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Review

Lipase from *Rhizomucor miehei* as an industrial biocatalyst in chemical process

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ABSTRACT

The lipase from *Rhizomucor miehei* (formerly *Mucor miehei*) (RML) is a commercially available enzyme in both soluble and immobilized form with very high activity and good stability under diverse conditions (anhydrous organic solvents, supercritical fluids, etc.). Although this lipase was initially produced to be used in food industry, in this review we will focus our attention on the application of this enzyme in organic chemistry, from biodiesel production to fine chemicals (mainly in enantio or regioselective or specific processes). After showing the enzyme features, some of the most efficient methods of RML immobilization will be commented (entrapping on reverse micelles, preparation of cross-linked RML aggregates or immobilization on pre-existing solids). Finally, the main uses of the enzyme in organic chemistry will be revised. The use of RML in the production of biodiesel will be analyzed, and compared to the performance of other lipases. The synthesis of esters of carboxylic acids as flavors is other example where RML has been successfully employed. Taking advantage of the wide specificity of the enzyme, mainly a high enantiospecificity, many examples of the use of RML in the resolution of racemic mixtures of chiral carboxylic acids, alcohols or esters will be presented. Special mention requires the use of the regioselectivity of RML, mainly the chemistry of sugars. Finally, more unusual uses of RML will be presented (anomalous substrates, novel uses, etc.). In general, this enzyme seems very adequate for esterification reactions due to its high stability in anhydrous media and good esterification activity.

Contents

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1. Lipases as biocatalyst in the industry

Lipases are among the most widely used enzymes in enzyme technology [1–4], because they recognize a wide variety of substrates and may catalyze many different reactions, such as hydrolysis or synthesis of esters bonds [5–7], alcoholysis [8], aminolysis [9–13], peroxidations [14–16], epoxidations [17–19] and interesterifications [20]. This lipase promiscuity makes that lipases had been used in many different reactions, finding applications, among other areas in pharmaceuticals and drugs production [21–24], in the production of biodiesel [25–27] or food modification [28–30].

The use of these enzymes as industrial biocatalysts needs to consider their peculiar mechanism of action. Lipases in nature hydrolyze very hydrophobic oils and fats, which will be found as emulsions or drops. To solve this situation, lipases have a common property: in homogenous medium, they have their active center secluded from the medium by a polypeptide chain called lid or flap [31–34]. 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* [35]) or, as in the case of the lipase from *Bacillus thermocatenulatus*, to be a quite complex structure involving a large percentage of the amino acids of the enzyme and forming a double lid [36]. In the presence of a drop of oil [37,38], the lid moves to let the interaction between its hydrophobic internal face and the hydrophobic residues that usually surround the lipase active center with this hydrophobic surface. This way, the lipase becomes adsorbed on this hydrophobic surface, and the active center is exposed to the reaction medium (open form) [31–38]. This mechanism of action is usually called "interfacial activation" of the lipases, and these drastic conformational changes should be considered in the use of these enzymes as biocatalyst. Other hydrophobic surfaces "mimic" these drops of oils, allowing the adsorption of the open form of the lipases via interfacial activation: hydrophobic supports [39–43], hydrophobic proteins [44] or even other "open" lipase molecule [45,46]. This last point may be a problem in the study and utilization of a lipase, because the properties of individual or associated lipase molecules may be very different [47–49].

When a lipase molecule is immobilized inside a porous support, it should be considered that it is protected from interactions with any external interfaces, and may not longer suffer interfacial activation by this external interfaces (at least in systems containing a large percentage of water). However, the enzyme can be with its stabilized open form if using a hydrophobic support, even in the absence of any additional external interface [39–43].

On the other hand, due to the extreme flexibility of the active center of lipases, their catalytic properties may be easily altered without inactivating the enzyme. Thus, lipase properties may be greatly modulated by slight changes in the reaction conditions [50–52], but also dramatic changes in the enzyme properties may be achieved by using different immobilization protocols [50–53] or by its chemical [54,55] or physical modification [56].

In this review, we will focus on the uses of the lipase from *Rhizomucor* (previously *Mucor*) *miehei* (RML) as catalyst on the energy and organic chemical industries, while the uses of the enzyme in food industries, to modify oils, fats or free fatty acids, will be presented in another paper [57].

1.1. Properties of RML

This extracellular enzyme was first described in 1973 [58]. The enzyme has a molecular size of 31,600 Da and a p*I* of 3.8 [59]. Few years later, the first use of the enzyme in food modification was described [60]. 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 several synthetic substrates including sorbitol esters of fatty acids [60]. The enzyme is currently commercially available from Novozymes in free form (Palatase 2000 L) or 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 [61–63]. In these first studies, it was reported that the immobilization altered the lipase specificity for different fatty acids [62].

RML was the first lipase whose structure was resolved (at 1.9 Å resolution [34]) (Fig. 1). Therefore, it has been the subject of many different studies [61–69] and its interfacial activation mechanism is well known [70–84].

The study of the hydrolysis of some hydrophobic esters (like 4-methylumbelliferyl oleate, 4-methylumbelliferyl palmitate and monoolein) catalyzed by RML in a homogenous mixture composed by a buffer and some different polar organic solvent was performed [85]. They suggested that the addition of a polar organic solvent to an aqueous solution will be an efficient method for changing the hydrolytic performance of RML [85], the solvents improved substrate solubility but also produced some changes in the enzyme structure.

Some additives may be useful for the management of RML. RML is activated by the presence of some detergents, even in organic media [86]. The detergents sodium cholate, Tween 80 or Tween are possible activators of the enzyme, increasing 2-fold RML activity in organic media. In contrast, RML activity was inhibited in the presence of Brij 58 (other nonionic detergent) or the cationic detergent cetyltrimethylammonium bromide (this was attributed to the inactivation of the enzyme by these detergents). Some studies trying to study the interactions of RML with detergents were performed. For example, using the amphoteric surfactant sodium N-(2-hydroxydodecyl)sarcosinate, whose charge may be controlled by adjusting the pH [87]. These studies concluded that only when the head group is positively charged does the surfactant bind with RML. However, the number of surfactant molecules that bind to each enzyme molecule 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

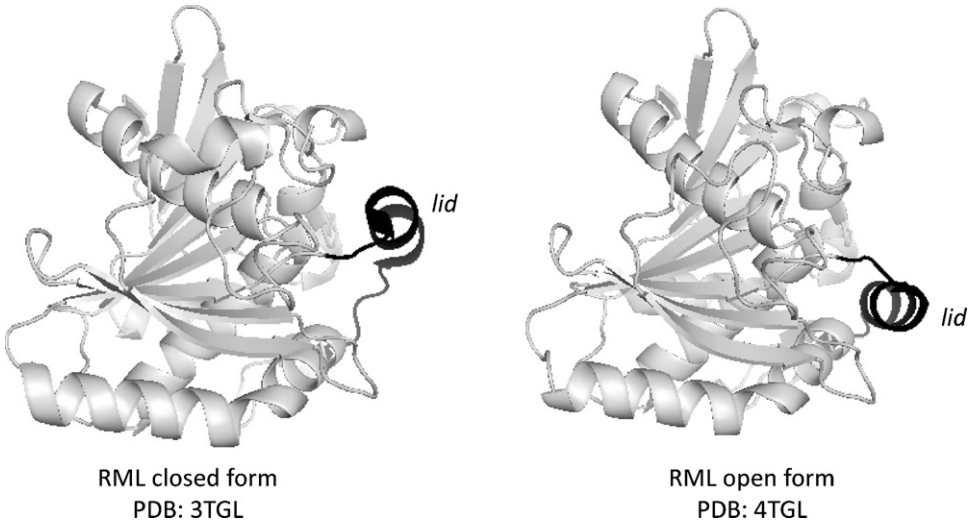

Fig. 1. Structure of open and closed forms of RML. The 3D structure was obtained from the Protein Data Bank (PDB) using Pymol vs. 0.99.

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 complexes with RML over a broad pH range (even below its isoelectric point). No such interactions were found for neither anionic nor nonionic surfactants [88]. The interaction between cationic surfactants and RML leads to a reduction of the reaction rate in hydrolysis of palm oil. The authors proposed that anionic and nonionic surfactants with bulky hydrophobic tails (to prevent that RML recognize them as substrates) are the preferred surfactants for microemulsion-based reactions with RML as catalyst [89].

RML tends to form bimolecular aggregates with reduced activity, dimmers that may be broken by detergents (e.g., Triton X-100) [47]. This should be other effect of detergents that needs to be considered when using this enzyme. The immobilization of the enzyme in the presence of detergents seems to be a good option of getting individual immobilized RML molecules [47].

2. Preparation of industrial biocatalysts with RML

In many instances RML has been modified using different strategies before being used as biocatalyst in different reactions and reactors.

For example, the soluble RML has been modified with activated polyethylene glycol that may react with different groups of the protein. This modification was performed with the aim to improve its catalytic properties in organic solvents, mainly by improving its solubility. While this modification hardly affected the enzyme activity in aqueous medium, it was found that the transesterification activity in organic medium (*o*-xylene, *tert*-butyl methyl ether, *tert*-butanol or 2-butanone) could be greatly improved [90]. The most effective modifier was found to be the least hydrophobic polymer, tryesyl-activated polyethylene glycol 2000 monomethyl ether, activating lipases up to 27-fold in organic solvents. Later, this modification was found to alter the activity and enantioselectivity in transesterification reactions using chiral alcohols in *o*-xylene and *tert*-butyl methyl ether [91]. Soluble RML was also chemically modified with sorbitan monostearate, a surfactant. In this case, the final activity of the modified enzyme was improved in both aqueous and organic media [92]. That way, this chemical modification seems to be very useful to improve RML properties.

However, in most cases enzymes require their previous immobilization to be used as industrial biocatalysts. Although the main objective of the immobilization is the reuse of a moderately expensive biocatalyst [93–95], a proper immobilization of the enzyme may permit to greatly improve its features. The most evident examples are the improvement of the enzyme stability via multipoint covalent attachment and prevention of intermolecular interactions of the enzyme with other components of the medium (avoiding autolysis, inactivation by interaction with bubbles, etc.), but also in some cases a proper immobilization may improve enzyme activity, selectivity or decrease inhibition [53,96].

RML has been submitted to many different immobilization protocols using various materials, from nylon membranes [97] to different silicates [98].

The immobilization of the enzyme on the own producing microorganism has been the object of several papers (Fig. 2). Zhang et al. showed how producing RML-α–agglutinin fusion protein RML can be expressed on the cell surface of *Saccharomyces cerevisiae*, with an even higher activity than the native enzyme [99]. The enzyme remained attached to the cell surface using a glycosylphosphatidylinositol (GPI) anchor. In another paper, the enzyme was displayed on the surface of *Pichia pastoris*, and a disulfide bond was introduced via site-directed mutagenesis [100]. This mutant enzyme was more stable than the native enzyme even in the free form. However, yeast surface improved the stability of RML, as well as amplified the thermostability through the engineered disulfide bond.

In another example, RML activity was improved by immobilization on biomass particles that acted as supports [101]. This immobilization technique avoids the use of any further support, producing an already immobilized enzyme, but may have some problems, like the low volumetric activity, the presence of many contaminant enzymes or the lack of versatility in the immobilization.

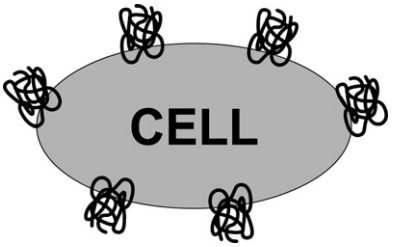

Fig. 2. Immobilization of RML on the producer microorganism.

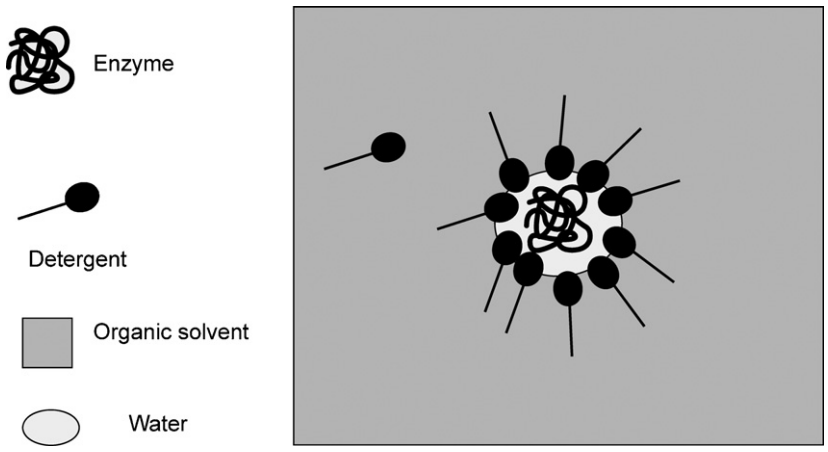

Fig. 3. Immobilization of RML by entrapping it in reverse micelles.

However, most examples are on the immobilization of free enzymes. This allows to use the pure enzyme, improving the volumetric activity and reducing side reactions, and is quite versatile.

RML has been immobilized in reverse micelles (Fig. 3). The catalytic activity of RML solubilized in the cetyltrimethylammonium bromide reverse micelles increased with increasing molar ratio of water to detergent [102]. RML was chemically modified with cellobiose and N-succinimidyl palmitate to alter the physical properties of the enzyme surface before immobilizing it into reversed micelles of OT aerosol (*bis*-(2-ethylhexyl)sulfosuccinate sodium salt) in isooctane [103]. Authors found that they can have individual molecules of RML or force the aggregation of RML molecules to give a tetramer by using different conditions and micelle sizes. The catalytic activity of RML in the micellar medium was found to be higher than that in aqueous solution. RML hydrophobization resulted in a decrease in the enzyme activation effect with an increase in the AOT concentration in comparison with the unmodified enzyme, while RML hydrophilization dramatically decreased the activity of RML tetramer when the AOT concentration was increased. Kinetic data indicated a mixed type of activation of both oligomeric forms of the native and the hydrophobized RML by AOT molecules and the noncompetitive type of the activation and AOT inhibition of the monomer and the tetramer of the hydrophilized RML, respectively [103]. However, the use of micelles at industrial level may present some problems, for example their use is restricted to a narrow range of conditions.

Another popular immobilization technique is the entrapment of the enzyme in a matrix (Fig. 4). This immobilization technique may be quite simple, however it is not expected that the entrapment should rigidify the enzyme. On the other hand, the generation of a defined environment surrounding the enzyme may have some positive effects [53]. For example, RML has been encapsulated in lecithin water-in-oil microemulsion-based organogels formulated with either hydroxy (propylmethyl) cellulose or gelatin, and used as catalysts for the esterification of lauric acid and 1-propanol in supercritical carbon dioxide with very good results [104].

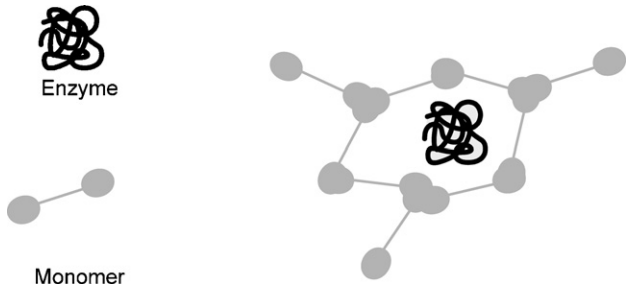

Fig. 4. Immobilization of RML by trapping of the enzyme in a gel or polymerized matrix.

In another paper, RML has been immobilized in lecithin microemulsion-based gels formed with agar and hydroxy (propylmethyl) cellulose. This preparation was used as catalyst of the esterification of 1-propanol with fatty acids in non-polar hydrocarbons, with over 85% yield [105]. RML has been also trapped in *bis*-(2-ethylhexyl)sulfosuccinate sodium salt as well as lecithin water-in-oil microemulsion-based organogels formulated with biopolymers such as agar and hydroxy(propylmethyl) cellulose, respectively [106]. Using these organogels, various esterification reactions in non-polar solvents as well as in solvent-free systems were performed. RML activity was influenced to some extent by the nature and the concentration of biopolymers used, while its stability was much higher than that observed in water/oil microemulsions. In another report, RML was successfully immobilized in hydroxy(propylmethyl) cellulose or agar gels containing lecithin or AOT microemulsions [107]. The preparation of biocompatible microemulsions based on R-(+)-limonene, water, and a mixture of lecithin and either 1-propanol or 1,2-propanediol as emulsifiers was also used to entrap RML [108]. The esterification of octanoic, dodecanoic, and hexadecanoic acids with short-chained alcohols used as co-surfactants for the formulation of the microemulsions was studied. The enzyme efficiency was affected by the chain length of the carboxylic acids and the nature of the alcohol [108]. RML entrapped in surfactant free microemulsion-like ternary systems consisting of *n*-hexane, short-chain alcohols (1-propanol or 2-methyl-2-propanol) and water, efficiently catalyzed the esterification of fatty acids or natural phenolic acids including cinnamic acid derivatives [109]. RML has been also entrap-immobilized on cellulose acetate-TiO_2 gel fiber by the sol–gel method [110]. This fiber-immobilized RML was stable in a phosphate buffer solution and easy to handle, but the expressed activity was quite low.

Other interesting technique to immobilize RML is the relatively new technology of cross-linked enzyme aggregates (CLEAs). This immobilization technique has as main advantage that it is not necessary to use any support; it consists in the precipitation of the enzyme and the cross-linking of the precipitated enzymes with some chemical cross-linker (usually glutaraldehyde) [111–114] (Fig. 5). The enzyme does not need to be pure and it is possible to coprecipitate the enzyme with a polymer to alter the environment of the enzyme [115,116]. For example, RML was precipitated with $(NH_4)_2SO_4$ in the presence of SDS, followed by cross-linking with glutaraldehyde affording CLEAs with two times the hydrolytic activity of the soluble enzymes [117]. These RML preparations

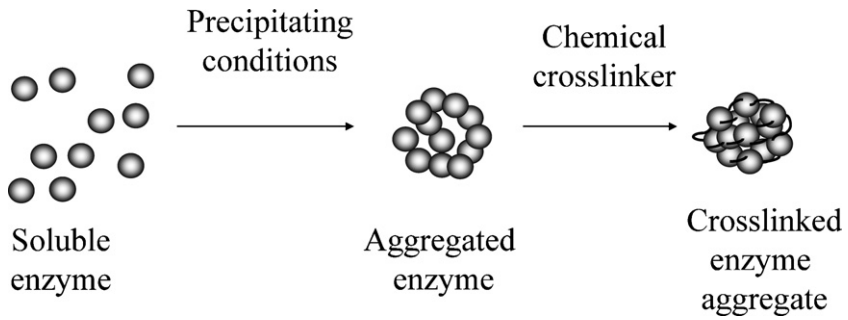

Fig. 5. Immobilization of enzymes via CLEA technology.

presented up to 10-fold enhanced activity in organic medium [117].

The use of pre-existing supports may afford some additional advantages: the final physical properties of the biocatalysts will be defined by the support properties, it is easy to control the enzyme support interaction, and enzymes may be stabilized via multipoint covalent attachment [53]. Its main drawback is that a support needs to be consumed.

A way to reduce the cost of the support is the use of reversible immobilization methods. For example, RML has been immobilized via ionic exchange on different supports like styrene-divinyl benzene polymer resin [118] or ultrafiltration polysulfone hollow fiber membrane chips [119]. RML was one of the examples in the use of supports coated with sulfate-dextran that permits to have a very strong but no distorting adsorption of the proteins [120]. After enzyme inactivation, the protein could be fully desorbed from the support, and then the support could be reused for several cycles. Moreover, the enzyme stability was significantly improved, mainly in the presence of organic solvents, perhaps as a consequence of the highly hydrophilic microenvironment of the support [121,122].

However, as discussed in the introduction, the most popular method to reversibly immobilize lipases in general, and RML in particular, is the use of hydrophobic supports [40]. These supports mimic the hydrophobic surface of the substrates, and the lipase becomes adsorbed on them via interfacial activation (Fig. 6) [40]. Thus, RML has been immobilized, hyperactivated and in some cases even purified by immobilization on a wide variety of hydrophobic supports or support coated with hydrophilic groups: agarose coated, acrylic resins, silicates, etc. [39,42,123–135]. This kind of immobilization has been described to generate a hydrophobic microenvironment around the active center of the enzyme that increases its specificity towards hydrophobic compounds, enabling to stop hydrolysis of multi-esters in a partial one [136]. Moreover, by changing the support and its hydrophobicity, it is possible to control the adsorption strength, activity and even selectivity of the lipase [137]. The adsorption of the lipase on these supports is very strong, making it possible to use the biocatalysts even in the presence of moderate concentrations of organic cosolvents, but full desorption could be achieved by incubation in detergents or sodium guanidine, leaving the support ready for a new load of enzyme [39,40]. Finally, in many instances this immobilization may produce the stabilization of the enzyme [134], making this protocol a very suitable for RML immobilization.

Another technique of enzyme immobilization is the covalent reaction between enzyme and support. We should bear in mind that covalent immobilization implies that after enzyme inactivation, both support and enzyme must be discarded. Therefore, this immobilization technology should be mainly used when a high stabilization of the enzyme is achieved by multipoint or multisubunit immobilization [53,138]. The study of the covalent immobilization of RML has been object of several papers [139,140].

We will comment here in some detail the use of a new kind of heterofunctional epoxy-disulfide support to immobilize chemically thiolated RML [141]. Their use allowed not only the specific immobilization of enzymes through their thiol groups via thioldisulfide interchange, but also enzyme stabilization via multipoint covalent attachment. The stabilization factor observed was around 12–15 when comparing the optimal derivative with just-thiol interchange immobilized counterpart [141]. This was the first assay that finally have driven to the stabilization of proteins by coupling site-directed mutagenesis to tailor-made thiol-epoxy supports to achieve the site-directed rigidification of enzymes via directed immobilization plus multipoint covalent attachment [142].

In another interesting example, RML was covalently immobilized onto a graft copolymer with a backbone of polyethylene and side chains of poly(hydroxyethylmethacrylate), partially hydrolyzed, to alter the support hydrophobicity and the lipase environment [143]. The synthesis of *n*-octyl oleate in the absence of solvents was used as a model reaction. Esterification activity of the immobilized enzyme was five times higher than using the free enzyme. The higher specific activity after immobilization, the purification effect of this process and the high operational and shelf

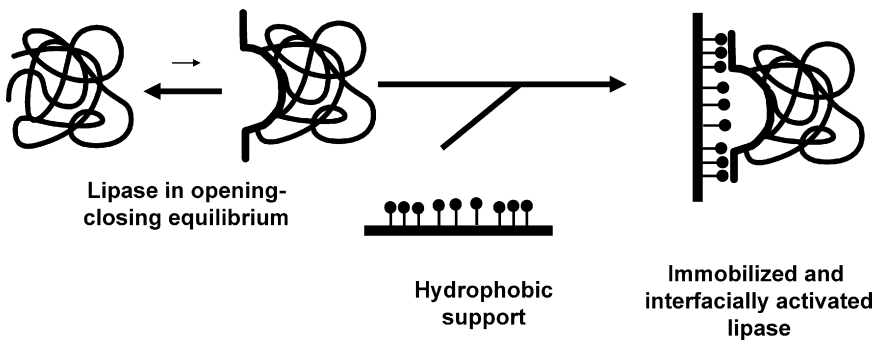

Fig. 6. Immobilization of lipases via interfacial activation on hydrophobic supports.

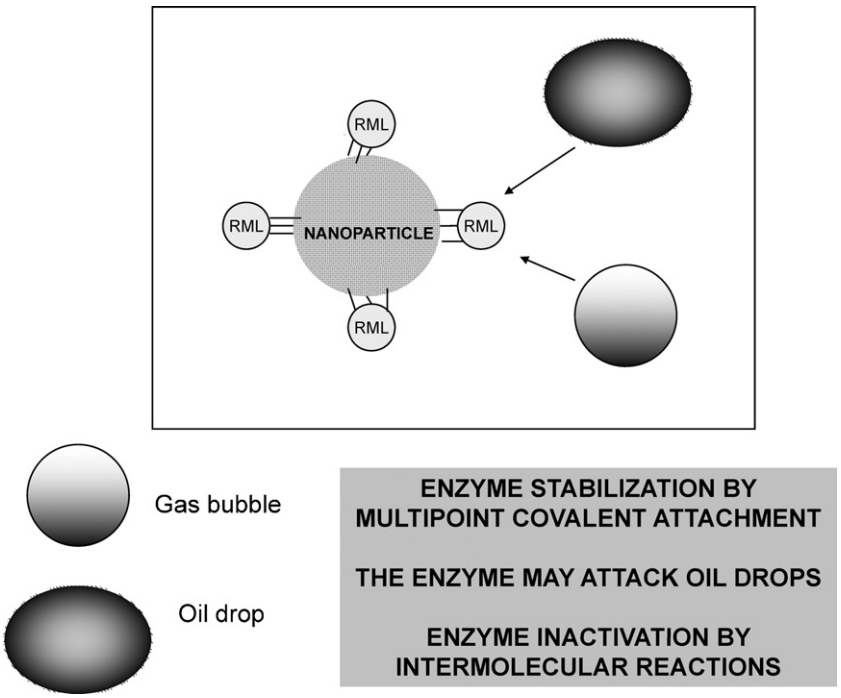

Fig. 7. Advantages and drawbacks of the immobilization of lipases on nanoparticles.

stabilities were mainly attributed to the properties of the copolymer [143].

The advances in nanoscience have made it possible to postulate that magnetic nanoparticles may be used to immobilize proteins in the near future. Thus, Bruno et al. have different reports on the covalent immobilization of RML on polysiloxane-polyvinyl alcohol magnetic particles, using it in the synthesis of flavor esters using heptane as solvent [144] or olive oil hydrolysis [145]. Immobilized enzyme showed to be more resistant than soluble RML when assays were performed out of the optimum temperature or pH [146]. Immobilization on nanoparticles offers some advantages regarding immobilization on porous supports, mainly the decrease of diffusion problems, and the possibility of using the enzymes over solid or no medium-soluble substrates [147]. Although enzymes could be stabilized via multipoint or multisubunit immobilization [53,138], it should be considered that the enzyme will be immobilized on the surface of the support, therefore becoming exposed to the medium (Fig. 7). As a positive effect, RML immobilized on the surface of nanoparticles can suffer interfacial activation by drops of substrates, acting not only on the soluble fraction. However, protein inactivation due to autoproteolysis, interaction with gas bubbles or drops of no water miscible solvent may occur [148,149]. These problems may be prevented if the enzyme is coated with a hydrophilic polymer, which will avoid direct interaction between the immobilized enzyme and any other macromolecule or surface [150].

3. Uses of RML as industrial biocatalyst

3.1. Biodiesel production via transesterification or alcoholysis of oils

Environmental problems associated with the petroleum industry and the foreseen lack of supply in the long term have converted biodiesel in an alternative to diesel, since it is made entirely from vegetable oil or animal fats, thus being renewable and biodegradable [151–153]. Biodiesel is produced by transforming triglycerides into fatty acid alkyl esters, in the presence of an alcohol, such as methanol or ethanol, in most cases using an acid or alkali as catalyst, with glycerol as a byproduct [154]. Alternatively, lipases can be used as biocatalysts in the biodiesel synthesis [25–27]. The main advantages of lipase catalyzed alcoholysis as compared to classical procedures are the mild reaction conditions, the isolation of glycerin without further purification and without the formation of chemical waste, and the ability of lipases to catalyze the

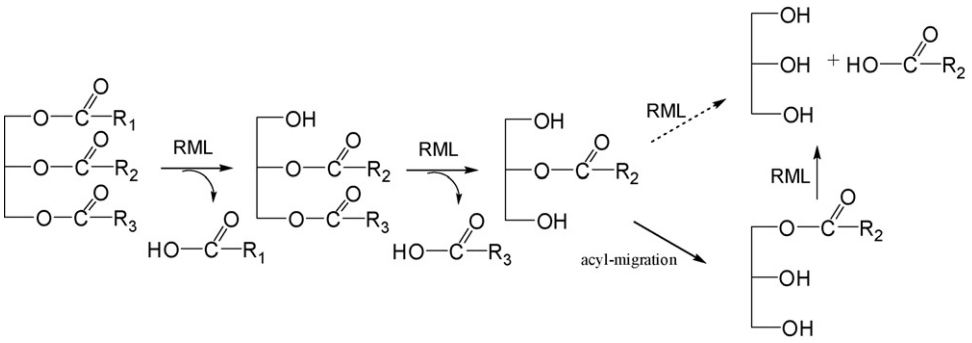

Fig. 8. General scheme of production of biodiesel catalyzed by RML.

esterification of free fatty acids, enabling the use of acidic oils. Moreover, separation and purification of enzymatically produced biodiesel are simplified due to the absence of soap and other byproducts. However, to turn the biocatalytic route economically competitive the development of stable and active biocatalysts is very important in order to improve conversions in the shortest possible time and to allow the reuse of enzyme for many batches [25–27]. The scheme of the reaction is represented in Fig. 8.

In one of the first papers using RML in this process, RML was found to be the most adequate enzyme among several lipases screened for their ability to transesterify triglycerides with short-chain primary alcohols to alkyl esters [155]. Conditions were established for converting tallow to short-chain alkyl esters at more than 90% conversion. These same conditions also proved effective for transesterifying vegetable oils and high fatty acid-containing feedstocks to their respective alkyl ester derivatives. In a further paper, the ethanolysis of sunflower oil with Lipozyme RM IM in a solvent-free medium afforded around 85% yield after optimization [156].

Later, transesterification of soybean oil and methanol catalyzed by Lipozyme RM IM was carried out. Under optimal conditions, a 92.2% weight conversion was achieved in solvent-free system [157] or 76.9% in *n*-hexane (using only 2.37 methanol/oil mole ratio) [158].

Methyl esters were also produced from sunflower oil in different solvents using different lipases. Yields using lipase from *Pseudomonas* were higher than those using RML [159] (90 and 80%, respectively). *n*-Hexane and petroleum ether seem to be the best solvents for this reaction [160]. In this medium, the transesterification could be conducted for at least 120 h during five batch runs without significant loss of enzyme activity.

Biodiesel production from triolein and short-chain alcohols through biocatalysis was performed using different lipases in solvent-free conditions, being also in this case the lipase from *Pseudomonas* more active than RML [161].

In other research involving several lipases, lipase from *Thermomyces lanuginosus* was found to give better yields than RML, but RML was the most stable lipase under the reaction conditions (presence of methanol) retaining its activity over 120 h [162]. RML also showed acceptable conversion levels using cottonseed oil and ethanol, 1-propanol, 1-butanol and isobutanol (50–65% conversion after 24 h) in solvent-free conditions. Isopropyl fatty acid esters obtained by enzymatic alcoholysis of natural vegetable oils can find application in cosmetics industry.

Enzymatic transesterification of sunflower oil in an aqueous-oil biphasic system was studied using RML among other lipases, but RML was discarded in favor of other enzymes [163].

A mathematical model taking into account the mechanism of the methanolysis reaction in hexane starting from the vegetable oil as substrate has been developed using RML [164]. Later, the authors tried to study the effect of the oils and the alcohol in a separate way [165]. Ping-Pong Bi-Bi mechanism with inhibition by both reactants was adopted from the experimental results. In a further paper, the study of the ethanolysis of a raffinate product obtained after distillation of olive oil catalyzed by three commercial lipases (including RML) was also performed to fix a kinetic model of the process [166]. Because the half-life of the enzyme is comparable to or even shorter than the half-life of the reaction, the intrinsic reaction rate and enzyme deactivation must both be considered in modeling the kinetics.

As previously commented, lipases have as an advantage that they can catalyze the production of esters from free acids. The kinetics of the production of biodiesel by esterification of butyric acid with methanol, catalyzed by RML was studied in *n*-hexane microaqueous and biphasic (*n*-hexane/water) [167]. In the microaqueous media, butyric acid did not inhibit the reaction in the range of initial concentrations considered. The initial rate of reaction increased as the initial water content increased up to 25% (v/v). However, the conversion was found to be higher at low initial water concentrations.The results found in literature suggest that the currently available biocatalyst of RML may compete in this reaction with other lipase preparations only from a stability point of view, yields and reaction rates seem to be better using other lipases (from *Pseudomonas* or *T. lanuginosus*). However, the use of improved RML preparations, bearing in mind the modulation that lipases may suffer by immobilization [53], may change the situation in the future.

3.2. Synthesis of esters from carboxylic acids or their esters

Together with biodiesel, some carboxylic esters may be employed in diverse areas. Organic esters are employed as solvents, fragrances, flavors, and precursors in a variety of industries. Particularly, aliphatic esters are used as flavors in food industry and aromatic esters in fragrance compositions. The use of biocatalysts provides an opportunity for carrying out reactions under milder conditions leading to better quality products suitable to be employed in fragrance and flavor industry [168]. The use of natural available substrates and enzymes is an essential part of the process design, because the products produced that way may obtain natural label. As may be observed below, RML has been successfully employed in many esterification reactions, its stability and activity under very low a_w makes this enzyme a very good candidate for this kind of reactions.

3.2.1. Synthesis of esters from aliphatic acids and alcohols

Considering that RML is an enzyme with good activity even at very low water activity (a_w) in different systems (organic solvents, supercritical fluids or ionic liquids), the enzyme is a good candidate to catalyze the synthesis of this kind of products via direct esterification (using free acids) or transesterification (using esters of the acids) [169,170], where the lower the water activity in the system, the higher the synthetic yields [171].

RML adsorbed on polymer beads retains substantial catalytic activity even after exhaustive drying [172]. Over 30% of the maximum RML activity (obtained at a_w 0.55) in the synthesis of dodecyl decanoate could be observed after drying the biocatalyst with anhydrous MgO ($a_w < 10^{-4}$). Further drying caused a further reduction in activity, but the remaining activity was still significant [172]. The activity/a_w profile was essentially the same with most supports tested: polypropylene, anion-exchange resin, celite, and anion-exchange modified silica [173]. A hydrophobic porous glass or a polyamide material support reduced the rate somewhat at intermediate a_w values.

The effect of the RML immobilization support on the enzyme properties has been studied in other papers. The specific activity, in the synthesis of methyl propionate, of immobilized RML adsorbed onto hydrophilic supports, compared to the free lipase, showed that enzyme esterification activity was altered by immobilization [174]. Non-polar solvents were shown to be less harmful for the biocatalyst than solvents with higher polarity. Diethyl ether was used as cosolvent of hexane to improve the solubility of substrates in the organic phase thus, increasing contact with the enzyme. Under optimal conditions, 97% yield could be achieved in a quite rapid way [174].

It should be remembered that the enzyme preparation may greatly affect the enzyme performance on these media [175], making it complex to compare results from different papers.

The comparison between biphasic and monophasic systems was studied in the synthesis of butyl butyrate from *n*-butanol and *n*-butyric acid in *n*-hexane, obtaining similar results [176]. In the biphasic system, the transfer of the ester to the organic phase per-

mitted to have high yields, higher than those obtaining producing ethyl butyrate, where the substrate–products differences in the partition were smaller [176]. Later, a microaqueous medium containing RML in suspension in hexane, a water–hexane two-phase system, and reverse micelles were compared in the same process [177]. From an applied point of view, the best performances were obtained with either microaqueous or liquid–liquid two-phase systems. The use of reverse micelles can be recommended only in particular conditions, such as low enzyme concentration; compatible with the specific constraints that involves the maintenance of a micellar system [177].

In a further study, the performance of RML in the myristic acid esterification in *n*-hexane was compared to that on supercritical carbon dioxide. It was found that RML was very stable in this medium [178]. After a first optimization, authors found higher activity in supercritical medium, but the moderate solubility of the myristic acid in that medium was a drawback to be considered in this reaction [178]. Further studies showed that the inhibition by ethanol was one problem of this reaction, and that an increase of carbon dioxide moisture content from 0 to 0.25% improves the conversion, whereas, beyond this value, RML activity is irreversibly altered [179].

A similar study of the enzyme in the synthesis of nonanyl acetate via transesterification reaction using nonanol and ethyl acetate showed that the transesterification rate in near-critical carbon dioxide proved to be much lower than in hexane at comparable conditions of temperature, water content, and substrate and enzyme concentration [180]. Again, the system was determined by the solubilization of the substrates in the near-critical carbon dioxide.

Some simple esters have interest, like hexyl acetate or hexyl butyrate that are a significant green notes flavor compounds and widely used in the food industry. Hexyl acetate was obtained by transesterification of hexanol with triacetin catalyzed by RML in *n*-hexane or in a solvent-free system, with an 86.6% molar conversion [181]. Later, a re-optimization, using response surface methodology, of the reaction, improved the yields to 98.2% molar conversion [182]. The reaction in supercritical carbon dioxide gave a lower yield (77%) [183]. Similarly, hexyl butyrate was produced via transesterification of hexanol and tributyrin in a solvent-free system catalyzed by Lipozyme RM IM with a yield over 95% [184]. Lipozyme RM IM was used in the production of butyl butyrate in *n*-hexane via esterification with yields around 40% [185].

Solvent-free systems and use of dry isooctane as solvent were compared in the production of various esters via esterification [186]. Using butanoic acid, the solvent-free system afforded lower reaction rates, while using octanoic acid reactions, rates were similar in both systems. Among the studied lipases, Lipozyme RM IM was more resistant to the harmful effect of butanoic acid. This negative effect of butanoic acid could be circumvented by two-step addition of acid substrate [186].

The use of vinyl acetate as activated acyl donor in the transesterification of benzyl alcohol catalyzed by Lipozyme RM IM gave 100% conversion in 10 min [187]. Unlike the chemical catalytic processes, it produced no undesirable side product. This reaction was also studied in ionic liquids, establishing that the activity of RML exponentially decreased with increasing Cl^- content in 1-octyl-3-methylimidazolium *bis*[(trifluoromethyl)sulfonyl] amide [188].

Esterification of oleic acid with oleyl alcohol was performed in supercritical fluids (carbon dioxide, *n*-butane, *n*-propane, *n*-propane/*n*-butane mixture) [189]. The addition of small amounts of water increases the conversion rate. When supercritical *n*-butane was used as reaction medium a decrease of conversion rate was observed.

It was shown that ethyl hexanoate can be synthesized by reaction of ethyl caprate and hexanoic acid in *n*-hexane via acidolysis using immobilized RML, with a yield over 95% [190].

3.2.2. Synthesis of isoamyl esters

Isoamyl esters are fruit flavors with high interest in food industry, and these compounds have been the targets of many studies using RML. Butyl acetate, isoamyl acetate and isoamyl valerate were prepared by RML-catalyzed esterification of free acids and alcohols carried out in non-aqueous systems using heptane and silica gel which removes water formed in the reaction [191]. Under optimum conditions using 40% butyl acetate, at 60 °C, within 48 h, 53% isoamyl acetate and 61% isoamyl valerate conversions were observed. The esterification of butyric acid and isoamyl alcohol in *n*-hexane was later optimized, achieving a 98% yield [192,193]. Large excesses of acid or alcohol reduced the yields due to inhibition of the enzyme. In the synthesis of isoamyl acetate, yields were over 99% [194,195], while in the synthesis of isoamyl isovalerate, yields were over 85%. Hydrophobic solvents including cyclohexane, *n*-hexane, *n*-heptane/isooctane were found the most suitable ones for this reaction [196,197]. The use of RML in the esterification reaction between isobutyric acid and isoamyl alcohol to synthesize isoamyl isobutyrate in *n*-hexane was also studied, obtaining a yield near to 90% [198]. RML was also employed to synthesize ethyl isovalerate by esterification in *n*-hexane with yields near to 50% [197]. A bi-substrate inhibition pattern was observed. It follows a Ping-Pong Bi-Bi mechanism with dead-end inhibition of enzyme by both the substrates [199].

3.2.3. Synthesis of fatty acid esters of hydroxy acids

Fatty acid esters of hydroxy acids like lactic and citric acids and alkyl lactates constitute a very interesting group of surfactants in food industry [200]. The esterification reaction between stearic acid and lactic acid using RML was optimized for maximum esterification using response surface methodology [201]. Stearoyl lactic acid ester formation was found to increase with incubation period and lactic acid (stearic acid) concentrations with maximum esterification of 26.9%. Esterification of lactic acid with palmitic acid catalyzed by immobilized RML has been also reported, with a yield over 40% under optimal conditions [202,203]. Methyl, ethyl, propyl, isopropyl and butyl lactates were worst inhibitors of RML than lactic acid. This resulted in slightly better yields, allowing the preparation of o-palmitoyl alkyl lactates [204]. The highest esterification rate was obtained when producing butyl lactate. Lipozyme RM IM was also employed in the esterification reaction between hydroxy-stearic acid and monohydric fatty alcohols (C_8–C_{18}). The yields of esters were in the range of 82–90% [205]. Hydroxy fatty acid esters of long-chain alcohols have potential applications from lubricants to cosmetics.

3.2.4. Synthesis of esters of terpenes

Esters of terpenes (e.g., citronellol and geraniol) have a large interest for the food industry as flavorings, and these compounds have been target of many investigations using RML. Transesterification of acetate esters and geraniol has been studied. Propyl acetate was found to be the best substrate for geranyl acetate synthesis, and inhibition by excess of geraniol was found [206]. The esterification of lauric acid with geraniol catalyzed by Lipozyme RM IM has been also performed. It was found that the inhibition exerted by water was predominantly a physical effect due to its accumulation around the enzyme. It was also found that the reaction was substrate inhibited by lauric acid, but not by geraniol [207]. Direct esterification of citronellol and geraniol with short-chain fatty acids catalyzed by free RML was performed with high yields in *n*-hexane [208]. The consumption of excess substrate by adding calculated amounts of acid gives a 10% yield enhancement, and leads to 100% pure terpenyl esters. This fed batch strategy was proposed as a way to prevent the damage of the enzyme by excess of acetic acid [209].

Optimization of reaction conditions for the esterification of geraniol from palmarosa oil with *n*-butyric acid was performed

[210]. The elimination of the water formed during the reaction by using sodium sulfate allowed to maintain the enzyme activity even after 5 reuses, keeping the 95% yield obtained in the reaction [210].

RML was found to give the best conversion yields, about 85%, on geranyl butyrate and valerate synthesis, by direct esterification in solvent-free system at 37 °C, among 5 studied lipases [211]. Yields ranging from 96 to 99% molar conversion were achieved after 6 h in the RML-catalyzed esterification of geraniol and citronellol with short and medium chain fatty acids at 55–60 °C [212]. Optimization of the esterification of citronellol and butyric acid reached to a yield of 98% [213]. In the synthesis of geranyl acetate and citronellyl acetate using a solvent-free system, RML permitted to obtain yields from 75 to 77% molar conversion [214]. RML-catalyzed synthesis of geranyl and citronellyl esters of mixed fatty acids has been also performed by alcoholysis of coconut oil, with yields higher than 50% [215].

Using supercritical fluids in the synthesis of geranyl acetate by transesterification catalyzed by RML, it was found that the increase of water content or temperature diminished enzyme stability [216]. Propyl acetate was found to be the best substrate for geranyl acetate synthesis. Considering the reaction rates and the maximum velocity of the reaction, the supercritical fluid system was found to be worse than the conventional organic solvents, such as hexane, for the reaction of transesterification between geraniol and propyl acetate [216].

3.2.5. Synthesis of other esters

Cis-3-hexen-1-yl acetate is another widely used fruity odor in the food industry, which has been produced by RML with yields near to 90% [217].

Other esters with more specific uses have been also produced using RML. The synthesis of amphiphilic molecules type fatty hydroxamic acids was one of the first examples of synthesis of unusual esters catalyzed by this enzyme [218]. The reaction was studied using both fatty acids in their free or methyl ester form, obtaining good results in both cases.

Estolides and particularly, estolide esters may be suitable as lubricants or lubricant additives. Esterification of estolides improved their properties (for example, lower viscosity and higher viscosity index) but slightly raised the melting point. Immobilized RML was chosen among 8 lipases to catalyze the esterification of mono- and polyestolide with fatty alcohols or α,ω-diols. Yields were >95% for fatty alcohol reactions and >60% for diol reactions [219]. In addition, the estolide linkage remained intact through the course of the esterification process (because the RML was unable to hydrolyze this bond).

Lesquerolic acid wax and α,ω-diol esters were synthesized at preparative scale by Lipozyme RM IM catalyzed esterification of lesquerolic acid or alcoholysis of lesquerella oil [220].

Esterification of organosilicon alcohol with different fatty acids in organic solvent was also accomplished using RML [221].

(*Z*)-3-Hexen-1-yl butyrate is an important flavor and fragrance compound as it represents the model of a natural herbaceous (green) note. The synthesis of (*Z*)-3-hexen-1-yl butyrate by direct esterification in *n*-hexane yielded 95% using Lipozyme RM IM, although Novozym 435 (immobilized lipase B from *C. antarctica*) gave higher reaction rates [222].

Several lipases were compared in the synthesis of different esters of pentanoic and stearic acids with acetylenic and olefinic alcohols (with different size) [223]. The esterification of C_{11}, C_{18}, and C_{22} acetylenic alcohols with pentanoic acid appeared to be generally unaffected by the presence of an acetylenic bond in the alcohol as relatively high yields of the corresponding esters (78–97%) were obtained. Esterification of short-chain acetylenic and olefinic alcohols was most efficiently achieved using RML or Novozyme 435, while in other cases reaction rates were very low. The same authors performed a similar study, using acetylenic olefinic acids of different size and *n*-butanol in *n*-hexane [224].

The esterification of some natural antioxidants such as cinnamic acid derivatives and ascorbic acid in non-aqueous media was also investigated [225]. The alcohol chain length affected the rate of esterification of cinnamic acids. Higher reaction rates were observed when the esterification was carried out with medium or long-chain alcohols. The acyl donor structure also influenced the reactions rates. Higher yields were observed for the esterification of cinnamic acid (59%) catalyzed by RML, while Novozym 435 presented better activity for the esterification of ascorbic acid. In a further research, oleyl *p*-coumarate and oleyl ferulate were produced in a solvent-free system, RML showing only a moderate activity compared to Novozym 435 [226,227].

Esterification of retinol reduces photodestruction and irritation problems characteristic of retinol. In this context, retinyl adipate, retinyl succinate, retinyl oleate and retinyl lactate (greatly appreciated by cosmetic manufacturer) were also produced by esterification catalyzed by RML [228].

Sterols, stanols and steroids have been converted in high to near-quantitative yields to the corresponding acyl esters via esterification with fatty acids and transesterification with methyl esters of fatty acids or triacylglycerols using Lipozyme RM IM. For example, sitostanol has been converted in high to near-quantitative extent to the corresponding long-chain acyl esters via esterification with oleic acid or transesterification with methyl oleate or trioleoylglycerol using Lipozyme RM IM in vacuo (20–40 mbar) at 80 °C [229]. Saturated sterols such as sitostanol and 5α-cholestan-3β-ol were the preferred substrates as compared to Δ^5-unsaturated cholesterol in transesterification reactions with methyl oleate, the enzyme activity was retained even after 10 repeated uses of the biocatalyst. Other cholesterol derivatives were produced in this work [229]. Later, sterols, stanols and steroids have been converted in high to near-quantitative yields to the corresponding acyl esters through the esterification with fatty acids and transesterification with methyl esters of fatty acids or triacylglycerols using Lipozyme RM IM. Esterification of canola phytosterols with oleic acid could be performed with RML, but at much lower rates than using Novozym 435 [230].

Esters of *m*- and *p*-cresols with organic acids having carbon chain lengths C_2–C_{18} have been prepared by using RML, *p*-cresyl laurate synthesis afforded over 80% using high amount of enzyme [231].

The enzymatic synthesis and hydrolysis of alkyl sebacates and *o*-, *m*-, *p*-phthalates via alcoholysis of dimethyl phthalates and dimethyl sebacate with 2-ethylhexanol and 3,5,5-trimethylhexanol in a solvent-free medium, using Lipozyme RM IM as catalyst have been carried out, although results were better using Novozym 435 [232].

Esterification of α-terpineol with acetic anhydride or propionic acid mediated by RML has been also described, obtaining better results using propionic acid than using acetic anhydride [233].

3.3. Resolution of racemic mixtures

Lipases may be used to resolve racemic mixtures of chiral esters (via hydrolytic routes) or chiral acids or alcohols (via synthetic routes) [22,23].

3.3.1. Resolution of racemic mixtures by hydrolysis

The resolution of racemic mixtures via hydrolysis catalyzed by RML is one of the uses of the enzyme, although there are not so many examples as resolution via synthetic strategies [234] (Fig. 9).

2-Methyloctanoic acid esters of *rac*-2,3-isopropylidene glycerol and *rac*-glycidol were hydrolyzed with several lipases, RML among them. The *S* configuration of the acid reacted faster [235]. The (*S*)-

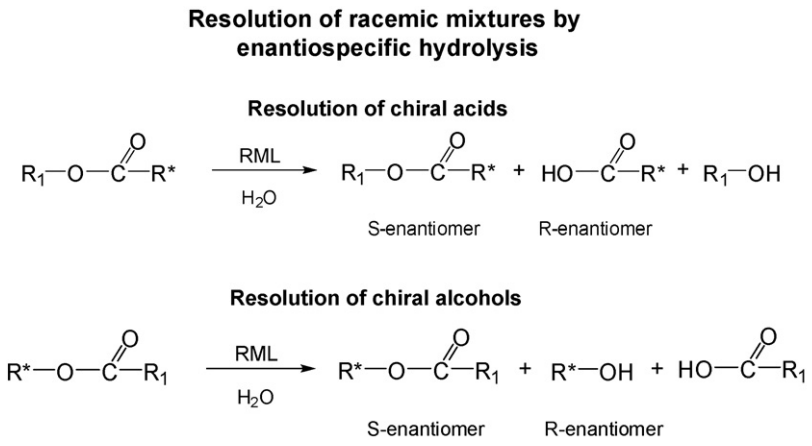

Fig. 9. General scheme of resolution of racemic mixtures via hydrolysis catalyzed by RML.

2,3-isopropylidene glycerol esters and (*R*)-glycidol esters reacted faster than did the esters of the enantiomeric alcohols. For RML, the most promising alcohol candidate for resolving 2-methyloctanoic acid by hydrolysis appear to be (*S*)-glycidol.

RML was also the best catalyst in the enantioselective hydrolysis of racemic oxathiolane, giving as a result the enantiomerically enriched residual ester of the correct absolute stereochemistry, (−)-*R*, for subsequent synthesis of the anti-viral agent lamivudine [236].

The hydrolysis of racemic 3-(4-methoxyphenyl)glycidic acid methyl ester by Lipozyme RM IM in supercritical CO_2 allowed to get a stereoisomeric excess of the (2*R*,3*S*)-form to reach 87% at 53% total conversion level [237]. The reaction rate in supercritical CO_2 was considerably faster than in the toluene/water-mixture.

Different racemic arylpropionic esters, precursors of therapeutically important non-steroidal anti-inflammatory drugs, were subjected to enantiospecific hydrolyses catalyzed by several lipases, in some cases RML reached a very high enantiomeric excesses (ee) [238].

On the contrary, kinetic resolution of 4-methylhexanoic acid methyl ester and 4-methyloctanoic acid methyl ester by transesterification using RML gave no discrimination for the last, and only an *E* value of 2 for the first [239], showing the unsuitability of the enzyme for this process.

Enantiopure (*R*)- and (*S*)-3-chloro-1-(2-methoxyphenoxy) propan-2-ol have been produced by kinetic resolution of the corresponding racemic butanoate by hydrolysis catalyzed by Lipozyme RM IM or Novozym 435 [240].

An irreversible resolution of ketoprofen prodrug was developed by RML-catalyzed hydrolysis in water/dioxane using the corresponding vinyl ester as activated substrate [241]. The product obtained, (*S*)-ketoprofen vinyl ester (ee > 99%) would be used as a potential prodrug and a useful monomer for preparing polymeric drugs.

In many cases, the resolution of a compound is intended via hydrolytic (using the ester) and synthetic (using the alcohols or the carboxylic acid) routes in the same paper. RML was found to be the optimal biocatalyst to obtain (*R*)- and (*S*)-2-octanol by kinetic resolution through hydrolysis of the racemic octanoate ester and by esterification of the racemic alcohol with octanoic acid [242]. In another example [243], enzyme-catalyzed reactions in organic media of *rac*-ketoprofen esters with different nucleophiles such as alcohols, amines, and water have been also reported, being again RML the best enzyme among the assayed ones [243]. The preferred substrate was the trifluoroethyl ester of *R*-ketoprofen and the optimal reaction media diisopropyl ether as solvent. The transesterification with 1-butanol gave 90% yield of (*S*)-ketoprofen (88% ee), the transesterification with 2-(2-pyridyl)ethanol gave 94% yield (92% ee), and the hydrolysis in wet organic solvent gave 93% yield and 97% ee.

Phosphocarnitine was conveniently obtained from easily available diethyl 3-chloro-2-oxopropanephosphonates, followed by subsequent reduction, RML mediated resolution, amination and dealkylation [244].

Considering the flexibility of the active center of lipases, it may be easy to alter the enzyme properties [49–57], but difficult to guess the final result of a given change. Several strategies have been intended to alter the enzyme enantioselectivity.

The construction, purification and enantioselectivity of two recombinant RML designed to catalyze the reaction of ring opening of oxazolin-5(4H)-ones has been studied, analyzing if active site models can help to design improved enzymes [245]. However, the predicted mutations did not eliminate enantioselectivity in the case of the *tert*-butyl oxazolinone, perhaps due to the small size of the isopropyl substituent. This work demonstrates the inherent difficulty in extending active site models to re-engineering protein function, as may be expected from the high flexibility of the active center of lipases.

In another approach, RML was chemically modified. The native enzyme prefers to hydrolyze S-enantiomer of 1-heptyl 2-methyldecanoate ($E(S) = 8.5$) but the *R*-enantiomer of phenyl 2-methyldecanoate ($E(R) = 2.9$). The specific chemical modification of arginines with 1,2-cyclohexanedione resulted in a decreased enantioselectivity ($E(R) = 2.0$), only when the phenyl ester was used as a substrate [246]. In contrast, treatment with phenylglyoxal showed a decreased enantioselectivity ($E(S) = 2.5$) only when the heptyl ester was used as a substrate. The presence of soluble guanidine, an arginine side chain analog, decreased the enantioselectivity with the heptyl ester ($E(S) = 1.9$) and increased the enantioselectivity with the aromatic ester ($E(R) = 4.4$). The enantioselectivities in the esterification of 2-methyldecanoic acid with 1-heptanol were unaffected by the lid modifications. This suggests the high complexity of the factors given a final enzyme performance.

RML was also used as a model of how the immobilization of the enzyme via different areas may alter its selectivity. RML was covalently immobilized on different epoxy resins (standard hydrophobic epoxy resins, epoxy-ethylenediamine, epoxy-iminodiacetic acid, epoxy-copper chelates) and adsorbed via interfacial activation on octadecyl-Sepabeads support (fully coated with very hydrophobic octadecyl groups) and used in the hydrolytic resolution of (*R*,*S*)-2-butyroyl-2-phenylacetic acid [247]. Different catalytic properties (activity, specificity, enantioselectivity) were found to depend on the particular support used. For example, the octadecyl-RML preparation was the only immobilized enzyme

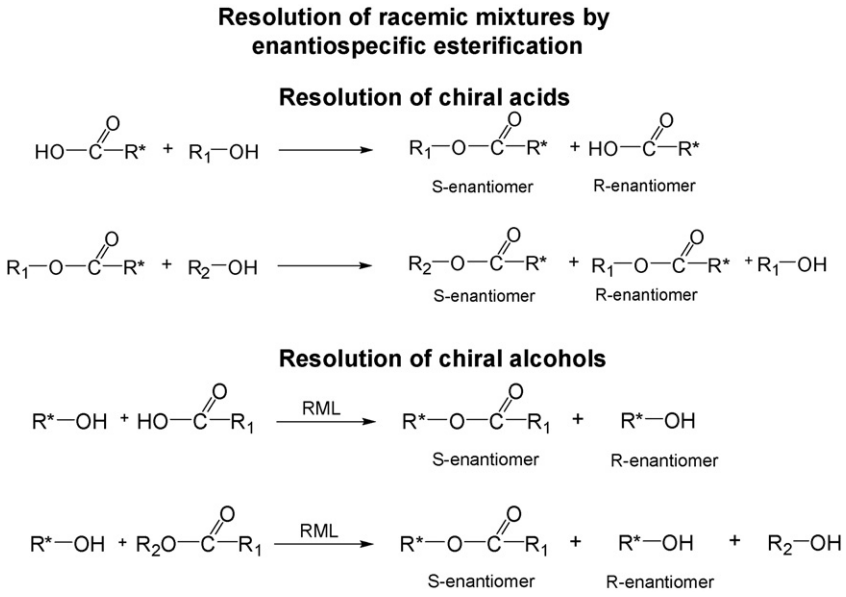

Fig. 10. General scheme of resolution of racemic mixtures via esterification catalyzed by RML.

derivative which exhibited significant enantioselectivity towards the *R* isomer (with *E* values ranging from 5 at 4 °C and pH 7 to 1.2 at pH 5 and 25 °C). The other immobilized preparations, in contrast, were S selective. Immobilization on iminodiacetic acid-Sepabeads afforded the catalyst with the highest enantioselectivity ($E(S) = 59$ under optimum conditions).

RML enantiospecificity towards racemic dicaprin spread as a monolayer at the air–water interface was investigated. It was increased by lowering the surface pressures (while decreasing the enzyme catalytic activity) [248].

Thus, RML has been successfully employed in many resolutions through hydrolysis of racemic mixtures. In any case, the full change of the enzyme properties by immobilization [247] or by lipase–lipase interactions if using soluble enzyme [47], makes complex to compare results from different laboratories.

3.3.2. Resolution of racemic mixtures by ester synthesis

In a previous review, it was stated that RML seems to be more suitable for resolution by esterifications and transesterifications reactions than hydrolysis [234]. Perhaps by this reason, there are many more examples of uses of RML via these routes than hydrolytic ones. The general scheme of the enantioselective esterifications is in Fig. 10.

Some mechanistic explanation of the enzyme properties may be formulated based on the knowledge of the enzyme structure. For example, the enantioselectivity of RML towards 1-phenylethanol, 2-hexanol and 1-phenylethanol acetate is controlled by the formation of a tetrahedral intermediate, whereas Michaelis complex formation has a much lower significance [249]. The stereoelectronic considerations and the molecular modeling using an X-ray structure of RML suggested that the catalyzed reactions proceed under stereoelectronic control [250]. The enantioselectivities for 1-phenylethanol, l-phenyl-2-propanol, and 1-cyclohexylethanol were estimated in terms of the RML-induced strain caused at the transition state. Again, results pointed that the enantioselectivity in the lipase-catalyzed transesterifications arises from the difference in V_{max} between the two enantiomers rather than from the difference in K_m. This indicates that the ability of RML to discriminate between the enantiomers at the transition state is high, while the ability to recognize the chirality in the binding step is poor. Furthermore, the difference in V_{max} between the enantiomers was found to result not from the enhanced reactivity of the (*R*)-enantiomers but from the reduced reactivity of the (*S*)-enantiomers [250].

Novozym 435 was found to be more active but less enantioselective than Lipozyme RM IM in the alcoholysis of dimethyl adipate by *rac* 2-ethylhexanol as well as neopentyl glycol [251]. The less reactive enantiomer was *R*-(−)-2-ethylhexanol. However, the racemization of di-2-ethylhexyl adipate decreased the observed enantiomeric excess.

In another example, the relative rates of enzyme-catalyzed esterification of the enantiomers of 2-octanol with various acids were determined for several commercial lipases, being RML the one with the highest enantioselectivity [252]. In the same paper, RML was employed to prepare 8-methyl-2-decanols with high configurational purity at the carbinol carbon.

Using RML, three different methods have been scaled-up for the resolution of *rac*-ketoprofen: transesterification with 1-butanol (90% yield of (*S*)-ketoprofen, 88% ee), transesterification with 2-(2-pyridyl)ethanol (94% yield, 92% ee), and hydrolysis in wet organic solvents (93% yield, 97% ee) [243].

Other research report showed that meso-compounds 1,2-dihydroxycyclopentane, 1,2-dihydroxycyclohexane and 1,2-dihydroxycycloheptane were desymmetrised by enantioselective esterification with vinyl acetate in *tert*-butyl methyl ether catalyzed by Lipozyme RM IM and other lipases [253]. The obtained products were (1*S*,2*R*)-acetate (+)-1,2-dihydroxycyclopentane, (1*R*,2*S*)-acetates (-)-1,2-dihydroxycyclohexane and (+)-1,2-dihydroxycycloheptane in good enantiomeric excesses and chemical yield.

The use of Lipozyme RM IM for the esterification of (±)-2-phenyl-4-*tert*-butyloxazolin-5(4H)-one in toluene containing *n*-butanol and a catalytic amount of triethylamine resulted in a 95% yield of (*S*)-N-benzoyl *tert*-leucine butyl ester (99.5% ee)[254]. The two step hydrolysis of this product (using Alcalase and 6N HCl, reflux) yielded homochiral L-(*S*)-*tert*-leucine.

Other paper shows the enantioselective acetylation of *cis*-1,2-dihydroxycyclohexa-3,5-diene with vinyl acetate in *tert*-butyl methyl ether. This reaction catalyzed by Lipozyme RM IM, yielded (1*R*,2*S*)-1-acetoxy-2-hydroxycyclohexa-3,5-diene in good chemical yield and with high enantiomeric excess [255].

The resolution of *rac*-suprofen by direct esterification with methanol in toluene, catalyzed by RML adsorbed in celite, gave (−)-(*R*)-suprofen with good optical purity [256].

Rac-2-hydroxymethyl-1-phenylthioferrocene and 2-hydroxymethyl-1-*tert*-butylthioferrocene were subjected to kinetic resolution via acetylation catalyzed by Lipozyme RM IM [257]. The obtained enantiopure thioethers underwent chemical oxygenation at the sulfur atom to give the corresponding ferrocenyl sulfoxides with settled central/planar chirality.

Conduritol and their precursors has been the subject of many modifications using RML. Lipozyme RM IM was used to catalyze the enantiomeric alcoholysis of (±)tetraacetylconduritol to give enantiopure (1*R*,2*R*,3*R*,4*R*)-tetrahydroxycyclohex-5-ene (−)-conduritol E, and the unreacted ester (1*S*,2*S*,3*S*,4*S*)-tetraacetyloxycyclohex-5-ene [258]. The latter was transformed by basic hydrolysis into the desired product in high yield and 95% ee. Later, other derivatives of conduritol E were produced using chemoenzymatic routes [259]. Two partial esters of conduritol E, (2*R*)-hydroxy-(1*R*,3*R*,4*R*)-triacetoxycyclohex-5-ene and (1*S*,2*S*)-dihydroxy-(3*S*,4*S*)-diacetoxycyclohex-5-ene, were chemically prepared and used for the enantioselective RML-catalyzed esterification of the resulting conduritol E diacetate. After, (±)1,2-diacetylconduritol E [260], through complementary use of Lipozyme RM IM and lipase from *Candida cylindracea*, was transformed in (1*S*)-1,2-diacetylconduritol E, (1*R*)-1,2-diacetylconduritol E, (1*S*)-1,2,4-triacetylconduritol E, (1*R*)-1,2,4-triacetylconduritol E, with high enantiomeric excesses and chemical yields. Several lipases, among them RML, catalyzed the alcoholysis of *rac*-conduritol-B peracetate, by *n*-butanol to give enantiopure (2*S*,3*S*)-diacetoxy-(1*R*,4*R*)-dihydroxycyclohex-5-ene and (1*S*,2*R*,3*R*,4*S*)-tetraacetoxy-cyclohex-5-ene in *t*-butylmethylether [261]. 2,3-Dibromocyclohex-5-en-1,4-diol (useful to produce conduritols) was resolved using RML operating in an organic solvent by alcoholysis [262].

Enantiospecific esterification of 2-arylpropionic acids catalyzed by RML has been also reported [263]. Lipozyme RM IM showed *S*-(+) enantiorecognition in all cases, except for (*R*,*S*)-ketoprofen, where *R*-(−) stereobias was observed.

In other example, an azole antifungal agent, SCH 56592, was produced using a chemoenzymatic approach [264]. RML was used to catalyze the diastereoselective acylation of 2-benzyloxy-3-pentanol to produce (2*S*,3*R*) ester in >97% diastereomeric excess.

An efficient stereocontrolled synthesis of (*S*)-*N*-benzyloxycarbonyl-serine and of its (*R*)-enantiomer was reported by the kinetic resolution of racemic 3-(hydroxymethyl)-1,4-benzodiazepins via acetylation by the immobilized Lipozyme RM IM giving a high enantiomer purity (ee around 99%) [265].

In other paper, 5-[4-(1-hydroxyethyl)phenyl]-10,15,20-triphenylporphyrin and its zinc complex were resolved using different lipases, including RML, via transesterification reactions with high enantioselectivities ($E > 100$)[266]. The enzyme recognizes the *R*-enantiomer giving, at first, the monoester (+). Using conditions where the spontaneous hydrolysis of the products does not occur, alcoholysis reached to 50% conversion giving enantiopure products.

The resolution of esters of 4-chloro-3-hydroxybutanoic acid by transesterification in organic solvents was investigated using various enzymes. RML was the most efficient lipase with the enantiomeric ratio being dependent upon of the nature of the alkoxy group of the ester and the reaction medium [267].

In another example, the one-step synthesis of optically active alpha-monobenzoyl glycerol by RML-catalyzed transesterification of benzoate derivatives with glycerol in 1,4-dioxane using vinyl benzoate as an acyl donor is described [268]. RML and CALB were the only enzymes able to catalyze the reaction, unfortunately being the results with RML not very good (yield 87%, ee 15%, *R*-enantiomer produced).

Enzymatic enantiomeric synthesis of (*S*)-2-methylbutanoic acid methyl ester (an apple and strawberry flavor) was studied. Among 20 lipases, Lipozyme RM IM exhibited one of the highest enzymatic activities and enantioselectivities [269].

The enantioselective resolution of ibuprofen by Lipozyme RM IM was performed using isooctane as solvent and butanol as esterificating agent [270]. After optimization, enantiomeric excess and total conversion values were 93.8 and 49.9%.

The alcoholysis of (±)-2-phenyl-4-benzyloxazol-5(4H)-one using 1-butanol as the nucleophile in low-water organic solvents was performed using several lipases [271]. Although the intrinsic selectivity of RML was lower than that found for CALB, both triethylamine and 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic Acid (CAPSO)/CAPSO·Na enhanced both the activity and selectivity of the enzyme in the production of the (*S*)-butyl ester.

Several lipases were screened for their ability to catalyze the enantioselective transesterification of 2-bromo-*o*-tolyl acetic acid [272]. Among the preparations tested, the best enantioselectivity in the production of *S*-octanol-2-bromo-*o*-tolyl acetic acid was obtained with immobilized RML ($E = 11.3$), which was more stereoselective than the free form.

RML was described to efficiently catalyze the enantioselective esterification of *rac*-1-(3-trifluoromethylphenyl)propan-2-ol [273]. The obtained *S* enantioforms are suitable to prepare both enantiomers of fenfluramine.

The preparation of chiral 1- and 2-hydroxyalkanephosphonates bearing a trifluoromethyl moiety with high enantiomeric excess via RML alcoholysis has been also reported [274].

In another paper, the synthesis of acyclovir and L-ascorbic acid with divinyladipate was performed with RML in different anhydrous organic solvents by transesterification reaction [275].

Direct enzymatic esterifications catalyzed by RML using some primary alcohols with a chiral center at the next carbon atom (2-methoxy-2-phenylethanol, 2-phenyl-1-propanol and 1-phenyl-1,2-ethanediol) and different carboxylic acids has been also studied [276]. It was found that RML has strict substrate selectivity towards both the acid and the alcohol, although CALB presented better *E* values.

Asymmetrization of prochiral 2-methylpropane-1,3-diol by RML catalyzed acylation with vinyl benzoate affords the corresponding (*S*)-monobenzoate (65% ee), that can be obtained enantiomerically pure in 40% yield by a sequential benzoylation procedure at 58% conversion of the diol to the corresponding dibenzoate [277].

RML was found to be the most efficient enzyme regarding enantiomeric excess (ee) and yield in the kinetic resolution of the racemic *cis*- and *trans*-isomers of 2-(4-methoxybenzyl)cyclohexanol by esterification with acetic acid [278].

The enzymatic kinetic resolution of atropisomeric (±)-3,3′-*bis*(hydroxymethyl)-2,2′-*bi*pyridine *N*,*N*-dioxide was performed via enzymatic enantioselective esterification in alcohol/vinyl acetate (20:80) [279]. Lipozyme RM IM was found to show good enantioselectivity with an (*aS*)-enantiopreference in the axial recognition, and allowed to efficiently perform the preparation of both enantioforms with ee >98%.

ILs were found to act as excellent non-aqueous reaction media for the RML-catalyzed kinetic resolution of *rac*-glycidol by using vinyl ester as acyl donor, allowing to improve the RML activity almost by a 100-fold factor when compared to toluene [280]. *R*-Glycidyl esters were preferentially obtained. In the same paper, supercritical CO_2 was also used with good results.

As in the hydrolytic reactions, enantioselectivity of RML may be greatly altered by the reaction conditions (solvent, temperature) or immobilization conditions. However, changes are difficult to predict.

For example, using RML lyophilized from buffer, the preparations were found to be much more enantioselective at 7 than at

45 °C in dioxane, nitromethane and acetonitrile, while in tetrahydrofuran, triethylamine, and pyridine the enantioselectivity is relatively unaffected by temperature [281]. The way of preparing the lyophilized lipases also greatly altered the influence of the experimental conditions on the enantioselectivity of the enzyme.

The effect of immobilization on activity, stability, and enantioselectivity of RML for the kinetic resolution of 1-phenyl ethanol with vinyl propionate in organic solvents was studied in other paper [282]. RML was trapped in photo-cross-linkable resins of the hydrophilic type ENT 1000. The immobilized enzyme retained 25% esterification activity of the native enzyme and also the high enantioselectivity of the free enzyme (200). The entrapped enzyme was successfully applied in a packed-bed reactor for 72 days of continuous resolution of enantiomerically pure 1-phenyl ethanol. After, the productivity was more than one magnitude higher than could be expected from the activity data, suggesting some favorable change in the enzyme during operation [282]. This may be again based on the flexibility of the active center of lipases in general and RML in particular.

RML freeze-dried powders rinsed with *n*-propanol was found to be more efficient that the non-rinsed enzyme preparation, when employed for kinetic resolution of (R,S)-β-citronellol using vinyl acetate as acylating reagent in solvent-free media [283]. This preparation gave 90% ee for (*R*)-(+)-β-citronellyl acetate at 45% conversion ($E = 42$).

Together to the good stability in anhydrous media, and the good esterification activity described above, RML shows a good enantioselectivity in esterification reactions, making this enzyme one of the most promising for this kind of reactions.

3.4. Use of the regioselectivity of RML

3.4.1. Hydrolytic reactions

There are some examples where the regioselectivity of RML to selectively hydrolyze one among several similar esters has been used to produce interesting compounds.

In one example, RML-catalyzed hydrolysis of 1,3-*bis*[3,5-*bis*(ethoxycarbonyl)-lH-pyrazol-l-yl]propane was used to produce 3-mono- and 3,3′-diacids [284]. These compounds are useful intermediates to synthesize podand and crown esters. In another instance, polyenyl derivatives of permethylated 6-amino-6-deoxy-β-cyclodextrin were obtained by polycondensation of acetaldehyde on permethylated 6-amino-6-deoxy-β-cyclodextrin [285]. The immobilized RML increased the rate of this unexpected reaction, as well as permethylated 6-amino-6-deoxy-β-cyclodextrin for the water uptake to form the enamine.

Preparation of 98% ee (*R*)-4-chloro-2-butanol was carried out by the enzymatic hydrolysis of chlorobutyl palmitates using several enzymes [286]. Lipozyme RM IM, using *tert*-butanol as solvent, hydrolyzed 91.1% of 3-chlorobutyl palmitate but also hydrolyzed 4-chloro-2-butyl palmitate (8.9%). Moreover, regioselective hydrolysis of crotepoxide has been studied employing various commercially available lipases being RML one of the enzymes that gave good regioselectivity and activity [287]. The presence of epoxide rings make interesting the use of mild biocatalytic routes to liberate hydroxyl groups. RML catalyzed reaction, though kinetically slow, afforded 3-deacetyl-7-debenzoyl crotepoxide as the only product.

3.4.2. Esterification reactions

There are also some papers devoted to the use of RML to catalyze esterification reactions of polyol or poly acid compounds, trying to reduce the number of products.

For example, modification of hydroxyl groups of β-cyclodextrin and its methyl and hydroxypropyl derivatives using RML in *n*-heptane at 50 °C has been carried out using many different carboxylic acids [288]. In another paper, the esterification of 2-chlorobutyric acid and 1,2-epoxy-5-hexene catalyzed by immobilized RML to produce 2-chloroesters was reported [289]. The selectivity presented by the biocatalyst towards the studied ester considerably decreased the final product distribution.

The RML-catalyzed esterification of one or two hydroxyl groups of an amino acid glyceryl ester derivative by lauric acid has been also described [290]. The method developed allowed to prepare glycerol-conjugates of arginine, aspartic acid, glutamic acid, asparagine, glutamine and tyrosine in isolated yields of the regioisomeric mixtures ranging from 22 to 69%

Immobilized RML showed high regioselectivity towards the secondary hydroxyl of methyl shikimate, which presents three hydroxyl groups with similar reactivity [291]. The reaction catalyzed by RML in acetone facilitated the single step synthesis of 5-*O*-acyl methyl shikimate derivatives in high yields. In other research reports, it was found that RML produced the regioselective acetylations of the eudesmane tetrol from vulgarin, yielding only the 12-acetyl derivative [292]. In another paper, RML-catalyzed esterification of propylene glycol (which bears primary and secondary alcohol groups) with different carboxylic acids was studied [293]. It was found, by using several solvents, that polarity could influence the product profile in situations in which multiple products of various polarities can be formed. Thus, production of primary monoester is increased in a relatively polar solvent such as *tert*-butyl methyl ether ($\log P = 1.4$) [293]. The RML-catalyzed monobenzoylation of 1,4-diols in an organic solvent has been also reported [294].

3.4.3. Acidolysis

In some cases, a free carboxylic acid is used to produce the breakage of an ester bond. For example, the regioselectivity of RML was used to carry out an acidolysis process. In this example, RML, selected among other lipases, was used to catalyze the acidolysis reaction between 1,2-diacyl-3-*O*-[β-galactopyranosyl]-(1-6)-*O*-[α-galactopyranosyl]-glycerol (DGDG) and heptadecanoic acid in toluene [295]. A mixture of DGMG, DGDG, acyl-DGMG and acyl-DGDG was obtained. The extra acyl group is bound to the primary hydroxyl of the digalactosyl moiety.

3.4.4. Transesterifications

Regioselective transesterifications catalyzed by RML is perhaps the most widely used strategy. This strategy uses alcohols as nucleophiles to break the ester bond. For example, the transesterification of methyl acrylate telomers by RML catalysis in toluene was found to be highly regioselective. Actually, only the ester functions of the end-groups (A and C functions) and those of the monomer units linked to the telogen segment (B function) were modified [296]. The reactivity of the latter was not expected according to data on the specificity of the RML. In a further paper, using other derivatives, it was concluded that the B function is still reactive whatever the structure of the incorporated telogen [297]. RML produced (+)-(1*R*,2*R*,3*S*,4*S*)-1-hydroxy-2,3,4-triacetoxy-5-cyclohexene and (+)-(1*R*,2*R*,3*S*,4*S*)-3,4-diacetoxy-1,2-dihydroxy-5-cyclohexene by transesterification of *meso*-conduritol D tetraacetate with *n*-butanol [298]. These compounds are of potential utility in the synthesis of cyclitols and aminocyclitols. Different morin acetates have been prepared by alcoholysis of peracetate morins in tetrahydrofuran with butanol, using RML as catalysts [299]. The enzyme liberates with comparable rates ester groups at position C-7 on A ring and C-4′ on B ring. Prolonging alcoholysis time, a morin ester having free OH groups at C-7 and C-4′, 3,5,2′-triacetylmorin, was produced.

RML regioselectively catalyzes the transesterification of 3,5-diethoxycarbonyl-pyrazole derivatives with octanol, producing good yields of new pyrazole esters with aliphatic and polyetheric

chains [300]. Later, RML was used in the transesterification of 16 aromatic and heteroaromatic esters with octanol in organic solvents [301]. The reactions took place in moderate to good yields and, in some cases, regioselectively.

Diester crowns have been prepared by regioselective RML-catalyzed transesterification with aliphatic primary alcohols [302]. The synthesized new ester crowns include a 1,3-bis(1H-pyrazol-1-yl)propane unit as a part of the macrocycle. Acyclic intermediates were also obtained. Later, the regioselective RML-catalyzed transesterification of the dipyrazolic tetraethylester with monomethylether polyethyleneglycols to produce podands (acyclic crown ethers) was described [303].

Long-chain 3-*O*-acylcatechins were prepared in high yield by alcoholysis with *n*-butanol of the corresponding penta acyl derivatives in the presence of Lipozyme RM IM [304]. In an alternative procedure, the mixed ester, tetraacetyl-3-*O*-acylcatechin, was synthesized and used as substrate for the same alcoholysis process that proceeds with higher reaction rate [304].

The polyol, trimethylolpropane (2-ethyl-2-hydroxymethyl-1,3-propanediol), and a mixture of rapeseed oil fatty acid methyl esters were transesterified by Lipozyme RM IM without additional organic solvent [305]. Yields over 90% were obtained for the triester, therefore, the reaction was not very regioselective.

In other cases, an activated acyl donor was used to attack the alcohol. In one paper, RML-catalyzed synthesis of fatty acid guaifenesin esters through transesterification was achieved in acetone [306]. Guaifenesin and vinyl fatty acid esters were used as substrates. Guaifenesin was regioselectively acylated at the primary hydroxyl groups and guaifenesin derivatives with long-chain acyl group were prepared in good yields (88%).

RML is able to catalyze the monobenzoylation of the primary hydroxy group of 1,2- 1,4- or 1,5-diols with vinyl benzoate in an organic solvent, the reaction proceeding with high regioselectivity and moderate enantioselectivity [307]. For example, RML-catalyzed benzoylation of 2-methyl-1,3-propanediol produced the enantiomerically pure (*S*)-monobenzoate with a 58% conversion yield.

RML was also used to convert irilone diacetate to 5-*O*-acetylirilone by transesterification with butanol [308]. In another paper, three vinyl thiamphenicol esters with different acyl donor carbon chain length (C_4, C_6, C_{10}) were regioselectively synthesized by Lipozyme RM IM in acetone to give 73, 81, 63% yield, respectively [309]. The products were valuable monomers for preparation of macromolecular antibiotics.

An efficient synthesis of 3′- and 5′-*O*-acyl-nucleoside derivatives has been developed from inosine and 2′-deoxyuridine by enzyme-catalyzed regioselective acylation with divinyl dicarboxylates [310]. In acetone, Lipozyme RM IM gave 5′-*O*-acyl-nucleoside products.

RML has also been described to be able to catalyze the benzoylation of the primary hydroxy group of 1,2-diols with vinyl benzoate in organic solvents [311]. RML-catalyzed benzoylation proceeded with high regioselectivity and moderate enantioselectivity, whereas in the dibenzoylation reaction activity of RML and stereoselectivity of the enzymatic process is strongly influenced by steric factors.

Immobilized RML catalyzed the regioselective acylation of the C-2 side chain of the C-alkyl resorcin[4]arene tetra-alcohol in the 1,2-alternate form in organic solvents using vinyl acetate as acylating reagent [312].

Protocatechuic aldehyde (3,4-dihydroxybenzaldehyde-PA) has been acetylated using acetic anhydride in presence of RML to give a mixture of 3-acetoxy and 3,4-diacetoxy derivatives in good yields [313]. RML shows better selectivity towards the production of 3-acetoxy-4-hydroxybenzaldehyde than the diacetoxy or the 4-acetoxy derivatives.

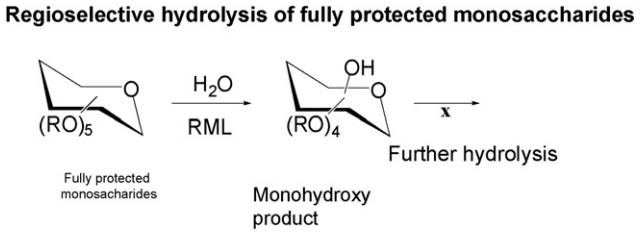

Fig. 11. General scheme of the regioselective hydrolysis of peracetylated sugars catalyzed by RML. After a first regioselective hydrolysis of just one ester bond, ideally the reaction should not further progress, yielding the monodeacetylated product.

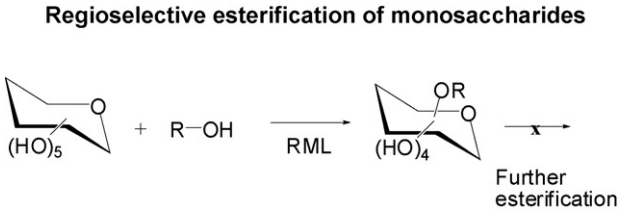

Fig. 12. General scheme of the regioselective protection of sugars via esterification catalyzed by RML. After a first regioselective esterification, ideally the reaction should not further progress, yielding the monoacetylated product.

3.4.5. *Modification of sugars*

The modification of sugars or sugars derivatives is perhaps one of the examples where RML has been more used. These modifications may be regioselective synthesis of esters in a determined position of the free sugars, or regioselective hydrolysis of one particular position of peracetylated sugars.

3.4.5.1. Regioselective hydrolysis of peracetylated sugars. The regioselective hydrolysis of one position of peracetylated sugars to give monodeprotected derivatives is one of the possibilities to get potentially interesting building blocks [314,315] (Fig. 11). RML has been used in some cases for this kind of reactions.

For example, 1,6-anhydro-2,3,4-tri-*O*-*n*-butanoyl-β-D-glucopyranose was hydrolyzed using several lipases, including RML [316]. The hydrolysis permitted to obtain 1,6-anhydro-2,3-di-*O*-*n*-butanoyl-β-D-glucopyranose. Using 1,6-anhydro-2,3,4-tri-*O*-*n*-butanoyl-β-D-galactopyranose the hydrolysis in position 2 seems to be preferred.

In another research paper, a highly efficient regioselective enzymatic preparation of penta-O-acetyl-1,5-anhydro-2-deoxy-3-hydroxy-4-O-β-galactopyranosyl-D-arabinohex-1-enitol was achieved [317]. This product was obtained in >99% conversion (>95% overall yield) by hydrolysis of per-*O*-acetylated lactal catalyzed by RML immobilized on octyl-agarose beads.

3.4.5.2. Regioselective protection of sugars. The regioselective synthesis of acetylated sugars is a simple way of achieving interesting building blocks having a sugar position protected and the other positions remaining as free hydroxyl groups [315] (Fig. 12). RML has been used in many instances in these reactions.

For example, some sugar-based emulsifiers have been produced using RML. In a first paper, the modified carbohydrate 1,2,3,4,-di-O-isopropylidene-galactopyranose was esterified in the C-6-position with corynomycolic acids (α-branched-β-hydroxy fatty acids, C_{30}–C_{40}), fatty acid esters or triglycerides in organic solvents [318]. Later, RML production of n-alkyl 6-O-acyl-α-D-glucopyranoside was showed in solvent-free media (molten fatty acid). High monoester conversion yields were obtained [319]. However, the diacid melting point is too high to allow such a reaction. Esterifying the diacid with 2 equivalents of 2,2,2-(+)-ichloroethanol

resulted in a lower meeting point. This increased the reactivity of the acyl moieties. The reaction was successfully performed in molten di-(2,2,2-trichloroethyl)adipate with *n*-alkyl 6-0-acyl-a-D-glucopyranoside and Lipozyme RM IM to yield different diesters. It is interesting to note that the use of an acetylated diester such as di-(2,2,2-*m*-chloroethyl) adipate allowed the rapid transesterification of butyl α-D-glucopyranoside in a regiospecific manner and opened the way for the subsequent chemical hydrolysis of the unreacted activated ester part [319].

The acylation of glucose with lauric acid in supercritical carbon dioxide catalyzed by RML has been also reported [320,321]. Synthesis of 6-O-acylate-α-D-glycopyranose from underivatized substrates in anhydrous *tert*-butanol was achieved using immobilized RML [322]. The catalytic activity of RML increased with increasing acyl donor chain length with a maximum for stearic acid of 0.45 μmol min^{-1} g. Using maltose as substrate, the catalytic activity decreased by a factor of 20, while with maltotriose no reaction was observed. Glucose palmitate was produced at 41.18% conversion in hexane at 40 °C, using molecular sieves to eliminate the produced water [323].

A combination of different lipases from *Pseudomonas cepacia*, *C. antarctica* B, *Candida rugosa* and RML, allowed the regioesterification of the free fructose allowing the synthesis of 1,6-di-*O*-acetyl-D-fructofuranose, 1,4,6-tri-*O*-acetyl-D-fructofuranose, 1,6-di-*O*-acetyl-4-*O*-benzoyl-D-fructofuranose and 1,6-di-*O*-benzoyl-D-fructofuranose [324].

A conversion of 80–93% was achieved for the esterification of oleic acid and fructose (or sucrose) catalyzed by Lipozyme RM IM at 65 °C using near-stoichiometric amounts of substrates [325]. The product consisted of mono- and diester at a ratio of 9:1 g g^{-1}. The main obstacle for achieving a high rate of reaction, the poor miscibility of the substrates, was overcome by taking advantage of the greatly increased solubility of fructose as the proportion of ester increased. Solvent (*t*-butanol) was present only during the first phase of the time course of the reaction to enhance fructose solubility and was allowed to evaporate away completely on reaching 25% conversion [325]. Lipozyme RM IM did not lose activity when employed for three successive fructose-oleate esterification batch reactions or, equivalently, for a 24-day reaction period.

Fructose-oleic acid esters were synthesized under solvent-free conditions at 65 °C in stirred-batch mode and using several different bioreactor systems, using Lipozyme RM IM as biocatalyst [326]. For a stirred-tank bioreactor and using fed-batch fructose addition, the conversion yield was over 80%. Using a packed-bed bioreactor, yields were over 84%.

The use of disaccharides made the reaction more complex, the substrate is bulkier, and there are more hydroxyl groups that may be modified. However, RML has been used with these substrates in some instances.

A range of lactose and maltose monoesters was prepared in overall yields of 48–77% from the corresponding sugar acetals and fatty acids [327]. Lactose tetra-acetal was found to be the best substrate despite potential steric hindrances, and up to 80% conversion was obtained in 24 h with this substrate. The reaction led to the exclusive formation of 6′ monoesters within the reaction time used. However, some diesters of 5′ were detected after 24 h. Later, lactose monolaurate and sucrose monolaurate were synthesized using vinyl laurate in 2-methyl-2-butanol and several lipases. RML was found to offer the best performance [328]. The optimal yield in the synthesis of lactose monolaurate was 99.3%. In another research, sucrose monoesters of a fatty acid were synthesized by using RML in a solvent-free system [329]. Adding barium hydroxide to control water activity, and using capric acid as the donor, sucrose, glucose, galactose, fructose, trehalose, mannose, maltose, and lactose were acylated in the primary hydroxyl group [329].

Enzymatic synthesis of fatty acid inosine esters was performed by Lipozyme RM IM-catalyzed transesterification reaction of inosine and vinyl fatty acid esters (from vinyl caprylate to vinyl stearate) in acetone [330]. Inosine was regioselectively acylated at the primary hydroxyl groups and inosine derivatives with long-chain acyl group were prepared in good yields.

Enzymatic N-acylation of N-methyl-glucamine (1-deoxy-1-methylamino-D-glucitol) in hexane using Lipozyme RM IM has been also described [331]. N-methyl-glucamine was solubilized by oleic acid addition which resulted in the formation of an ion-pair between acid and amine function. The reaction yield never exceeded 50% of acid conversion. The chemoselectivity of the reaction between oleic acid and N-methyl-glucamine towards amide or ester synthesis was under the control of acid/amine ratio.

Unsaturated fatty acid α-butylglucoside esters were prepared by enzymatic esterification of α-butylglucoside in non-aqueous media [332]. In pure molten substrates, using oleic acid, the removal of water under reduced pressure enabled yields higher than 95%. RML did not exhibit a very high regioselectivity for the primary hydroxyl group in this reaction. The biocatalyst could be recycled more than 10 batches without any significant activity loss [332]. Using a mixture of unsaturated fatty acids containing more than 60% of linoleic acid, the final percentage of diesters reached 21%, the highest percentage when compared to other lipases (e.g., Novozyme 435 gave only 3%) [333].

Alkyl glycoside fatty acid esters were synthesized by lipase-catalyzed transesterification of methyl glucoside, methyl galactoside, and octyl glucoside as the carbohydrate starting material and methyl oleate, eicosapentaenoic acid, and docosahexaenoic acid as the acyl donors [334]. The experiments were carried out in benzenelpyridine using lipases from various sources as biocatalysts, being the results obtained with the lipase B from *C. antarctica* better than using RML.

The regioselective acylation of cholesteryl β-D-glucoside, at the C-6 of the glucose moiety, was achieved using RML (among other lipases) in organic solvents [335]. With palmitic acid as an acyl, 63% production of 6′-O-palmitoyl derivative was obtained. High yields (64–92%) were also obtained with fatty acids 6:0–22:0 and 16:1 (n-7). The synthesis of cholesteryl (6′-*O*-palmitoyl)glucoside was also achieved by transesterification, using mono-, di- and tripalmitoylglycerols or methyl and ethyl palmitate as acyl sources. Using RML transesterification between methyl palmitate (80 mM) and cholesteryl glucoside (1 mM) proceeded after 24 h with a nearly quantitative yield (97%).

Lipozyme RM IM was found to catalyze the formation of 6′-O-(2-bromomyristoyl)-4-*O*-(3′,4′-O-isopropylidene-β-D-galactopyranosyl)-2,3:5,6-di-*O*-isopropylidene-1,1-di-*O*-methyl-D-glucose at preparatively useful rates [336]. The products obtained after enzymatic transformation were chemically dimerized with dicarboxylic acids, and after deprotection, a trimeric sugar ester surfactant was prepared in a similar fashion in just one step by reacting 6-*O*-(2-bromomyristl) methyl-α-D-glucoside with 1,3,5-tris (4-carboxybutyloxy) benzene.

Some nice multi-enzymatic processes have been described. Tetraethylene glycol β-D-glucoside, tetraethylene glycol β-D-xyloside, and methoxy triethyleneglycol β-D-glucoside, were prepared by almond β-glucoside-catalyzed (trans)glycosylation carried out in supersaturated solutions of glucose or p-nitrophenyl β-D-xyloside and the respective polyethylene glycols [337]. The products were further modified by enzymatic esterification with lipase from *C. antarctica* B and RML. RML showed a much greater selectivity for the primary hydroxyl group on the polyethylene glycol chain of the glucoside substrate, thus enabling us to obtain exclusively the corresponding monoester, ω-*O*-oleoyl tetraethylene glycol β-D-glucoside.

Synthesis of aminoesters of different sugars: Aminoesters of carbohydrates as may be potential drugs, as for example inhibitors of angiotensin converting enzyme [338]. Enzymatic synthesis of L-alanyl, L-leucyl and L-phenylalanyl esters of D-glucose was carried out in a non-polar solvent using several lipases, RML among them [339]. Yields of ester up to >99% were achieved. The product was a mixture of 6-*O*-, 3-*O*- and 2-*O*-monoesters and 2,6-di-*O*- and 3,6-di-*O*-esters. Later, L-phenylalanyl ester of D-glucose with unprotected L-phenylalanine and D-glucose was produced using RML [340,341]. Maximum yield was 92.4%. 5 different products were formed: three different L-phenylalanyl-D-glucose monoesters (6-*O*: 24.1%, 3-*O*: 23.3% and 2-*O*: 19.2%) and two different diesters (2,6-di-*O*: 16.6% and 3,6-di-*O*: 16.8%). In the reaction, RML followed a Ping-Pong Bi-Bi mechanism, in which L-alanine and D-glucose bind in subsequent steps releasing water and L-alanyl-D-glucose, with competitive substrate inhibition by D-glucose at higher concentrations leading to the formation of dead-end RML D-glucose complexes [342,343]. L-Alanyl-D-glucose, L-valyl-D-glucose, L-phenylalanyl-D-glucose and L-phenylalanyl-lactose esters were synthesized enzymatically using RML among other lipases [338].

Other research groups studied other carbohydrates in this field, together with the D-glucose, like aldohexoses (D-galactose and D-mannose), ketohexose (D-fructose), pentoses (D-arabinose and D-ribose) and disaccharides (lactose, maltose and sucrose), in organic solvents, using RML [344]. Yields ranged between 8 and 56%, while regioselectivity was only moderate.

RML seems to be one of the most promising lipases in sugar modifications, mainly via esterification reactions.

3.5. Modification of polymers

3.5.1. Hydrolysis of polymers

Lipases may be used in the hydrolysis of different polymeric materials to accelerate its degradation in bioremediation. RML is not among the most used for this purpose (for example compared with lipase from *T. lanuginosus*) [345], but several examples may be found in the literature. High molar mass random poly(butylene succinate-co-butylene sebacate) and poly(butylene succinate-co-butylene adipate), with different composition were subjected to enzymatic hydrolysis by RML [346]. The enzymatic hydrolysis of films of these polymers produced a mixture of water-soluble monomers and co-oligomers. RML prefers cleaving sebacic ester bonds in poly(butylene succinate-co-butylene sebacate), whereas succinic ester bonds appear to be hydrolyzed faster than adipic ester bonds in poly(butylene succinate-co-butylene adipate). Similar experiments were performed using soil buried polymers [347].

The activity of RML in the degradation of poly(ε-caprolactone) was found to be better than of other 3 lipases [348]. RML was able to catalyze the degradation of poly(ε-caprolactone) with a maximum conversion degree of about 70% only after 1 h, in toluene, within the temperature range of 40–60 °C. The biocatalyst was nearly stable at 40 °C, whereas the enzyme half-life was less than 2 h at 60 °C.

The hydrolysis of carbonate surfactants derived from tetra(ethylene glycol) esters and alkylchloroformate catalyzed by RML has been also studied [349,350]. The enzyme is able to hydrolyze these bonds, but at a quite slow rate.

3.5.2. Synthesis of polymers

The preparation of polymers is another of the application of lipases, and RML has been used to this goal in some instances. RML polymerization of *bis*(2,2,2-trifluoroethyl) sebacate and aliphatic diols has been performed [351]. The removal of the 2,2,2-trifluoroethanol formed was necessary to shift the thermodynamic equilibrium of the reaction. Among the four lipases studied, RML was the best biocatalyst for polytransesterification and diphenyl ether was the best solvent for the polymerization. A degree of polymerization of 184 was obtained under optimal conditions. Using unmodified sebacid acid a degree of polymerization of 167 was achieved [352]. Later, by controlling the addition of lipase, this was increased to 196 [353]. Molecular dynamics simulations and electrostatic potential calculations were used to study the structure of a RML–substrate complex in this reaction [354]. During the simulation, catalytically important hydrogen bonds were formed more easily, when the acid was placed in the hydrophobic end and the ester in the hydrophilic end of the active center. In a further work, it was demonstrated that also *bis*(2-chloroethyl) esters of succinic, fumaric, and maleic acid, and *bis*(2,2,2-trifluoroethyl) dodecanedioate can be polymerized, being RML the best enzyme among those studied to catalyze these polytransesterifications [355]. RML-catalyzed polymerization of divinyl adipate and divinyl sebacate with glycols has been also studied; in this case although RML can catalyze the reaction, the lipase from *P. cepacia* gave the best results [356]. The alcoholysis reaction of dimethyl sebacate with neopentyl glycol and 2-ethylhexanol was also performed to produce oligomeric esters [357]. It was found that the reactions conducted in the presence of biocatalyst proceed with high yield, 95%, under mild conditions.

The direct enzymatic polymerization of lactonic sophorolipids (SLs) was investigated with four lipases. Results (using isopropyl ether as the reaction medium) showed that RML presented the highest conversion, up to 97% [358].

Thioesterification of 1,12-dodecanedioic acid with 1,6-hexanedithiol and 1,8-octanedithiol catalyzed by RML produced poly thioesters with good yield (69%) [359]. Transthioesterification of diethyl 1,12-dodecanedioate with 1,6-hexanedithiol led to the formation of more than 66% yield of polythioesters. Some intermediates of the reaction were elucidated in this study [359].

Branched-chain polythioesters were formed in good yield (over 87%) by chemoenzymatic reactions including thiyl radical-induced addition of 1,6-hexanedithiol to the double bond of dimethyl 1,18-octadec-9-enedioate and transthioesterification of polyfunctional dimethyl 1,18-octadec-9-enedioate with bifunctional 1,6-hexanedithiol catalyzed by immobilized RML [360].

Polyesters of poly(ricinoleic acid) and trimethylolpropane, pentaerythritol, or dimer diol, examples of lipophilic star polymers, were synthesized by bulk polymerization at 70 °C using different lipases, being RML one of the ones that gave best results: the average degree of polymerization for its poly(ricinoleyl) chains being 5.4 ± 0.5 under optimal conditions [361]. The rate-limiting step in the formation of poly(ricinoleic acid) was the propagation.

3.5.3. Synthesis of propylene glycol based nonionic detergents

Propylene glycol monoesters of docosahexaenoic acid and eicosapentaenoic acid are potentially health-beneficial water-in-oil emulsifiers useful in the food industry. Lipozyme RM IM was found to be the most promising biocatalyst to produce both propylene glycol monoesters [362]. The anhydrous enzyme and 9 (*n*-hexane)/1 (*t*-butyl) presented, after optimization, over 95% conversion yield in both cases. The enzyme still retained over 60% of its original activity after 10 days of batch-type operation.

Nonionic surface-active molecules were made from acid oils such as mustard, sunflower, rice bran, soybean, coconut, and polyethylene glycols of varying molecular weights (200, 300, 400, 600), using processes based on lipase-catalyzed hydrolysis with *C. cylindracea* lipase and esterification with RML [363].

In other study, polyethylene glycol esters using castor oil fatty acid (85% ricinoleic acid) was performed using RML as biocatalyst [364]. Conversion of olyethylene glycol to esters was in the range of 86–94%, depending on the molecular size of polymer.

Later, the direct esterification of propylene glycol with lauric acid was investigated, with a 96% molar conversion [365].

3.6. Other RML applications

3.6.1. Non-conventional reactions

It is also possible to find in the literature some examples where RML is used in non-conventional reactions. For example, it has been described that RML catalyzes the lactonization reaction of 15-hydroxypentadecanoic and 16-hydroxyhexadecanoic acids to appropriate macrocyclic mono- and oligolactones [366]. The synthesis of isoniazid (an anti-tuberculosis drug) from ethyl isonicotinate and hydrazine hydrate was studied in non-aqueous media via lipase-catalyzed hydrazinolysis under both conventional heating and microwave irradiation by using different lipases [367]. Lipozyme RM IM was found to be less effective than Novozym 435 in this reaction. In another example, complete hydrolysis of 1-pyrroline ester was successful performed using Lipozyme RM IM under stirred or ultrasound condition [368]. Fatty esters containing a pyrrolidine or *N*-methyl pyrrolidine system in the alkyl chain were not hydrolyzed unless conducted in an ultrasonic bath. The hydrolytic activities of the enzymes appeared to be strongly affected by the stereochemistry of the N-heterocyclic ring system [368]. An esterification process was developed for the direct synthesis of 2-hydroxy-5-hexenyl 2-chlorobutyrate ester from 2-chlorobutyric acid by using the epoxide 1,2-epoxy-5-hexene and RML as the biocatalyst in a batch reactor [369].

The promotion of amide bonds is one of the examples of this non-conventional reactions using RML. Moderate yields of fatty amides were obtained using an immobilized RML preparation as catalyst, using primary alkyl amines and fatty acid methyl esters or triglycerides as substrates [370]. 25% yield synthesis of N-oleoyltaurine (a fatty amide surfactant) was achieved by condensation of taurine and oleic acid in organic media using Lipozyme RM IM [371]. RML readily accept N-Cbz-amino acids as substrates and catalyze their esterification/amidation with long-chain alcohols and α,ω-diols/α,ω-diamines in excellent yield [372]. The resulting alkanediyl-α,ω-bis-(N-Cbz-amino acid) can be either deprotected using conventional procedures to attain a number of multifunctional bola-amphiphiles or further modified to obtain a range of amino acid-based gemini surfactants. Lipozyme RM IM was used to catalyze the acylation of the amino acid L-lysine with the free fatty acids, palmitic and oleic acids, to synthesize N-ε-palmitoyllysine and N-ε-oleoyllysine, respectively [373]. Palmitoyl vanillylamide was synthesized through lipase-catalyzed amidation in supercritical carbon dioxide, being Lipozyme RM IM the most active among the 5 analyzed lipases [374].

One of the most unexpected reactions detected using RML was the hydrolysis of N-acetylalanine, as there are reports that state that lipases cannot hydrolyze amide bonds [375].

However, the most abundant examples of non-conventional uses of RML are in reactions involving thiol groups. In fact, enzyme-catalyzed esterifications of thio and carboxylic acid analogues and thiotransesterifications were studied using Lipozyme RM IM and thio acids are found to react with butanol at rates much faster than those for the corresponding carboxylic acids [376]. Enzymatic reactions of butanol and (*S*)-3-(acetylthio)-2-methylpropionic acid were found to occur by a complex mechanism leading to the formation of multiple products. This reaction appears to occur in a sequential way, following a parallel-consecutive mechanism yielding initially the carboxylic acid butyl ester and later the deacetylated thiomethylpropionic acid butyl ester [376]. Esterification of the 3-, 4-, 5-, 9-, and 12-thiastearic acids with *n*-butanol in *n*-hexane using Lipozyme RM IM as the biocatalyst gave the corresponding butyl esters in 80–95% yield [377]. Interesterification of triolein with methyl 9-thiastearate showed the incorporation of 9-thiastearoyl chain at only one of the α-positions of triolein.

The thioesterification between oleic acid and butanethiol in *n*-hexane, solvent-free systems or supercritical carbon dioxide catalyzed by Lipozyme RM IM was also accomplished [378].

Long-chain acyl thioesters (thio wax esters) have been prepared in high (80% to more than 90%) yields by solvent-free esterification of fatty acids (lauric, myristic, palmitic and stearic acids) with long-chain thiols, such as decane thiol, dodecane thiol, tetradecane thiol and hexadecane thiol, catalyzed by RML [379,380]. Palmitic acid hexadecylthioester and other long-chain acyl thioesters have been prepared in high yield (80–85%, purity >98%) by solvent-free lipase-catalyzed thioesterification of fatty acids with alkanethiols in vacuum [381]. Later, the same group prepared saturated long-chain acyl thioesters in high yield (80–85%) and purity (>98%) by RML-catalyzed solvent-free thioesterification of saturated fatty acids with 1-alkanethiols in vacuum, although lipase B from *C. antarctica* was more effective [382]. Transthioesterification of fatty acid methyl esters with 1-alkanethiols was less effective than thioesterification for the preparation of acyl thioesters. In the presence of antioxidants such as 2,6-*di*-*t*-butyl-4-methylphenol and octyl gallate thioesterification of oleic and elaidic acids with 1-tetradecanthiol as well as transthioesterification of methyl linoleate with 1-tetradecanthiol led to geometrically uniform thioesters without thiyl radical-induced side reactions.

On the contrary, in other research studies Lipozyme RM IM was found to be more effective than Novozym 435 or Lipozyme TL IM on the preparation of 1-S-mono-palmitoyl-hexanedithiol and 1-*S*-mono-palmitoyl-octanedithiol (80–90%) in a solvent-free system via thioesterification of palmitic acid with the corresponding α,ω-alkanedithiols in vacuum [383]. Similarly, 1,6-*di*-*S*-palmitoyl-hexanedithiol and 1,8-di-S-palmitoyl-octanedithiol were prepared in moderate yield (50–60%) by solvent-free RML thioesterification of palmitic acid with 1-S-mono-palmitoyl-hexanedithiol and 1-S-mono-palmitoyl-octanedithiol, respectively. In a further research, medium- and long-chain dialkyl 3,3′-thiodipropionate antioxidants such as dioctyl 3,3′-thiodipropionate, didodecyl 3,3′-thiodipropionate, dihexadecyl 3,3′-thiodipropionate, and di-(cis-9-octadecenyl) 3,3′-thiodipropionate were prepared in high yield by RML-catalyzed esterification and transesterification of 3,3′-thiodipropionic acid and its dimethyl ester, respectively, with the corresponding medium- or long-chain 1-alkanols, i.e., 1-octanol, 1-dodecanol, 1-hexadecanol, and *cis*-9-octadecen-1-ol without solvents, although Novozyme 435 was far more active [384].

3.6.2. RML as a biosensor

Enzymes are used in many cases to design biosensors, because of the high enzyme specificity of this biocatalyst [385]. Although the specificity of lipases is not too narrow, there are examples of the use of these enzymes as biosensors, and RML is not an exception. Lipase-catalyzed hydrolysis and alcoholysis of ester bonds in vitamin A and E esters was used to facilitate their determination in different food formulations [386]. Two vitamin esters, retinyl palmitate and α-tocopheryl acetate were used as model compounds and two food formulations, milk powder and infant formula, were used as model matrices. Among several lipases used, Lipozyme RM IM showed considerable activity towards retinyl palmitate. There was no observed activity with α-tocopheryl acetate using any of the enzyme preparations. In supercritical fluid, Novozyme 435 was the enzyme exhibiting the highest activity. Due to the high number of substrates that may be recognized by RML, the suitability of the enzyme to be used under very different conditions, the number of applications in this area will very likely increase along the time.

4. Conclusion

Although RML has been produced and used mainly in oils and fats modification [57], this lipase is a very long time known enzyme that have been successfully applied in many reactions in organic chemistry.

The high resistance of the enzyme under anhydrous conditions and its high activity in esterification reactions have made that RML had been used mainly in synthetic process. Moreover, in these synthetic processes is where the enzyme manifested its best regioselectivity and enantiospecificity [234]. Therefore, the enzyme may have very good prospects of application in many areas of the chemistry.

In fact, RML is a very useful enzyme in most of the processes reviewed in this paper, not as popular as those from yeast of the genus *Candida*, but in some instances with even better properties. However, the enzyme looks less appropriate than other lipases as catalyst of transesterifications. In the case of the biodiesel, it seems that the lipases from *T. lanuginosus* [345] or *C. antarctica* may offer better results, however depending on the alcohol used [388], RML could become the best choice.

Nevertheless, it should be considered that most of the results presented in this review are based on the use of just one RML preparation, in many instances just under one experimental condition. Considering that lipase may be easily altered in their properties by any alteration in the medium or immobilization protocol, it may be expected that the current results may be easily improved by just using this "conformational engineering" technique [50–56].

Using soluble RML, the fact that the enzyme tends to form bimolecular aggregates even at very low concentrations or to be adsorbed to any hydrophobic component of the crude extract must be carefully considered [45–48]. These aggregates have very different properties when compared to the monomeric form of the lipase. This makes the study of the enzyme very complex and difficult to reproduce or understand the differences between different researches [45–48]. Mild immobilization protocols (ideally via just one point and using long and inert spacer arms) [387], performed in the presence of detergents to break the dimers [45–48], may be one simple way to get monomeric and pseudo-native lipase structures, that may facilitate comparison between several lipases. On the other hand, the use of hydrophobic supports may be a good option to have interfacially activated and monomeric RML molecules [39–41,53].

The application of the new tools (including genetic, chemical and physical modifications) to RML (simplified by the deep knowledge of this lipase) should enhance the performance of RML in the current applications and open new opportunities for the tuning of the RML properties, and that way further increase the industrial use of this interesting enzyme.

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