybean oil [311], coconut oil [312], groundnut oil [313], and olive oil [314]. Simple triglycerides like tricaprylin [312] and tristearin [315,316] were reacted with conjugated linoleic acids. Two regioisomers of structured triglycerides containing one mole of long-chain conjugated linoleic acid (L) and two moles of medium-chain caprylic acid (M) have been produced (MLM and LMM) [317]. A commercially available free fatty acids mixture containing 9-*cis*, 11-*trans* (9c,11t)- and 10t,12c-conjugated linoleic acid was selected as long-chain fatty acid, and caprylic acid was selected as medium-chain fatty acid. MLM was synthesized by acidolysis of acylglycerols containing two medium-chain fatty acid isomers with caprylic acid catalyzed by Lipozyme RM IM. LMM isomer was synthesized by acidolysis of tricaprylin with conjugated linoleic acid. Regiospecific analyses of MLM and LMM indicated that the 2-positions of MLM and LMM were 95.1 mol% conjugated linoleic acid isomers and 98.3 mol% caprylic acid, respectively [314].

7.4. Modification of phospholipids

Acidolysis of synthetic dimyristoyl phosphatidylcholine with oleic acid RML under solvent-free conditions could be carried out as efficiently as in toluene, but the degree of hydrolysis was lower than in toluene [318]. The maximum yield of modified phosphatidylcholine was 35% of the original phospholipid.

Soy lecithin was modified using lauric acid and oleic acid by RML [319]. Oleic acid was more incorporated into soy lecithin. The main fatty acids replaced were palmitic and linoleic acids.

Lipozyme RM IM was employed to catalyze the acidolysis reaction of 1,2-diacyl-*sn*-glycero-3-phosphatidylcholine with *n*-3 polyunsaturated fatty acids under solvent-free conditions [320]. Phospholipids were successfully modified by RML-catalyzed acidolysis to incorporate EPA and DHA [321]. The best results were achieved using phosphatidylcholine and EPA in hexane.

7.5. Other acidolysis reactions catalyzed by RML

7.5.1. Modification of triolein or olive oil

RML-catalyzed acidolysisof triolein with caproic or butyric acids was performed to produce reduced-calorie structured lipids (the most efficient among the 9 lipases assayed) [322]. In other papers, triolein was submitted to acidolysis with caprylic acid [323,324].

Lipozyme RM IM-catalyzed acidolysis of olive oil and caprylic acid was performed in a bench-scale packed bed bioreactor to produce structured lipids [325]. Olive oil is characterized by four major clusters of triacylglycerol species with equivalent carbon number of C44, C46, C48, and C50. Three monosubstituted products and two disubstituted products were detected after the reaction. Monosubstituted products had equivalent carbon number of C36, C38, and C40, and disubstituted products had equivalent carbon number of C30 and C32. Structured lipids produced at optimal conditions had 7.2% caprylic acid, 69.6% oleic acid, 21.7% linoleic acid and 1.5% palmitic acid at the *sn*-2 position [325].

7.5.2. Modification of soy bean

In order to decrease the content of linoleoyl moiety in soybean oil, soybean oil that contains 22.8% oleoyl, 54.8% linoleoyl, and 7.1% α-linolenoyl moieties was submitted to acidolysis in *n*-hexane with oleic acid or α-linolenic acid, using Lipozyme RM IM as catalyst [326]. The products using oleic acid contained 50.8% oleoyl, 38.8% linoleoyl, and 5.4% α-linolenoyl moieties. Using α-linolenic, the reaction products contained 13.5% oleoyl, 40.8% linoleoyl, and 40.4% α-linolenoyl. In a further investigation, the stability of the biocatalyst was analyzed under those conditions [327]. The catalytic activity of Lipozyme RM IM decayed in a first-order fashion. Based on these deactivation kinetics, the flow rate of the feed stream was simulated for the operation of a continuous, packed bed reactor at 37 °C that produces an oil of a fixed composition of oleoyl moiety.

RML-catalyzed acidolysis of triacylglycerols of soybean oil with oleic acid in organic solvent has been also studied [328]. At 50 °C, about 49% oleic acid incorporation was observed. However, at this temperature, the modification of the *sn*-2 position was higher than at 40 °C (with slightly lower modification yield).

7.5.3. Modification of canola oil

A structured lipid was synthesized from canola oil and caprylic acid via interesterification catalyzed by RML [329]. Cold-spreadable butter was made by blending butterfat with the product of the reaction. The new butterfat blend had lower contents of hypercholesterolemic fatty acids and lower atherogenic index as compared to pure butter or butterfat-canola oil.

Structured triacylglycerols from canola oil were produced by its enzymatic acidolysis with caprylic acid in a packed bed bioreactor, using Lipozyme RM IM [330]. Chocolate-flavored nutritional beverages formulated with structured lipid were synthesized from canola oil and caprylic acid [331]. This product may be more readily metabolized in the body while providing essential fatty acids.

Another structured lipid was synthesized from canola oil and caprylic acid with RML [332]. Cold-spreadable butter was made by blending butterfat with this structured lipid. The structured lipids can counterbalance the hypercholesterolemic attributes of butterfat and improve the cold-spreadability of the new spread without any adverse effect on its flavor attributes.

7.5.4. Modification of other oils

Structured lipids were synthesized by acidolysis of peanut oil with caprylic acid in a stirred-batch reactor catalyzed by RML in the absence of organic solvent [332]. Sesame oil was modified to incorporate capric acids with RML in *n*-hexane or in a solvent-free system [333] and to incorporate caprylic acid by RML-catalyzed acidolysis in a bench-scale continuous packed bed reactor [334]. In another paper, a similar study was performed using rice bran oil [335]. The same oil was used to produce structured lipids rich in stearic acid by acidolysis with stearic acid using Lipozyme RM IM [336]. The structured lipids prepared retained their beneficial nutraceuticals while they did not contain any *trans* fatty acids.

Triglycerides containing stearic acid at the *sn*-2 position may become nutritional and low-calorie fats. Two different structured lipids were synthesized by acidolysis of tristearin with caprylic acid or oleic acid catalyzed by RML in the presence of *n*-hexane [337]. Oleic acid incorporation was higher than that of caprylic acid while positional analysis confirmed that stearic acid remained at the *sn*-2 position.

Production of structured triacylglycerols containing short-chain fatty acids by Lipozyme RM IM-catalyzed acidolysis between rapeseed oil and caproic acid was performed in a batch reactor in a solvent-free system [338]. The same oil was submitted to acidolysis with capric acid [339]. Triacylglycerols after reaction contained mainly oleic, linoleic and linolenic acids (about 90%) in the internal *sn*-2 position, whereas capric acid was mostly in the external *sn*-1,3-positions (approximately 40%).

Structured lipids were synthesized by acidolysis of perilla oil with caprylic acid using Lipozyme RM IM [340]. Beef tallow was reacted with stearic acid, using RML, to produce structured triglycerides useful to formulate dark chocolate [341]. The modified tallows did not present a detrimental effect on the crystallization of cocoa butter after proper tempering of the chocolates, while the modified lipids reduced bloom rates.

Non-used oils extracted from the inner skin of *Strychnos madagascariensis* fruit shell, *Trichelia emetica* seed and *Ximenia caffra* seed were utilized as starting materials in RML-catalyzed production of cocoa butter equivalents using acidolysis, by incorporating stearic acid or palmitic acid into the *sn*-1- and 3-positions of the triglycerides [342]. Seal (*Phoca groenlandica*) blubber oil was submitted to acidolysis with lauric acid, being RML the best enzyme among those assayed [343].

Structured lipids were synthesized by the acidolysis of corn oil by caprylic acid in supercritical carbon dioxide with Lipozyme RM IM [344]. An interesting paper reported the synthesis of resembling human milk fat analog containing palmitic, oleic, stearic, and linoleic acids, by RML acidolysis between tripalmitin, hazelnut oil fatty acids, and stearic acid in *n*-hexane [345]. Lipozyme RM IM acidolysis of refined, bleached and deodorized palm olein with caprylic acid was carried out in a continuous packed bed bioreactor to produce structured lipid that can confer metabolic benefits when consumed [346].

8. Multireaction strategies to produce structured lipids

In some cases, the starting substrates have several compounds that may be modified by the lipase to produce the target compound (e.g., acid oils will present glycerides and free fatty acids). Thus, acid coconut, soybean, mustard, sunflower, and rice bran oils were used for the purpose of making various monohydric alcohol esters of fatty acids [347]. Fatty acid oils were converted into fatty acid esters of short- and long-chain alcohols like C4, C8, C10, C12, C16, and C18 in high yields by simultaneous esterification and alcoholysis reactions with RML.

In some cases, the objective of the reactions is to facilitate the purification of other products. That is the case of the isolation of tocopherols and sterols from sunflower oil deodorizer distillate. To improve their purification by distillation, the neutral glycerides of the deodorizer distillates were hydrolyzed by lipase from *Candida cylindracea* [348]. The total fatty acids (initial free fatty acids plus those from neutral glycerides) were converted into butyl esters

with RML. The fraction collected at 230–260 °C at 1.00 mmHg for 15 min was rich in tocopherols (about 30%) and sterols (about 36%). The overall recovery of tocopherols and sterols after hydrolysis, esterification and distillation were around 70% and 42%, respectively, of the original content in sunflower oil deodorizer distillate.

In other cases the oil is submitted to several consecutive reactions, catalyzed by different lipases, to achieve the final product. For example, the synthesis of *sn*-1,3-diacylglycerols in two steps without isolation of the intermediates was performed. In the first step, Novozym 435, a non-specific lipase, was used in the alcoholysis of extra virgin olive oil with ethanol to produce glycerol and fatty acids ethyl esters [349]. In the second step the ethanolysis products have been re-esterified using Lipozyme RM IM to produce *sn*-1,3-diacylglycerols.

A highly efficient enzymatic method for the synthesis of regioisomerically pure 1,3-dicapryloyl-2-docosahexaenoyl glycerol in two steps has been also proposed [350]. 2-Monoglyceride was obtained by ethanolysis of tridocosahexaenoylglycerol with Novozym 435. After, Lipozyme RM IM was used in the reesterification of 2-monodocosahexaenoylglycerol with ethylcaprylate. Good results were obtained also for the synthesis of 1,3-dicapryloyl-2-eicosapentaenoylglycerol by the same method. The same triglyceride was synthesized enzymatically from bonito oil in a two-step process: (i) ethanolysis of bonito oil to 2-monoacylglycerols and fatty acid ethyl esters, catalyzed by Novozym 435 and (ii) reesterification of 2-monoacylglycerols with ethyl caprylate, catalyzed by Lipozyme RM IM to give structured triglycerides [351].

The low discrimination of RML between *cis*9, *trans*11 or the *trans*10, *cis*12 isomer of conjugated linoleic acid was used to introduce these acids on palm oil [352]. Using a synthetic fatty acid mixture that contained both isomers in similar percentage, the lipase from *Geotrichum candidum* esterified the *cis*9, *trans*11 isomer more rapidly than the *trans*10, *cis*12 isomer, producing an ester fraction with a content of 91% *cis*9, *trans*11 CLA and an unreacted free fatty acid fraction consisting of 82% *trans*10, *cis*12 isomer. The components of the reaction mixture were separated by molecular distillation. Each enriched fraction was then incorporated into different palm oil triglycerides by interesterification or acidolysis catalyzed by RML [352].

The enzymatic synthesis of phosphatidylcholine with decanoic acid in the *sn*-1 and hexanoic acid in the *sn*-2 position has been achieved in several steps: (i) treatment of egg yolk with phospholipase A_2 to hydrolyze egg yolk phosphatidylcholine to 1-acyl lysophosphatidylcholine; (ii) esterification of previous product with hexanoic acid catalyzed by phospholipase A_2 to yield phosphatidylcholine with hexanoic acid in the *sn*-2-positions; (iii) removal of the fatty acid in the *sn*-1-position by RML-catalyzed ethanolysis to yield 2-hexanoyl lysophosphatidylcholine; and finally (iv) introduction of decanoic acid in this position by lipase-catalyzed esterification of 2-hexanoyl lysophosphatidylcholine to yield 1-decanoyl-2-hexanoyl-phosphatidylcholine [353]. The method is applicable for the synthesis of other mixed-acid phosphatidylcholine species as well.

In other cases, processes that combine enzymatic and physical techniques have been studied for concentrating and separating eicosapentaenoic acid and docosahexaenoic acid from fish oil [354]. Lipase from *Candida rugosa* has been used in hydrolysis reactions of fish oils to concentrate these acids in the glyceride fraction. By controlling the degree of hydrolysis, two products have been obtained, one enriched in total *n*-3 acids (50%), and other enriched in docosahexaenoic acid (40%) and depleted in eicosapentaenoic acid (7%). The glyceride fraction from these reactions was recovered by evaporation and converted back to triglycerides by partial enzymatic hydrolysis, followed by enzymatic esterification, catalyzed by RML [354].

Structured triglycerides of the ABA-type, containing one type of fatty acid (A) in the *sn*-1 and *sn*-3-positions and a second type of fatty acid (B) in the *sn*-2 position of the glycerol, were synthesized using RML, in a two-step process [355,356]. In the first step, a triglyceride of the B-type was subjected to an alcoholysis reaction in an organic solvent yielding the corresponding 2-monoglyceride. Using this strategy, 2-monopalmitin was obtained. Esterification of 2-monopalmitin with oleic acid resulted in the formation of 1,3-oleyl-2-palmitoylglycerol (an important structured triglyceride in infant nutrition) in up to 72% yield containing 94% palmitic acid in the *sn*-2 position. The same methodology was used to incorporate caprylic acid in *sn*-1- and 3-positions and an oleic or linoleic acid in the *sn*-2 position of glycerol [357]. Pure 2-monoglycerides were synthesized by alcoholysis of triglycerides (triolein, trilinolein, or peanut oil) in organic solvents with RML. Coconut and palm kernel fatty acid distillates can be utilized to produce medium-chain glycerides by a combination of lipase-catalyzed hydrolysis and esterification reactions [358].

Regiospecific ethanolysis of trioleoylglycerol, tridocosahexaenoylglycerol, trieicosapentaenoylglycerol, triarachidonoylglycerol, tri-α-linolenoylglycerol, and trilinoleoylglycerol with Novozym 435 was studied to produce 2-monoglycerides [359]. Purified monoglycerides were re-esterified with caprylic acid by Lipozyme RM IM to afford symmetrical structured triglycerides. The regioisomeric purity of both COC and CDC was 100%.

9. Conclusions

RML is a lipase that was firstly designed and mainly produced for oils and fats modifications [55–58], therefore it has found many uses in food manufacturing by the different strategies showed in this review.

Their use in industry is mainly due to the high stability of the enzyme under anhydrous systems, where it presents advantages when compared to other lipases [82–85]. These advantages are reinforced by its high esterification activity in anhydrous media, that way RML may be the choice lipase mainly in esterifications or in any reaction that proceed via esterification in some of the initial steps (e.g., acidolysis and interesterification).

However, the enzyme properties are not so adequate in transesterifications or alcoholysis, where other enzymes may offer better performace e.g., that from *Thermomyces laguginosus* [54].

Moreover, it should be considered that these reactions are extremely complex, because the number of possible products may be very large, enzymatic and chemical (mainly acyl migration) processes may be simultaneously occurring, and also lipase properties may be affected by small changes in the reaction medium or enzyme preparation.

Regarding the selectivity of the reaction, the enzyme is described as *sn*-1,3-specific. Between these two positions, the enzyme exhibits a *sn*-1 or *sn*-3 preference depending on the reaction. In hydrolysis, the enzyme prefers the *sn*-1-position [102], that means that in acidolysis and interesterification (where the first step is the hydrolysis of the triglyceride) the enzyme is mainly *sn*-1 specific. However, in esterification, the enzyme exhibits a slight *sn*-3 preference [147]. In any case, the enzyme does not recognize position *sn*-2 significantly. However, acyl migration may produce the modification of this position, allowing the full modification of the oil (both in hydrolysis or synthetic processes).

This fact, together with the fatty acid specificity exhibited by the enzyme, needs to be considered before selecting RML among other lipases in any reaction. Furthermore, these specificities should be included in any kinetic modeling if it should be accurate enough. Moreover, this makes extremely complex to compare results from different researches, the acceleration of acyl migration may be not

controlled and this chemical process may explain many of the observed results.

Although not as evident as in the case of the use of the enzyme in enantio or regioselective reactions in organic chemistry [54,360,361], the features of this enzyme in the modifications of fat or oils may be altered by the immobilization protocol used [58,182,197,281], and therefore this should be also considered in the evaluation of the enzyme (trying to include several immobilized forms of the lipase before taking a decision regarding the suitability or not of the enzyme for a reaction). Using soluble RML, the enzyme tends to form bimolecular aggregates even at very low concentrations or to be adsorbed to any hydrophobic component of the crude extract, this must be carefully considered [51–53] (Figs. 2 and 3). These aggregates have very different properties when compared to the monomeric form of the lipase. This makes evaluation of the soluble enzyme very complex. Mild immobilization protocols (ideally via just one point and using long and inert spacer arms) [362] in the presence of detergents to break the dimers [51–53], 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 RML molecules [36–38,45].

The growing demand of healthier foods by the human society permits to assume that the employment of RML in this area will increase in the near future [1–4]. On the other hand, the use of the many tools available to improve the properties of the enzyme may help to further enhance the enzyme selectivity or specificity, mainly considering the deep knowledge of the enzyme properties [67–89].

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