Rhizopus oryzae Lipase

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Lipases are biocatalysts with a significant potential to enable a shift from current pollutant manufacturing processes to environmentally sustainable approaches. The main reason of this prospect is their catalytic versatility as they carry out several industrially relevant reactions as hydrolysis of fats in water/lipid interface and synthesis reactions in solvent-free or non-aqueous media such as transesterification, interesterification and esterification. Because of the outstanding traits of *Rhizopus oryzae* lipase (ROL), 1,3-specificity, high enantioselectivity and stability in organic media, its application in energy, food and pharmaceutical industrial sector has been widely studied. Significant advances have been made in the biochemical characterisation of ROL particularly in how its activity and stability are affected by the presence of its prosequence. In addition, native and heterologous production of ROL, the latter in cell factories like *Escherichia coli*, *Saccharomyces cerevisiae* and *Komagataella phaffii* (*Pichia pastoris*), have been thoroughly described. Therefore, in this review, we summarise the current knowledge about *R. oryzae* lipase (i) biochemical characteristics, (ii) production strategies and (iii) potential industrial applications.

Pichia pastoris | biocatalysis | lipase | biodiesel | flavour | structured lipid | enzyme | Rhizopus oryzae | immobilisation | biotechnology

1. Introduction

Rhizopus oryzae is broadly employed in industry because it can carry out the synthesis of a great variety of products like organic acids (lactic and fumaric acids), volatile compounds and enzymes (cellulases, proteases, tannases, xylanasas, pyruvate decarboxylases, lipases etc.,) [1][2][3][4]. Concretely, according to Web of Knowledge data, *R. oryzae* lipase (ROL) is one of the most studied enzymes of this fungi. There are three major commercial formulations of this lipase (Table 1) and more than 200 scientific works have been published in the last 5 years highlighting the relevance of this enzyme. Therefore, the aim of this entry is to provide a complete overview of ROL in terms of biochemical properties, enzyme native and heterologous production and its industrial applications.

Table 1. Major commercial suppliers of *Rhizopus oryzae* lipase and some lipase properties [5].

Supplier	Name	Application	Lipase Properties
Amano	Lipase DF “Amano” 15	Oil and fats	Optimum pH range 6–7; stable pH range 4–7, optimum temperature

			range 35–40 °C, relatively specific to fatty acids
Sigma	Lipase from *R. oryzae* (no. 62305)	Oil and fats	Optimum pH 8, optimum temperature 40 °C
Sigma	Lipase, immobilised on Immobead 150 from *R. oryzae* (no. 89445)	Pharmaceutical and bioenergy	Optimum pH 7.5, optimum temperature 40 °C

2. Biochemical Properties

R. oryzae lipase (ROL) is a protein synthesised as a precursor form containing a presequence of 26 amino acids, followed by a prosequence of 97 attached to the N-terminal of a 269 amino acids mature sequence (Figure 1) [6]. All known lipases from *Rhizopus* genus follow the same identical structure even though some amino acidic substitutions can be detected when their primary sequences are compared, not only between different species but also between different isolated strains of the same species (Figure 2). For instance, Ben Salah et al. [7] addressed the presence of several substitutions in the sequences of *Rhizopus* lipases published by his group and Sayari et al. [8], Beer et al. [6], Derewenda et al. [9] and Khono et al. [10].

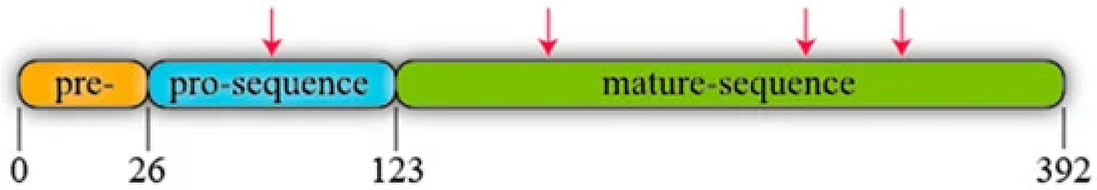

Figure 1. Schematic representation of *R. oryzae* lipase (ROL). Arrows stand for potential N-glycosylation points.

ROL contains four potential N-glycosylations sites (Figure 1) that follow the consensus sequence Asn-X-Ser/Thr, where X is any amino acid instead of proline. One of these putative sites is found in the prosequence where modifications in glycosylation patterns have been described to have an effect on protein secretion [11]. For instance, Yu et al. [12] added two extra N-glycosylation sites to ROL prosequence and expressed this mutant in *Komagataella phaffii* (*Pichia pastoris*). The extracellular activity and total protein were 218- and 6.25-fold higher respectively in the strain harbouring the two extra N-glycosylation sites than in the non-modified one highlighting the relevance of glycosylation.

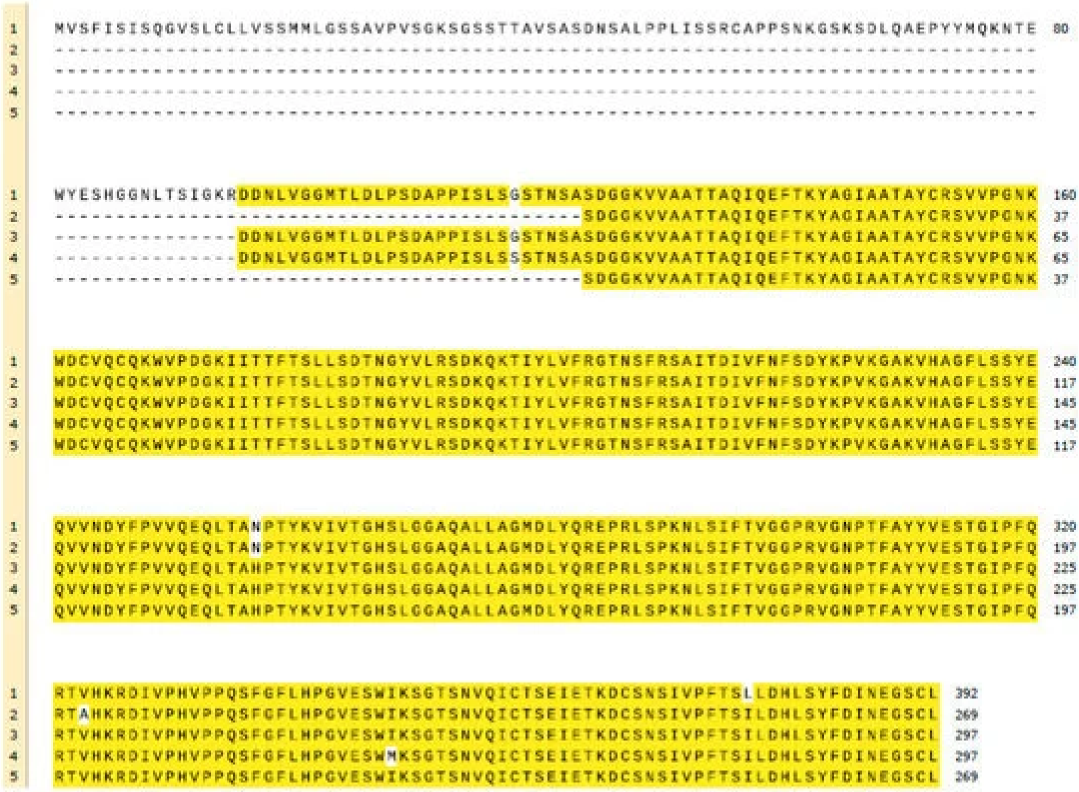

Figure 2. Multiple alignment of the sequences published by (1) Beer et al. (ROL) [6], (2) Ben Salah et al. (ROL) [7], (3) Sayari et al. (ROL) [8], (4) Khono et al. (*Rhizopus niveus* lipase) [10] and (5) Derewenda et al. (*Rhizopus delemar* lipase) [9]. Matching amino acids are highlighted in yellow, mismatching in white. BLAST from U.S. National Library of Medicine and Snapgene have been used for the creation of this figure.

The presequence of ROL has been described to act as signal peptide promoting enzyme secretion, while the prosequence has been reported to exhibit diverse functions that are still under research. Beer et al. [13] depicted the significance of the latter in lowering lipase toxicity during its synthesis and in acting as intramolecular chaperone enabling the proper folding of the enzyme. In fact, genetically modified *E. coli* strains producing heterologous ROL without the prosequence resulted in cell lysis. To date, a large number of prosequences of different enzymes have also been identified to function as intramolecular chaperone and to assist the folding of their respective proteins [14]. In addition, several scientific works have related ROL prosequence with the translocation of the protein across the endoplasmic reticulum membrane, enhancement of free lipase stability and changes in enzyme substrate specificity. Nevertheless, the mechanisms that allow these traits are yet unknown despite the broad research carried out [15][16][17][18][19][20]. In any case, both the presequence and the prosequence are expected to be proteolytically removed to form the mature lipase. In spite of this, the native microorganism secretes a lipase that is attached to the N-terminal of mature sequence the 28 C-terminal amino acids of the prosequence (proROL), which then are cleaved via limited proteolysis catalysed by extracellular proteases [6][10][21]. However, some studies have indicated that the presence of these 28 amino acids of the prosequence alongside the mature sequence is enough for some of the presumed features of the prosequence to occur. For instance, higher free lipase stability and changes in enzyme specificity have been described when the 28 amino acids of the prosequence were expressed together with the mature sequence in *K. phaffii* [22]. In addition, these amino acids have also enabled lowering the toxicity of ROL production in *E.coli* [13] and they have been related to direct proteins to secretory pathway in *Aspergillus oryzae* [23].

The mature sequence of *R. oryzae* lipase (rROL) is constituted by 269 amino acids and the protein formed by them has a molecular weight (MW) of 29.542 kDa and a isoelectric point (pI) of 8—calculated by Expasy Proteomics Server [7]. These results agree with the published experimental data (Table 2) in which MW and pI values around 29 kDa and 8 have been respectively reported [8][10][24][25]. However, variations in these values can be found because of the presence of the 28 amino acids of the prosequence described above [22][10][20][26][27]. In this case, MW increases to 32 kDa and pI decreases roughly to 7, highlighting the average acid nature of these 28 amino acids. Besides, the production of a lipase including the whole prosequence and close to 40 kDa has also been described (entire-proROL) [6][15].

Table 2. Biochemical properties and substrate specificity of different published works dealing with ROL.

Lipase Name [1]	MW (kDa)	Isoelectric Point	pH Optimum	T Optimum (°C)	Substrate Specificity	Ref.
rROL	29		8/7.25 [2]	30/40 [2]	C12>C10>C8>C4 [4]	[22]
proROL	32		7.25	40	C8>C12>C10>C4 [4]	[22]
rROL	30		8.5			[6]
entire-proROL	40		8			[6]
pre-entire-proROL [3]	42		8			[6]
rROL	29		8	37		[7]

rROL	29					[8]
proROL	32					[8]
proROL	34		6–6.5	35		[10]
rROL	30		6	40		[10]
proROL	35		9	40	C16>C18>C12>C8>C4 [5] C16>C12>C8>C18>C4 [6]	[18]
proROL	32	6.9				[20]
rROL	30	9.3	8.25	30	C8>C10>C6>C4>C12>C16,C14>C2 [6]	[24]
proROL	35		5.2	30	C12>C10>C8>C6>C16>C5>C4>C3>C2 [4]	[25]
proROL	32	7.6	7.5	35	C8>C6>C4>C2 [6]	[26]
rROL	29				C12>C10>C8>C6>C4>C3>C2 [4] C8>C10>C18>C4>C6 [6]	[28]
proROL	34				C2>C3>C8>C6>C12>C10>C4 [4] C8>C10>C4>C6>C18 [6]	[28]
proROL			8	40		[29]

rROL	30.3	8.6	8–8.5	30		[30]
proROL			8.5	30		[31]
proROL	37		8.5	40		[32]
rROL	29		8			[33]
ROL	17	4.2	7	40		[34]
ROL			7	40		[35]
ROL			6	45	C8>C4>C6>C2 [6] C8>C12>C14>C16>C18 [5]	[36]
proROL	32		7	35		[37]
ROL			6	30	C7,C8,C12,C16>C2,C3,C4,C18 [5]	[38]
ROL			7.5	50		[39]
proROL	32		7.5	30–40		[40]
ROL	14.45	6.5	9	30–40	C16>C18>C12>C8>C4>C2 [4]	[41]
			8.3	35–37		[42]
proROL	35				C10>C14>C12>C8>C6>C4>C16 [6]	[15]

entire-proROL	46		[15]

[1] Names are based on the established nomenclature in this review. ROL indicates that the lipase cannot be classified under the determined parameters in this work. [2] ... substrate specificity analysis. Saturated methyl esters are just considered; [3] monotriacylglycerols were employed for substrate specificity analysis.

The 3D structure of the lipase from *R. oryzae* [9][10] (Figure 3) and several microorganisms more, such as *Geotrichum candidum* [43], *Candida rugosa* [44], *Pseudomonas glumae* [45] and *Penicillium camemberti* [46] have been crystallographically resolved and showed that all lipases have a common α/β hydrolase fold structure that can also be found in other hydrolases. Regarding ROL, it contains nine α-helixes and eight β-strands forming a molecule that it is stabilised by three disulfide bonds between residues 29–269, 40–43 and 235–244 [10]. In addition, this structure contains three key components that can be also found in most lipases besides ROL, the lid, the active site and the oxyanion hole [47]. The lid is an amphiphilic loop—also called flap—that covers the active site preventing the access of the substrate while the enzyme is in aqueous medium [48]. The active site, in turn, is mainly responsible for carrying out enzyme catalysis and consists, in all α/β hydrolases, of a highly conserved catalytic triad formed by a nucleophilic, a catalytic acidic and a histidine residues. In lipases, this triad is composed of nucleophilic serine residue and an aspartic or glutamic acid residue that it is bonded to a histidine; hence, lipases are classified as serine hydrolases. In the specific case of ROL, the lid domain is a short α-helix structure formed by six amino acids (FRSAIT) and the active site is formed by three amino acids Ser^{145}, Asp^{204} and His^{257} [9][10][49][50]. The function of these two elements is crucial during catalysis in which the lipase binds to the water/lipid interface and the lid opening occurs by a concomitant structural change in the substrate-binding site that enables the coupling of the substrate to the active site—lid-closed and partially opened 3D structures of *Rhizopus delemar* (=*oryzae*) lipase have been described by Derewenda et al. [9]. The structural change undergone is known as ‘interfacial activation’ and it is a unique property of lipases that enables them to hydrolyse insoluble esters and to distinguish them from esterases that can hydrolyse water-soluble esters [47][51][52][53]. It must be highlighted that the 28 amino acids of the prosequence introduced above have been deemed to interfere in this process as they are located next to the lid region and contain 50% hydrophobic residues. Therefore, this sequence extends the hydrophobic patch created in the open lipase by the open lid and the catalytic crevice influencing the interaction with the lipidic substrate [8]. This role might explain some of the assumed properties of these 28 amino acids, however, the mechanism remains unknown. Additionally, together with the catalytic triad and the lid, the oxyanion hole plays an important role and it is also a highly conserved sequence that largely influences the catalytic efficiency of the enzyme. During the hydrolysis reaction, a negatively charged tetrahedral intermediate is generated and it gets stabilised by hydrogen binding with the oxyanion hole [47][54][55]. This function has been described to be presumably performed by the hydroxyl and main-chain amide groups of Thr^{83} in ROL [9][10][56].

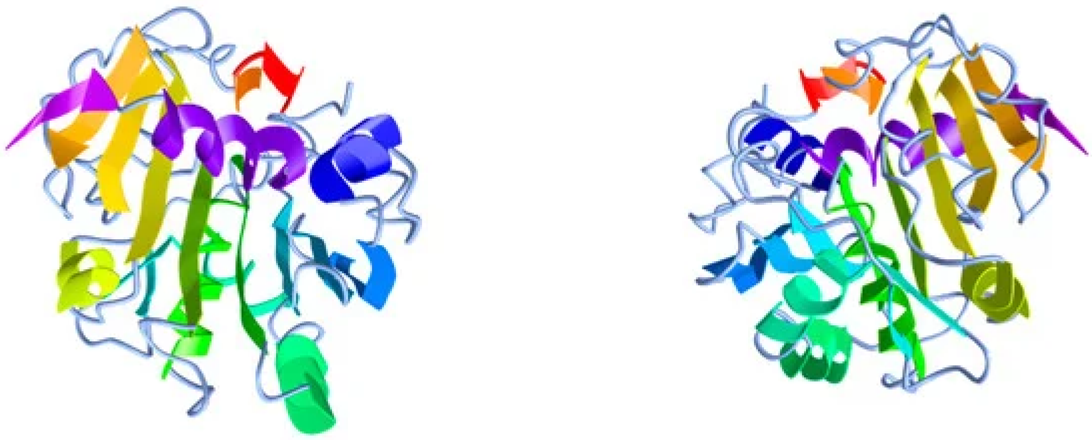

Figure 3. Three-dimensional structure of *R. delemar* (= *oryzae*) lipase from two different points of view. PDB ID: 1TIC. Image obtained from iCn3D web-based 3D structure viewer.

Due to the relevance of the lipolytic activity of this enzyme, it has been widely researched in order to know how it is affected by the conditions of reaction medium. Guillen et al. [28] described that ionic strength has a remarkable impact. Actually, the relative activity of ROL in 200 mM Tris-HCl was reported to be twice the activity observed in 400 mM. Moreover, as all enzymes, ROL activity is highly influenced by the pH and temperature. Optimum activity pH values of 8 have been principally reported [6][7][24][26][28][57][29][30][31][32][33]. However, other studies have also stated more acid [25][58][35][36][37][38][39][59] and basic [18][41][42] optimums. Regarding temperature, most of the published optimum values can be found between 30 and 45 °C. In fact, 40 °C has been the most commonly reported optimum [22][10][18][28][29][32][58][35][59][41] although lower [7][10][25][26][28][30][31][37][38] and higher [36][39] values have also been described. Nevertheless, for both pH and temperature, as can be observed in Table 2, some of the differences are based on the presence of the 28 amino acids of the prosequence. In this line, Kohno et al. [10] reported these differences and afterwards, other works [22][60][28] described similar results highlighting the relevance of these amino acids in lipase catalytic performance.

The presence of metal ions in reaction medium has been extensively studied as they play different and important roles influencing the structure and activity of enzymes. These ions may bind to some of the amino acid side chains of the lipase and participate in catalysis, interfere with the bonds between amino acid side chains and cause denaturation of the active site or alter enzyme activity by stabilising or destabilising enzyme conformation [37][61][62][63]. Amongst the different published works some contradictory information can be found. Nevertheless, there are some metal ions that have been clearly described to enhance or worsen ROL and other lipases performance. Wang et al. [18] and other authors [34][37][41] found that Ca^{2+} increases ROL activity as it might create electrostatic interactions that mask the repulsions either between the enzyme and its emulsified substrate or between the enzyme and product-free fatty acids [30]. On the other hand, Hg^{2+} has been reported to act like a ROL activity inhibitor suggesting that thiol groups are required for the adequate function of the enzyme [25]. Similar results have been reported with other lipases from *Pseudomonas aeruginosa* AAU2 [64], *Galactomyces geotrichum* Y05 [65], *Yarrowia lipolytica* [66] and *Candida rugosa* [67]. In addition, no significant effects have been observed with the chelating agent EDTA, indicating that ROL activity is independent of any metal, hence, it is not a metalloprotein [25][41].

ROL activity has also been analysed in presence of amino-acid-modifying agents in order to elucidate the relevance of the different amino acids in protein catalytic performance. *N*-Bromosuccinimide (NBS), which acts over tryptophan residues, has been reported to strongly inhibit enzyme activity indicating that the protein might have a tryptophan residue involved in its activity [25][34]. In the case of phenylmethylsulfonyl fluoride (PMSF), a serine protease inhibitor whose activity is related to serine residues modification, no clear results have been reported. Kantak et al. [34] indicated that this agent has a relevant effect while Hiol et al. [26] stated exactly the opposite. However, these differences might be caused by the different disposition of the lipase lid during the assay, that is, if it was open or not, it could allow or not the interaction of PMSF with the serine residue of the active site [68].

ROL activity—as most lipases from *Rhizopus* genus—has a strong 1,3-regiospecificity that makes its activity interesting for several industrial processes such as fat and oil modification for structured lipids production [5][4][26][36]. Nevertheless, Li et al. [69] reported, while studying ROL methanolysis performance, that this lipase was not regiospecific although showed a preference to 1,3-positions. These results were lately confirmed with *Rhizopus arrhizus (=oryzae)* lipase [70]. However, Okumura et al. and Song et al. [38][71] stated that *Rhizopus delemar (=oryzae)* and *R. oryzae* lipases, respectively, do not hydrolyse the ester bond in position 2. Afterwards, Canet et al. and Cao et al. [72][73] proved that mature ROL exhibits a negligible activity on 2-monoolein highlighting that the lipase has a strong 1,3-regioespecificity. The observed dissimilarities amongst different works might be due to the different employed reaction conditions that could enhance spontaneous acyl-migration, or the presence of the 28 amino acids of the prosequence that has already been described to have an effect on lipase specificity [22][13]. Besides 1,3-regiospecificity, substrate specificity of ROL has been also widely studied. Many of the published works are based on the employment of *p*-nitrophenol esters of different carbon-chain length. For instance, ROL isolated and characterised by Adak et al. [41] was reported to be more specific to long carbon-chain *p*-nitrophenol esters, concretely to *p*-nitrophenol palmitate (C16). Guillen et al. [28] reported a similar trend for rROL produced in *K. phaffii* and, although a higher specificity to short carbon-chain *p*-nitrophenol esters was detected for proROL, the presence of esterases in the commercial product was concluded to be the reason. In fact, Tako et al. [25] also observed that the longer the carbon-chain, the higher the specificity of ROL. However, in this last case, the maximum was obtained with *p*-nitrophenol dodecanoate (C12) and not palmitate. ROL substrate specificity has been also analysed with homotriacylglycerols, that is, triacylglycerols in which the three fatty acids are identical. C8 and C10 homotriacylglycerols—triacylglycerols containing three C8 and C10 fatty acids respectively—are preferably hydrolysed by ROL while it barely acts over C2 and C4 homotriacylglycerols. In contrast to some of the published works, some authors have also described that no significant differences were observed with those substrates between rROL and proROL [15][24][26][28].

Lipases are widely known for their capacity to carry out synthesis reactions in non-aqueous mediums. In fact, as previously mentioned, this capacity makes them relevant for many industrial processes in which these reactions are needed, or the solubility of substrates/products requires the use of organic solvents. Therefore, the higher the lipase stability in these solvents, the more suitable the lipase for industrial applications [74]. ROL has been extensively described as a tolerant enzyme to non-aqueous solvents [18][26][31], particularly in alkanes and long-chain alcohols such as hexane and dodecanol respectively. However, polar solvents like acetone or short-chain alcohols have an important negative effect on the enzyme because they strip off the crucial bound water from the enzyme's surface [75]. In some cases, it is remarkable the different results that can be obtained between the stability of the enzyme in a solvent, such as methanol and ethanol, and the operational stability employing that solvents as substrate. For instance, methanol has proven to be more detrimental than ethanol during biodiesel synthesis while during stability assays exactly the opposite result was obtained [22].

3. *Rhizopus oryzae* Lipase Production and Bioprocess Engineering

First attempts of ROL production were made with the original fungi isolated from palm fruit [26][31]. *R. oryzae* secretes, as previously mentioned, one form of lipase with a molecular weight close to 32 kDa—the mature sequence including 28 amino acids of its prosequence. However, a second form of ROL with a molecular weight around 29 kDa was detected after keeping the supernatant at 6 °C for few days; i.e., the lipase form corresponding to the loss of the 28 amino acids [8]. Consequently, the distinct lipases derived from *R. oryzae* described in the literature are originated because of the different proteolytic processing and not because of the presence of different genes [6].

To increase ROL industrial production, its expression in a cell factory is mandatory. This way, production cost, bioprocess engineering and downstream complexity are minimised [5].

In *Escherichia coli*, the presence of disulphide bonds in ROL structure and the lack of the necessary enzymes to process fungal maturation signals were the main causes that led to the production of enzymatically inactive protein as insoluble aggregates [6]. Thereafter, active lipase was obtained at lab scale by subjecting these aggregates to a refolding process. However, the large-scale production was not implemented due to the high cost of the procedure [13]. Despite that, Di Lorenzo et al. [76], achieved the production of an active and soluble ROL and proROL using the *E. coli* Origami (DE3) strain and pET-11d expression system. The final specific activities of both enzymes were quite similar but the yield of proROL production was higher than ROL, likely because of the toxic effect of the latter towards the host cells.

To avoid the inherent problems of prokaryotic cell factories producing eukaryotic proteins, particularly those related to post-translational processing, eukaryotic cell factories were tested for ROL production.

The extracellular production of ROL has been studied in *S. cerevisiae* and *K. phaffii* (*P. pastoris*) by expressing essentially three different genes. A gene encoding the prosequence of 97 amino acids fused to the N-terminal of the mature lipase region of 269 amino acids (proROL-gene), a gene encoding a truncated prosequence of its 28 C-terminal amino acids fused to the N-terminal of the mature lipase region (28proROL-gene) and a gene encoding the mature lipase (rROL-gene). Regardless of proROL-gene or 28proROL-gene expression, a protein with only 28 amino acids of the prosequence plus the mature lipase (proROL) was detected. Exceptionally, the complete prosequence plus the mature lipase region (entire-proROL) was also reported with proROL-gene construction. With respect to the rROL-gene construction, jus the mature lipase (rROL) was obtained.

First attempts of producing ROL in eukaryotic platforms were made with the widely used cell factory *S. cerevisiae*. Takahasi et al. [15] reported that *S. cerevisiae* secreted two active lipases when it was transformed with the proROL-gene fused to the pre-α-factor, the entire-proROL and proROL—the lipase formed after Kex2-like protease cleavage of the prosequence. In parallel, when *S. cerevisiae* strains were transformed with rROL-gene fused to the pre-α or prepro-α factor encoding gene, almost no activity was detected, highlighting the mentioned relevance of ROL prosequence during lipase production [16][19][21].

A summary of the results obtained with these cell factories is shown in Table 3.

Table 3. Summary of E. coli and *S. cerevisiae* cell factories expressing *Rhizopus oryzae lipase.*

Cell Factory	Promotor/Vector	Lipase	Production	Lipolytic Activity	Reference
E. coli Origami DE3	pET11	proROL	Intracellular	166 U mL^{-1}	[76]
	pET22	proROL	Intracellular	82 U mL^{-1}	
S. cerevisiae	UPR-ICL	rROL	Extracellular	0.29 U $flask^{-1}$	[15]
	UPR-ICL	proROL	Extracellular	191 U $flask^{-1}$	

3.1. *Komogataella phaffii* Cell Factory

Unlike the reported results with *S. cerevisiae*, when proROL-gene was expressed in *K. phaffii* cell factory, only proROL was detected in the medium, which might indicate that the activity of the Kex2-like protease is higher in this cell factory than in *S. cerevisiae* [17]. Moreover, rROL-gene was satisfactorily expressed and the corresponding lipase was detected in the supernatant [24].

This appropriate performance on ROL secretion, jointly with the well-known excellent characteristics of *K. phaffii*, make this yeast the most suitable cell factory for heterologous ROL production [77][78][79][80]. In addition, *K. phaffi* does not produce endogenous extracellular lipases or esterases [81]. Thus, downstream processes might be easier and cheaper. However, two of the bottlenecks of *K. phaffii* cell factory are transformed clones screening and selecting the best operational strategy to maximise production. To minimise this problem, the use of microbioreactor devices has been successfully implemented [82]. Further information about *K. phaffii* as cell factory for ROL production was summarised by López-Fernández et al. [83]

3.2. Whole cells

Hama et al. reported that rROL and proROL are located in different regions in *R. oryzae* cells, proROL in the cell wall and rROL bound to the cell membrane. Besides, these cells have been successfully employed as whole cells biocatalysts (WCB) in many relevant biotransformations, for instance, for enzymatic biodiesel production [84][85]. It must be highlighted that the fatty acid composition of the membrane has been reported to influence lipase activity and stability during biodiesel reactions [86].

Modified *S. cerevisiae* strains producing ROL have also been used as WCB [87][88]. Matsumoto et al. [87] reported the intracellular production of proROL in *S. cerevisiae* under the 5′ upstream region of the isocitrate lyase gene of *Candida tropicalis* (*UPR-ICL*). Additionally, the expression of the lipase under the constitutive promoter glyceraldehyde-3-phosphate dehydrogenase was also studied. However, this system did not improve the results obtained with *UPR-ICL*.

proROL was successfully expressed under P_{AOX} control and displayed on Mut^{+} phenotype *K. phaffii* cell surface using the Flo1P anchor system previously developed in *S. cerevisiae*. The obtained WCB showed higher thermal stability than free enzyme [89]. Additionally, a similar approach using Sed1p anchor protein was studied in a Mut^{S} phenotype. In the same sense, the obtained biocatalysts was stable in a wide range of temperatures and pH [90].

4. Industrial Applications of *Rhizopus oryzae* Lipase

Its 1,3-regiospecificity and catalytic versatility make ROL appropriate for improving the sustainability of food, pharmaceutical and energy industry [5][91][92].

4.1. Biodiesel Production

Because of petroleum depletion and environmental concerns, in the past decade, biodiesel (mono-alkyl esters of long chain fatty acids) is gathering significant interest as a renewable, biodegradable and more environmentally friendly alternative to fossil fuels. Biodiesel can be classified into three different generations based on the source from which it is derived. First-generation biodiesel is synthesised with edible-oils such as soybean or sunflower oils. Therefore, it might cause the so-called “food vs. fuel” ethical issue because of the use of food and agricultural lands for biofuel production [93]. In order to prevent this problem, alternative substrates have emerged for biodiesel synthesis, leading to second- and third-generation biodiesel production. The former uses non-edible oils that are not considered for human consumption and are produced from crops that, even if they require lands, are generally poor lands not useful for agriculture. Meanwhile, third-generation biodiesel completely avoids ethical issues by using microbial lipids and oleaginous wastes such as oils from microalgae or oleaginous yeasts and waste cooking oils (WCO) respectively [94][95][96][97]. Additionally, there is a fourth-generation biodiesel that is at its preliminary research stages and is based on man-made biological tools, that is, biodiesel producing genetically modified microorganisms [98][99].

Typically, these alternative substrates, those yielding second- and third-generation biodiesel, have a higher free fatty acid (FFA) content, which can make biodiesel production through chemical synthesis—the most common process for current industrial biodiesel production—more complex because a previous operation of FFA neutralisation is required to avoid soap formation, an usual side reaction when substrates with high FFA content and basic catalysts are employed [95][100][101]. In this context, enzymatic biodiesel synthesis with lipases arouses as an alternative owing to its several advantages such as the milder reaction conditions, less water consumption, easier downstream and particularly, the absence of side reactions and consequently the capacity of employing substrates with high FFA content [102][103]. In fact, substrates with initial high amounts of FFA have been reported to enhance enzymatic biodiesel synthesis reaction rate and biocatalysts operational stability [102][104][105]. Given all the advantages, numerous lipases have been studied in this biotransformation with significant results, such as the lipases of *Candida rugosa* [106][107], *Candida antartica* [108][109] and *Burkholderia cepacia* [110][111].

In the seeking for the best lipase to make enzymatic biodiesel feasible at industrial scale, lipases' regiospecificity has become a crucial trait. Non-specific enzymes produce mono-alkyl esters and glycerol, which is an undesired by-product of the transesterification reaction that has been described to hinder reaction progress or even affect negatively on enzymes stability and biodiesel downstream [112]. Conversely, 1,3-regioespecific lipases, avoid glycerol formation by producing 2-monoacylglycerol which acts as lubricant and in certain amount, upgrades biodiesel characteristics [113][114][115]. Furthermore, monoacylglycerols can improve the cost-effectiveness of a biodiesel biorefinery as they are more valuable products than glycerol because of their utility in pharmaceutical and food industry as emulsifiers [116][117][118]. Consequently, ROL has been widely studied in biodiesel production because of its regiospecificity.

Considering biodiesel ethical issues, even if several studies have employed ROL with edible oils such as olive [119], rapeseed [120][121], soybean [122][123][124][125] and sunflower [126][127] oils—commonly as model substrates for research—most of the published works have focused on the use of alternative substrates (Table 4). *Jatropha curcas* oil is one of the non-edible oils with higher potential for second-generation biodiesel production, probably because of the easy cultivation process and worldwide spread of the plant [128]. Rodrigues et al. [129] reported yields close to the theoretical 100%—real 66% considering ROL 1,3-regioespecificity—and high operational stability of the biocatalysts. In Table 4 are detailed other studies with promising results using this substrate as well as other non-edible oils like *Pistacia chinensis bge* oil [130], Tung oil [32], *Calophyllum inophyllum* oil [131] and alperujo oil (olive pomace) [115].

Table 4. Summary of biodiesel production with *Rhizopus oryzae* lipase as main biocatalyst.

Substrates	Lipase	Immobilisation Technique	Reactor Type	Stepwise Addition	Biodiesel Generation	Yield-Conversion/Op. Stability	Ref.

OO + MeOH	rROL	IA onto ReliZyme^{TM} OD 403M	PBR	Yes	1st	Y: PBR 49.1% OS: second batch 44.8%	[119]
OO + MeOH	rROL	IA onto ReliZyme^{TM} OD 403M	STR	Yes	1st	Y: STR 33.56% OS: second batch 7.7%	[119]
RO + MeOH	proROL	WCB over agar plate	SLLB	No	1st	No biodiesel production	[120]
RO + EtOH	proROL	WCB over agar plate	SLLB	No	1st	No biodiesel production	[120]
RO + MeOH	proROL	WCB over agar plate	SGLB	No	1st	Y: 58%	[120]
RO + EtOH	proROL	WCB over agar plate	SGLB	No	1st	Y: 72%	[120]
Crude CO + MeOH	proROL	Free enzymes	BR	Yes	1st	Y: 68.56%	[121]
Crude CO + MeOH	proROL-CRL	Free enzymes	BR	Yes	1st	Y: 84.25%	[121]
Crude CO + MeOH	proROL-CRL	CI onto functionalised silica gel	BR	Yes	1st	Y: 88.9%	[121]
SYO + MeOH	proROL	WCB immobilised into BSPs	BR	Yes	1st	Y: 82.2% OS: after 6 cycles almost all activity loss	[122]

SYO + MeOH	proROL	CI WCB immobilised onto BSPs	BR	Yes	1^{st}	Y: 92.2% OS: after 6 cycles no loss of activity	[122]
SYO + EtOH	proROL	IA onto microporous resin NKA (polystyrene)	BR	Yes	1^{st}	Y: 58.5%	[123]
SYO + EtOH	proROL-CRL	IA onto microporous resin NKA (polystyrene)	BR	Yes	1^{st}	Y: 80.8%	[123]
SYO + EtOH	proROL-Novozyme 435	proROL: IA onto microporous resin NKA (polystyrene). Novozyme 435: IA onto Lewatit VP OC 1600	BR	Yes	1^{st}	Y: 98.5% OS: after 20 cycles Y decreased to 78.3%	[123]
SYO + EtOH	proROL-PFL	IA onto microporous resin NKA (polystyrene)	BR	Yes	1^{st}	Y: 55.8%	[123]
SYO + MeOH	proROL	CI onto magnetic chitosan microspheres	MSFBR	Yes	1^{st}	Y: 91.3% OS: after 6 reaction cycles Y decreased to around 80%	[124]
SYO + MeoH	proROL	WCB immobilised into BSPs	BR	Yes	1^{st}	Y: over 90% OS: after 10 reaction cycles Y decreased to 10%	[125]

SYO + MeOH	proROL	WCB immobilised into BSPs	PBR	Yes	1st	Y: over 90% OS: after 10 reaction cycles Y decreased to 80%	[125]
SO + EtOH	proROL	CI onto modified sepiolite with *p*-hydroxybenzaldehyde linker	BR	No	1st	C: 84.3% OS: after 9 cycles C decreased to 21.4%	[126]]
SO + EtOH	proROL	CI onto modified sepiolite with benzylamine-terephthalic aldehyde linker	BR	No	1st		[126]
SO + EtOH	proROL	IE onto demineralised sepiolite	BR	No	1st	Y: 90.2% OS: proROL IE after 9 cycles C decreased to 18.1%	[126]
Pistacia chinensis bge seed oil + MeOH	rROL	CI onto Amberlite IRA-93	BR	Yes	2nd	Y: 92% OS: after 8 cycles Y decreased to 60%	[130]
Pistacia chinensis bge seed oil + MeOH	rROL	IA microporous resin HPD-400	BR	Yes	2nd	Y: 94% OS: after 8 cycles Y decreased to 50%	[130]
Calophyllum inophyllum linn	proROL	WCB immobilised into BSPs	PBR	Yes	2nd	Y: 92% OS: after 6 cycles Y	[131]

oil + MeOH						decreased a 4.9%	
Oil extracted from *Nannochloropsis gaditana* + MeOH	proROL	WCB	BR	Yes	3rd	Y: 83% OS: after 3 cycles Y decreased to 71%	[132]
Oil extracted from *Nannochloropsis gaditana* + MeOH	proROL	WCB immobilised into BSPs	BR	Yes	3rd	Y: 70% OS: second cycle Y decreased to 43%	[132]
Oil extracted from *Nannochloropsis gaditana* + MeOH	proROL	WCB immobilised into BSPs	BR	Yes	3rd	Y: 83% OS: after 3 cycles Y decreased to 71%	[133]
Oil extracted from *Nannochloropsis gaditana* + MeOH	proROL	WCB	TPB	No	3rd	Y: 58%	[134]
Oil extracted from *Nannochloropsis gaditana* + EtOH	proROL	WCB	TPB	No	3rd	Y: 92%	[134]
Oil extracted from	proROL	WCB	TPB	No	3rd	Y: 58%	[134]

Botryococcus braunii + MeOH							
Oil extracted from *Botryococcus braunii* + EtOH	proROL	WCB	TPB	No	3^{rd}	Y: 68%	[134]
Oil extracted from *Chlorella vulgaris* + MeOH	proROL	Free enzyme	BR	Yes	3^{rd}	C: 75%	[135]
Oil extracted from *Chlorella vulgaris* + MeOH	proROL	IA onto MNP	BR	Yes	3^{rd}	C: 46% OS: after 5 cycles decreased to 10%	[135]
Oil extracted from *Chlorella vulgaris* + MeOH	proROL	CI onto AP modified MNP	BR	Yes	3^{rd}	C: 53% OS: after 5 cycles C decreased to 25%	[135]
Oil extracted from *Chlorella vulgaris* + MeOH	proROL	CI onto AP-GA modified MNP	BR	Yes	3^{rd}	C: 69.8% OS: after 5 cycles C decreased to 45%	[135]
Sludge palm oil + MeOH	proROL	IE into alginate-polyvinyl alcohol beads	BR	No	3^{rd}	Y: 91.30% OS: no activity loss after 15 cycles	[136]
Oil extracted from SCG + MeOH	*R. delemar*	Free enzyme	BR	No	3^{rd}	Y: 18%	[137]

	(= *oryzae)* lipase						
WCO + MeOH	proROL	Free enzyme	BR		3rd	Y: 93%	[138]
WCO + iso-propanol	proROL	Free enzyme	BR		3rd	Y: 86.8%	[138]
WCO + iso-butanol	proROL	Free enzyme	BR		3rd	Y: 80.2%	[138]
WCO + iso-amyl alcohol	proROL	Free enzyme	BR		3rd	Y: 64%	[138]
WCO + MeOH	proROL	WCB IE into calcium alginate beads	BR		3rd	Y: 84%	[138]
WCO + iso-propanol	proROL	WCB IE into calcium alginate beads	BR		3rd	Y: 71%	[138]
WCO + iso-butanol	proROL	WCB IE into calcium alginate beads	BR		3rd	Y: 62%	[138]
WCO+ iso-amyl alcohol	proROL	WCB IE into calcium alginate beads	BR		3rd	Y: 43%	[138]
JO + MeOH	proROL	WCB IE into sodium alginate beads	BR	No	2nd	Y: 80.5% OS: after 6 cycles Y decreased to 61.5%	[139]
KO + MeOH	proROL	WCB IE into sodium alginate beads	BR	No	2nd	Y: 78.3% OS: after 6 cycles Y	[139]

						decreased to 63.4%	
SYO + MeOH	proROL	WCB	BR	Yes	1st	Y: 80% OS: after 3 cycles Y decreased to 18%	[140]
SYO + MeOH	proROL	WCB immobilised into BSPs	BR	Yes	1st	Y: 82% OS: after 10 cycles Y decreased to 10%	[140]
SYO + MeOH	proROL	CI WCB immobilised into BSPs	BR	Yes	1st	Y: 74% OS: after 35 cycles Y decreased to 65%	[140]
SYO + MeOH	proROL	WCB immobilised into BSPs	BR	Yes	1st	Y: 82% OS: after 6 cycles Y decreased to 48%	[141]
SYO + MeOH	proROL	CI WCB immobilised into BSPs	BR	Yes	1st	Y: 80% OS: after 6 cycles Y decreased to 70%	[141]
ALO + MeOH	rROL	IA onto rice husk	BR	Yes	2nd		[142]
ALO + MeOH	rROL	IA onto ReliZyme^{TM} OD403	BR	Yes	2nd	Y: 64.5% OS: after 7 cycles Y decreased to 41.3%	[142]

Crude microbial oil from *Candida* sp. LEB-M3 + MeOH	rROL	IA onto ReliZyme^{TM} OD403	BR	Yes	3rd	Y: 38% OS: after 7 cycles Y decreased to 26.6%	[143]
Neutralised microbial oil from *Candida* sp. LEB-M3 + MeOH	rROL	IA onto ReliZyme^{TM} OD403	BR	Yes	3rd	Y: 38%	[143]
OO + MeOH	rROL	IA onto ReliZyme^{TM} OD403	BR	Yes	1st	Y: 54.3% OS: after 7 cycles Y decreased to 40%	[143]
OA + MeOH	rROL	IA onto ReliZyme^{TM} OD403	BR	Yes	1st	Y: 68%	[143]
RO + EtOH	proROL	IA onto microporous resin NKA	BR	No	1st	Y: above 98% OS: After 10 cycles Y decreased to 60%	[144]
JO + MeOH	proROL-CRL	WCB (proROL) and free enzyme (CRL) IE into sodium alginate beads	PBR	No	2nd	Y: 84.2%	[145]
KO + MeOH	proROL-CRL	WCB (proROL) and free enzyme (CRL) IE into sodium alginate beads	PBR	No	2nd	Y: 81%	[145]

WCO + MeOH	proROL	WCB IE into sodium alginate beads	BR	No	3rd	Y: 94.01%	[146]
WCO + Methyl acetate	proROL	WCB IE into sodium alginate beads	BR	No	3rd	Y: 91.11%	[146]
WCO + Ethyl acetate	proROL	WCB IE into sodium alginate beads	BR	No	3rd	Y: 90.06	[146]
WCO + MeOH	proROL	IE into sodium alginate beads	BR	No	3rd	Y: 83%	[146]
WCO + Methyl acetate	proROL	IE into sodium alginate beads	BR	No	3rd	Y: 80%	[146]
WCO + Ethyl acetate	proROL	IE into sodium alginate beads	BR	No	3rd	Y: 78%	[146]
Oil extracted from *Chlorella vulgaris* + MeOH	proROL	IA into MNP	BR	Yes	3rd	Y: 45% OS: after 5 cycles Y decreased to 10%	[147]
Oil extracted from *Chlorella vulgaris* + MeOH	proROL	IA into MGO	BR	Yes	3rd	Y: 51% OS: after 5 cycles Y decreased to 16%	[147]
Oil extracted from *Chlorella vulgaris* + MeOH	proROL	IA into MGO-AP	BR	Yes	3rd	Y: 54% OS: after 5 cycles Y decreased to 25%	[147]

Oil extracted from *Chlorella vulgaris* + MeOH	proROL	CI into MGO-AP-GA	BR	Yes	3rd	Y: 68% OS: after 5 cycles Y decreased to 58.77%	[147]
Cottonseed oil + MeOH	proROL	WCB immobilised into BSPs	BR	Yes	1st	Y: 27.9%	[148]
Rubber seed oil + MeOHe	proROL	Free enzyme	BR	Yes	2nd	Y: 31%	[149]
Rubber seed oil + Ethyl acetate	proROL	Free enzyme	BR	No	2nd	Y: 33.3%	[149]
SYO + MeOH	proROL-CRL	CI onto silica gel pretreated with AP and GA	BR	Yes	1st	Y: 99.99% OS: after 20 cycles Y decreased to 85%	[150]
RO deodoriser distillate + MeOH	proROL	Free enzyme	BR	Yes	1st	Y: 93.07%	[151]
RO deodoriser distillate + MeOH	proROL-CRL	Free enzyme	BR	Yes	1st	Y: 98.16%	[151]
ALO + MeOH	rROL	CI onto ET, AP and GA pretreated ReliZyme™ HFA403	BR	Yes	2nd	Y: 57.16% OS: after 5 cycles Y decreased a 12.31%	[115]
ALO + EtOH	rROL	CI onto ET, AP and GA pretreated	BR	Yes	2nd	Y: 60.25% OS: after 7 cycles Y	[115]

		ReliZyme^{TM} HFA403				decreased a 11.89%	
Triolein + MeOH	rROL	Free enzyme	BR	No	1^{st}	Y: 71.2%	[72]
Triolein + EtOH	rROL	Free enzyme	BR	No	1^{st}	Y: 64.2%	[72]
Triolein + MeOH	rROL	IA onto RelyZyme^{TM} OD403S	BR	No	1^{st}	Y: 82.6%	[72]
Triolein + EtOH	rROL	IA onto RelyZyme^{TM} OD403S	BR	No	1^{st}	Y:100.7%	[72]
JO + MeOH	rROL	IA onto Lewatit VP OC 1600	BR	Yes	2^{nd}	Y: 61% OS: after 10 cycles Y decreased a 40%	[152]
JO + MeOH	rROL	IA onto Lifetech^{TM} ECR1030M	BR	Yes	2^{nd}	Y: 63% OS: after 10 cycles Y decreased a 40%	[152]
JO + MeOH	rROL	IA onto Lifetech^{TM} AP1090M	BR	Yes	2^{nd}	Y: 55% OS: after 10 cycles Y decreased a 25%	[152]
JO + MeOH	rROL	CI onto Lifetech^{TM} ECR8285M	BR	Yes	2^{nd}	Y: 63% OS: after 10 cycles Y decreased a 60%	[152]

JO + MeOH	rROL	CI onto Amberlita IRA 96	BR	Yes	2^{nd}	Y: 68% OS: after 10 cycles Y decreased a 20%	[152]
OO + MeOH	prorROL	IA onto Amberlite XAD 761	BR	No	1^{st}	Y: 77%	[153]
OO + EtOH	prorROL	IA onto Amberlite XAD 761	BR	No	1^{st}	Y: 62%	[153]
OO + Propanol	prorROL	IA onto Amberlite XAD 761	BR	No	1^{st}	Y: 46%	[153]
OO + Butanol	prorROL	IA onto Amberlite XAD 761	BR	No	1^{st}	Y: 18%	[153]
SYO + MeOH	prorROL	IA onto Amberlite XAD 761	BR	No	1^{st}	Y: 50%	[153]
SYO + EtOH	prorROL	IA onto Amberlite XAD 761	BR	No	1^{st}	Y: 46%	[153]
SYO + Propanol	prorROL	IA onto Amberlite XAD 761	BR	No	1^{st}	Y: 35%	[153]
SYO + Butanol	prorROL	IA onto Amberlite XAD 761	BR	No	1^{st}	Y: 10%	[153]
CO + MeOH	prorROL	IA onto Amberlite XAD 761	BR	No	1^{st}	Y: 70%	[153]

CO + EtOH	prorROL	IA onto Amberlite XAD 761	BR	No	1st	Y: 56%	[153]
CO + Propanol	prorROL	IA onto Amberlite XAD 761	BR	No	1st	Y: 43%	[153]
CO + Butanol	prorROL	IA onto Amberlite XAD 761	BR	No	1st	Y: 16%	[153]
SO + MeOH	prorROL	IA onto Amberlite XAD 761	BR	No	1st	Y: 32%	[153]
SO + EtOH	prorROL	IA onto Amberlite XAD 761	BR	No	1st	Y: 28%	[153]
SO + Propanol	prorROL	IA onto Amberlite XAD 761	BR	No	1st	Y: 17%	[153]
SO + Butanol	prorROL	IA onto Amberlite XAD 761	BR	No	1st	Y: 7%	[153]
Algal oil + MeOH	prorROL	IA onto Amberlite XAD 761	BR	No	3rd	Y: 63%	[153]
Algal oil + EtOH	prorROL	IA onto Amberlite XAD 761	BR	No	3rd	Y: 55%	[153]
Algal oil + Propanol	prorROL	IA onto Amberlite XAD 761	BR	No	3rd	Y: 40%	[153]
Algal oil + Butanol	prorROL	IA onto Amberlite XAD 761	BR	No	3rd	Y: 13%	[153]

ALO + MeOH	rROL	CI onto AP and GA treated ReliZyme^{TM} HFA403	BR	Yes	2nd	Y: 28.62% OS: after 9 cycles, Y decreased a 43%	[104]
JO + MeOH	proROL	WCB immobilised into BSPs	BR	Yes	2nd	Y: 88.6% OS: after 6 cycles Y decreased a 21%	[154]
OA + MeOH	proROL	WCB immobilised into BSPs	BR	No	1st	Y: 80% OS: after 8 cycles, almost no activity loss.	[155]
Rice bran oil + MeOH	proROL	IA onto rod-like mesoporous silica	BR	No	1st	Y: 81.7% OS: after 3 cycles Y decreased to 67.7%	[156]
JO + MeOH	proROL	IE into polyvinyl alcohol—alginate matrix	BR	No	2nd	Yield: 87.1%	[157]
ALO + MeOH	rROL	IA Octadecyl-Sepabeads	BR	Yes	2nd	Y: 58.31% OS: after 2 cycles Y decreased to 54.67%	[158]
Tung oil + MeOH	proROL	CI onto Amberlite IRA 93	BR	Yes	2nd	Y: 91.9% OS: after 6 cycles Y decreased to 85.1%	[32]

Babassu oil + EtOH	proROL	WCB immobilised into BSPs	BR	No	1st	Y: 74.15%	[159]

Regarding third-generation biodiesel, microalgae and waste oils have been the most studied substrates. The former has several advantages that make the overall process of biodiesel production more environmentally friendly as microalgae oil production involves atmospheric CO_2 fixation and can use domestic wastewater like growth substrate facilitating its posterior treatment. However, the main drawbacks for microalgae oil employment are the scale-up of photobioreactors and lipids extraction [160][161]. Nevertheless, ROL has been satisfactorily employed with this substrate, for instance, with oils extracted from *Nannochloropsis gaditana* [132][133][134], *Botryococcus braunii* [134] and *Chlorella vulgaris* [135]. Actually, with the last one, fatty acid methyl esters (FAME) conversions over 70% were obtained indicating ROL suitability for biodiesel production with microalgae oil. Additionally, oils extracted from oleaginous yeasts, such as *Candida* sp. LEB-M3, have been also employed. The use of yeasts becomes important in biodiesel refineries as they might grow in the glycerol coming from this biofuel production [143]. Regarding waste oils, they have a significant potential in biodiesel industry because of their relevance in circular economy strategies, which aim to avoid residue generation by seeking new applications to waste [162][163]. Moreover, considering the tight economic competition between biodiesel and fossil fuels, cheap raw materials are required. In fact, the cost of the feedstocks is more than the 70% of the total cost of biodiesel. Thus, oleaginous wastes might help lowering these percentage and making enzymatic biodiesel production feasible [164]. Sludge from palm oil [136] and spent coffee grounds [137]can be found amongst some of the oleaginous residues studied in biodiesel production with ROL. However, waste cooking oil is the foremost substrate of this category because it is inexpensive and, through its employment in biodiesel synthesis, public institutions avoid the great cost of its management [165][166]. Relevant results have been published with WCO, for instance, Bharathiraja et al. [138] reported maximum triglyceride conversion of 94%. Nevertheless, not many studies dealing with ROL and WCO have been published and, considering the great relevance of this substrate for biodiesel industry, it could be a possible research target for future projects.

Biocatalysts operational stability, reusability and price are related and essential traits that must be considered in enzymatic biodiesel production because of the high cost of enzymes and the tight economic competition with conventional diesel. Some approaches have focused on cutting prices of the enzymes through heterologous production, as it has been explained in the previous section. Besides, other strategies have centred on lipase immobilisation. This technique allows enzyme reutilisation and generally enhances enzyme stability [167][168][169]. In the following paragraphs, the different immobilisation strategies assessed with ROL in biodiesel production will be introduced.

Earlier attempts of employing this enzyme in biodiesel synthesis were principally based on whole-cell biocatalysts (WCBs). Thus, the enzyme acts confined in its natural cellular environment, which protects the lipase from inactivation and degradation. Moreover, as no downstream processes of the biocatalyst are needed, its final cost is considerably lowered [170]. Syed et al. [139] immobilised lipase-producing *R. oryzae* cells into alginate beads and employed them in biodiesel production with jatropha and karanja oil. A response surface optimisation was applied and under the best conditions, biodiesel yields of 73.5% and 72.5% with each respective oil were obtained. In addition, operational stability of the biocatalyst was evaluated and after six cycles, just an activity loss of 20% was reported. Even if free cells, without immobilisation into alginate beads, could have been used in biodiesel production, Sun et al. [140] stated the suitability of cell immobilisation to avoid enzyme leakage and denaturation. This author immobilised *R. oryzae* fungus cells onto biomass support particles (BSPs) and obtained higher operational stability than using free cells. Moreover, to further minimise the enzyme leakage and deactivation, the crosslinking agent glutaraldehyde was used for immobilised cells treatment. The crosslinked biocatalyst obtained better FAMEs yields and operational stability. In the same sense, glutaraldehyde treatment of WCBs—also called WCBs stabilisation—was reported by Ban et al. [141] as well. Lately, He et al. [122] employed this strategy too and obtained a ROL biocatalyst with increased operational stability. After six reactions cycles, more than 90% of initial activity was maintained. However, WCBs show higher complexity in being reused and worse conversion rates than free lipases immobilised onto acrylic resins [170]. For instance, Bharathiraja et al. [138] published that WCBs exhibit worse reaction rate than immobilised purified proROL because of diffusional problems. Therefore, considering these inconveniences and how heterologous production of ROL has been improved, the use of free ROL and its subsequent immobilisation have gained importance amongst the published works.

Traditionally, lipases have been immobilised through adsorption, particularly onto hydrophobic supports—generally acrylic resins with hydrophobic superficial groups such as octadecyl or divinylbenzene—because of the presence of a large hydrophobic patch around the catalytic triad of the lipases that enables an easy immobilisation and might lead to their hyperactivation [171][172]. However, during biodiesel enzymatic synthesis, highly non-polar reaction mediums are employed that might cause enzyme desorption and in consequence, poor biocatalyst operational stability [51]. Nevertheless, some authors have used ROL with this immobilisation technique and obtained outstanding stability results. For instance, Bonet-Ragel et al. [142] reported that after six consecutive reaction cycles, the biocatalysts retained more than the 60% of the initial activity, in accordance with the results published by Duarte et al. [143] and Su et al. [144]. Moreover, in order to overcome the potential enzyme leakage when adsorption techniques are used, some published works have treated the obtained biocatalysts with crosslinking agents like glutaraldehyde, as it was previously explained for WCBs [130][173]. Notwithstanding these mentioned works and other listed in Table 4, ROL entrapment and covalent immobilisation are the most common immobilisation techniques. The former has been used not only with free ROL but with WCBs because it is an easy, fast and cheap immobilisation technique [174]. The most common entrapment strategies are based on polyvinyl alcohol and alginate employment [145][146][157][175]. Muanruksa et al. [136] obtained outstanding results with free proROL immobilised into alginate-polyvinyl alcohol beads. Esterification degrees over 90% were reported and the biocatalyst exhibited a high operational stability, 15 reaction cycles were done with almost no loss of activity.

Regarding covalent immobilisation, since the binding forces between the lipase and the supports are strong, obtained biocatalysts tend to show high stability, high resistance to extreme pH and temperature conditions and almost no enzyme leakage. However, these strong links between the enzyme and the support, as well as the harsh conditions employed during immobilisation process, might have a negative impact on the enzyme activity [176][177]. In any case, there are several studies that employ this immobilisation technique in biodiesel synthesis. Nematian et al. [147] immobilised proROL onto a superparamagnetic nanostructure and described that amongst the three different biocatalysts studied—two based on lipase-support electrostatic interactions and the third one on covalent-linkage—the covalently immobilised proROL showed higher conversion and operational stability. Bonet-Ragel et al. [115] covalently immobilised rROL onto glutaraldehyde pre-treated epoxide acrylic resins and studied its reaction performance and operational stability in biodiesel synthesis with methanol and ethanol as acyl-acceptors. Under the best conditions, yields close to the theoretical 100% were obtained after 360 min for methanol and 260 min for ethanol. In addition, regarding operational stability, no significant activity loss was observed after five consecutive reaction cycles with both alcohols. Besides, Luna et al. [126]described similar operational stability results with ethanol and sunflower oil as substrates, indicating that covalent immobilisation is an adequate technique for biodiesel synthesis with ROL.

In terms of operational strategy in biodiesel synthesis, although ROL has been described as a suitable industrial and solvent-tolerant enzyme, some improvements have been reported to obtain better reaction yields, higher stability or enhance the scale-up of the bioprocess. One of the most commonly employed approach is based on the stepwise addition of the alcohol as the interaction between the lipase and the alcohol is the main enzyme-deactivating factor [178][179]. Several authors have published works in which ROL and stepwise addition strategy have been employed [115][148]. Additionally, other authors have focused on seeking the most adequate acyl-acceptor—the one that has fewer negative effect on the enzyme—by testing different alcohols [138][175] and even the short-esters of the corresponding alcohols performing interesterification reactions [146][149]. Besides, regarding solvents employment, their absence in solvent-free systems has aroused as an interesting operational alternative because of the minimisation of biodiesel downstream processes and the avoidance of hazardous solvents, making the overall biotransformations more cost-effective and environmentally friendly [104][142][149][152].

Lately, the joint employment of both 1,3-regiospecific and non-specific lipases have been researched in order to accelerate biodiesel reaction rates and obtain higher yields [121]. Lee et al. [150] reported yields close to 100% in 2-h reaction and outstanding operational stabilities when using proROL and *Candida rugosa* lipase (CRL). Actually, the conversion yield was still 85% after 20 reaction cycles. In line with these results, Zeng et al. [151] described higher biodiesel production rates when employing together proROL and CRL.

Regarding the scale-up of biodiesel production using ROL, Canet et al. [119] compared packed bed reactor (PBR) with stirred tank reactor (STR) in biodiesel synthesis with rROL immobilised through hydrophobic adsorption. Results showed a higher reaction rate with STR than PBR but, just the opposite outcome when operational stability was the analysed trait. Other authors have also employed PBRs [125][131][145] or even more genuine reactors such as the magnetically stabilised fluidised bed reactor [124] or three-phase bioreactors [120]. However, there are not many works related to the scale-up of biodiesel production with ROL considering the vast amount of research papers published dealing with this biocatalyst. Therefore, more research in this field could be relevant for future projects.

4.2. Structured Lipids Production

Fats and oils are consumed in daily diets as an important source of energy, essential fatty acids and fat-soluble nutrients. Their functional, nutritional and organoleptic properties depend on their composition in saturated and polyunsaturated fatty acids, fatty acid chain length and on the distribution of the different fatty acids in the triacylglycerols (TAGs) (position *sn*-1, *sn*-3 or *sn*-2). Therefore, by modifying the fatty acids composition or its profile, lipids with improved properties might be obtained, the so-called structured lipids (SL). Currently, there are various SLs of commercial interest whose properties have been widely described (Table 5), (i) low caloric and dietetic triacylglycerols that include TAGs with medium-chains (MMM) and TAGs with short- and medium-chain fatty acids in *sn*-1 and *sn*-3 and a long-chain fatty acids in *sn*-2 position, SLS and MLM respectively; (ii) human milk fat substitutes (HMFS), (iii) cocoa butter equivalents (CBE), (iv) *trans*- free plastic fats, (v) triacylglycerols rich in specific long-chain and polyunsaturated fatty acids (PUFAs) and recently, even (vi) diacylglycerols (DAG) and monoacylglycerols (MAG) have been considered as SLs [180][181].

Table 5. Definition and properties of the main commercially relevant structured lipids.

SL Type	Definition	Properties	Ref.
Low caloric and dietetic TAGs	Present lower caloric value than conventional oils and fats. SLS-, MLM- and MMM- type TAGs.	M and S fatty acids present lower caloric value than their counterparts L. M fatty acids have lower tendency to get accumulated. Released M fatty acids can be directly absorbed and provide readily energy in the liver.	[180][181][182][183]
Human milk fat substitutes (HMFS)	Mimic the fatty acid profile of human milk. Contain oleic (30–35%), palmitic (20–30%), linoleic (7–14%) and stearic acids (5.7–8%). Palmitic acid mainly in *sn*-2 position.	Promote palmitic acid absorption as 2-monoacylpalmitate Promote calcium absorption	[180][181][184][185]

Cocoa butter equivalents (CBE)	Mimic the scarce natural cocoa butter Mainly formed by saturated fatty acids (stearic and palmitic acids) in *sn*-1,3 and monounsaturated fatty acids (oleic acid) in *sn*-2 position.	Desirable polymorph is *β* form Similar organoleptic properties to cocoa butter	[180][181][186][187]
Trans-free plastic fats	Mimic *trans* fatty acids containing hydrogenated vegetable oils.	Avoid potential cardiovascular diseases caused by *trans* fatty acids.	[181][188][189]
TAGs rich in specific long-chain and polyunsaturated fatty acids (PUFAs)	Modified TAGs containing a combination of n-3 and n-6 PUFAs to enhance nutritional values. Mainly eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) are employed.	EPA decreases blood viscosity, platelets aggregation and promotes vasodilation. DHA promotes sensorial and neuronal maturation in babies.	[180][190]
MAGs and DAGs	Modified lipids containing one or two fatty acids linked to a glycerol	Non-ionic surfactants capable of using as emulsifiers in the food industry. 1,3-DAGs reduce serum TAGs level and supress body fat accumulation	[116][118][181][191]

SLs production can be carried out through chemical or enzymatical processes, the latter having several advantages when compared to chemical catalysis [192]. Hence, in the same way as stated for biodiesel synthesis in the previous section, enzymatically catalysed reactions allow milder reaction conditions what in this case, as well as lowering energy consumption, might lead to a reduction in the loss of original attributes of temperature-sensitive substrates and products. Moreover, through enzymatic catalysis, the use of deleterious solvents can be avoided enabling a safer and more environmentally friendly food production. However, the most remarkable advantage of lipase employment in this biotransformation is their specificity and selectivity [193][194]. Concretely, 1,3-regiospecific lipases like ROL arouse a keen interest because of their capacity to only modify the *sn*-1 and *sn*-3 positions of TAGs—even though acyl-migration phenomena might occur depending on reaction conditions.

Table 6 shows a summary of the latest published works about SLs synthesis employing ROL. Nunes et al. [195] produced MLM-type SLs by acidolysis of olive oil with capric and caprylic acids. The employed biocatalysts were rROL produced in *K. phaffii* and commercial native ROL (proROL), both of them covalently immobilised onto Eupergit© C and modified Sepiolite. Noticeably, rROL showed a better performance than the native lipase, the percentages of incorporated capric and caprylic acids were higher as well as the operational stability. In spite of the use of pure or commercial substrates, oleaginous wastes or even non-commercially profitable oils might also be employed for MLM-type SLs synthesis with ROL. For instance, Mota et al. [196] described how low-calorie SLs of MLM-type can be produced using oil extracted from spent coffee grounds and oil from olive pomace with proROL immobilised onto magnetic nanoparticles. In the same line, Costa et al. [197] synthesised MLM-type SLs with the oil extracted from grapeseeds of *Vitis vinifera* L., which are a by-product of the wine industry. Moreover, instead of residual oils, Nagao et al. [198] employed the oil from the oleaginous microorganism *Mortierella alpina* to produce MLMs rich in arachidonic acid, a precursor of several hormones.

Table 6. Summary of structured lipids production with *Rhizopus oryzae* lipase as main biotcatalyst.

Product	Substrates	Reaction Type	Lipase	Immobilisation Technique	ID/OS	Ref.
MLM	OO + CRA	Acidolysis	proROL/rROL	CI onto Eupergit®C/sepiolite ($AlPO_4$-sepiolite)	ID: 21.6%. OS: half-life 159 h	[195]
MLM	OO + CA	Acidolysis	proROL/rROL	CI onto Eupergit®C/sepiolite ($AlPO_4$-sepiolite)	ID: 34.82%. OS: half-life 136 h	[195]
MLM	SCG + CA	Acidolysis	proROL	CI onto GA treated MNP	ID: 50%	[196]
MLM	SCG + ethyl caprate	Interesterification	proROL	CI onto GA treated MNP	ID: 26%	[196]

MLM	OP + CA	Acidolysis	proROL	CI onto GA treated MNP	ID: 51% OS: 6.8 batches	[196]
MLM	OP + ethyl caprate	Interesterification	proROL	CI onto GA treated MNP	ID: 46%. OS: 9.1 batches	[196]
MLM	Grapeseed oil + CRA	Acidolysis	rROL	CI onto Amberlite IRA 96	ID: 54%. OS: half-life 166 h	[197]
MLM	Grapeseed oil + CA	Acidolysis	rROL	CI onto Amberlite IRA 96	ID: 69% OS: half-life 118 h	[197]
MLM	TGA58F + CA	Acidolysis	proROL	IA onto Dowex WBA	ID: 64.6%	[198]
MLM	TGA40 + CA	Acidolysis	proROL	IA onto Dowex WBA	ID: 62.8%	[198]
MLM	TGA55E + CA	Acidolysis	proROL	IA onto Dowex WBA	ID: 64.8% OS: 90 days in PBR[1] dropped 10%	[198]
MLM	OO + CRA	Acidolysis	rROL	CI onto Eupergit® C/IA onto Lewatit VP OC 1600	OS: half time 2.4 batches (54.3 h) with Eupergit®C	[199]
MLM	OO + CA	Acidolysis	rROL	CI onto Eupergit® C/IA onto Lewatit VP OC 1600	OS: half time 10.2 batches (234 h) with Lewatit VP OC 1600	[199]

MLM	OO + CRA	Acidolysis	rROL	CI onto Eupergit® C	ID: 15.5%	[200]
MLM	OO + CA	Acidolysis	rROL	CI onto Eupergit® C	ID: 33.3%	[200]
MLM	OO + CRA	Acidolysis	rROL	CI onto Amberlite IRA 96	ID: 76.9	[201]
MLM	OO + CA	Acidolysis	rROL	CI onto Amberlite IRA 96	ID: 85.6%	[201]
HMFS	PA enriched TAGs + OA enriched mixtures	Acidolysis	proROL	IA onto Accurel® MP-1000	ID: OA in *sn*-1,3 67.2% - PA in *sn*-2 67.8%. OS: no activity loss in 10 uses (190 h)	[202]
HMFS	Lard + FFA from EPAX 1050TG	Acidolysis	rROL	CI onto Accurel® MP-1000	ID: 24 mol%. OS: after 4 batches, 55% of original activity	[203]
HMFS	Tripalmitin + FFA from camelina oil	Acidolysis	rROL	AI onto Relizyme™ OD403/S/CI onto Lewatit VP OC 1600	ID: 52%	[204]
TAGs rich in PUFAs	cod liver + tuna oil + ethanol.	Alcoholysis	proROL	IA onto Accurel® MP-1000	Alcoholysis ID: 72% OS: after 6 cycles,	[205]

					complete deactivation.	
	2-MAG from alcoholysis + CRA	Esterification	proROL	IA onto Accurel® MP-1000	ID: 95%. OS: after 5 cycles, no activity loss.	[205]
TAGs rich in PUFAs	Tuna oil + CRA	Acidolysis	proROL	IA onto Accurel® MP-1000	OS: over one week	[206]
	cod liver oil + ethanol 96%	Alcoholysis	proROL	IA onto Accurel® MP-1000	Alcoholysis Y: 78%. OS: after 3 cycles, a 57% decrease	[207]
TAGs rich in PUFAs	cod liver oil + 1-butanol	Alcoholysis	proROL	IA onto Accurel® MP-1000	Alcoholysis Y: 78%. OS: after 3 cycles, no activity decrease	[207]
	Esterification: 2-MAG from alcoholysis + CRA	Esterification	proROL	IA onto Accurel® MP-1000	Esterification Y: 71%.	[207]
TAGs rich in PUFAs	Fish oil + CRA	Acidolysis	proROL	Non-immobilised	ID: 2.5%	[208]

HMFS	Milkfat + SYO	Interesterification	proROL	EI into polysiloxane-PVA	ID: 8.14%. OS: after 10 batches, no activity loss	[209]
CBE	SO + SA-PA mixtures	Acidolysis	proROL	IA onto Accurel® MP-1000		[210]

Regarding HMFS, Esteban et al. [202] used several commercial lipases, amongst them proROL immobilised onto Accurel® MP-1000, to produce a TAG rich in palmitic acid in *sn*-2 and oleic acid in *sn*-1,3; the so called OPO, which is the main component of human milk TAGs. proROL showed the best performance in oleic acid incorporation and exhibited a high operational stability, after ten reuse cycles almost no activity loss was found. Simões et al. [203] also tested different lipases for HMFS production and reported that rROL immobilised onto Accurel® MP-1000 showed a similar performance to Novozymes 435 and Lipozyme RM IM in acidolysis reaction between lard and FFA mixture from fish oil rich in docosahexaenoic acid. Besides, Faustino et al. [204] immobilised rROL produced in *K. phaffii* onto two different supports, Lewatit VP OC 1600 and Relizyme OD403/S, and applied the formed biocatalysts in the production of HMFS rich in polyunsaturated fatty acids (PUFAs). The acidolysis reaction was carried out in solvent-free system between tripalmitin and FFAs (mainly linoleic and linolenic acids) from camelina oil, which proved to be a good source of PUFAs. According to the authors, the results obtained with rROL immobilised onto Lewatit VP OC 1600 were comparable to the commonly used commercial lipase Lipozyme RM IM.

Triacylglycerols rich in long-chain and polyunsaturated fatty acids have also been produced with ROL. In most of the cases, these SLs' production is based on a two-step process in order to minimise the acyl migration phenomena [211]. In the first step, through alcoholysis reaction, 2-monoacylglycerols (2-MAGs) are obtained from oils containing TAGs rich in PUFAs or long-chain fatty acids in the mentioned *sn*-2 position, usually fish oils. Then, these 2-MAGs are esterified with other relevant FFA to obtain the nutritionally interesting TAGs rich in PUFAs. For instance, Muñio et al. [205] studied the performance of different commercial lipases, including proROL immobilised onto Accurel® MP-1000, in the process of alcoholysis of tuna and cod oil to obtain 2-MAGs and then, carry out their subsequent esterification with capric acid. In alcoholysis reaction the commercial lipase Novozyme 435 showed a better operational stability than Lipase D (commercial proROL), although the latter exhibited higher reaction yield. During esterification reaction, Lipase D obtained the highest SLs percentage (over 90%) in the mixture. Moreover, no loss in proROL activity was observed after at least five reaction cycles. Hita et al. [206] and Rodriguez et al. [207] reported similar results with immobilised proROL.

With respect to CBE, although Ray et al. [210] described the kinetics of the acidolysis of high oleic sunflower oil with stearic–palmitic acid mixtures that, after further fractionation of the product, could be potentially used in CBE formulations, ROL has not been extensively used for CBE production. Therefore, this subject might be a great research target for future projects, as well as DAG and MAG synthesis, which have not been specifically treated but just as a minor topic during other products synthesis, like biodiesel.

4.3. Flavour Esters Production

Flavour and aromatic esters are widely found in nature and have pleasant organoleptic attributes, including fruity, floral, spicy, creamy or nutty aromas. These traits made them suitable as ingredients for food, beverages, cosmetics, pharmaceuticals, chemicals and personal care products, like perfumes, body lotions, shampoos and other toiletries [212][213]. In general, most of the flavour and fragrance compounds are produced through extraction from their natural source, usually fruits, plants and flowers. However, they are found in the environment in low concentrations making the extraction a costly process and not viable to fulfil their growing demand. Therefore, chemical and enzymatic synthesis procedures have aroused to solve flavour esters scarcity [214][215]. Noticeably, the latter exhibits a significant advantage—notwithstanding the already explained benefits of enzymatic synthesis over chemical one in the previous sections—which is the capacity to label the obtained products as natural according to European Legislation (EC 1334/2008) if and when the employed reactants are also natural. Thereby, the use of enzymes satisfies consumers trend towards natural products and boosts economic value of the obtained flavour esters [212]. In fact, as well as ROL, other lipases have been used for flavour esters production, for instance, the commercial Novozym® 435 (*Candida antartica* lipase B) [214][215], *Candida rugosa* lipase [216][217] and *Burkholderia cepacia* lipase [218].

Ethyl butyrate is an important component of many fruit flavours such as pineapple, passion fruit and strawberry [219]. The enzymatic synthesis of this compound can be carried out through esterification of butyric acid and ethanol. Guillen et al. [220] immobilised rROL onto three different supports, EP100, Eupergit®CM and Octadecyl-Sepabeads to test them in this esterification reaction. In terms of reaction rate and yield, rROL immobilised onto EP100 showed the best performance. However, rROL immobilised onto Octadecyl-Sepabeads exhibited the highest operational stability. Consequently, this biocatalyst was used for further research in which the effects of butyric acid and ethanol concentration were studied through DoE strategy to maximise the reaction rate and final yield [221]. The obtained results indicated that the suitable acid:alcohol ratio for maximum yield was 1.45 and that the higher the butyric acid concentration the higher the reaction rate. However, as previously described by Grosso et al. [222], elevated concentrations of butyric acid led to enzyme deactivation.

Butyl acetate is another flavour ester with resembling organoleptic properties to pineapple flavour whose production with ROL was reported by Ben Salah et al. [223]. The synthesis of this compound was carried out through esterification reaction of butanol and acetic acid with immobilised proROL onto Celite 545—as preliminary results of the reaction with free enzyme showed poor yield and they were clearly exceeded by the immobilised biocatalyst. According to the authors, solvent-free reaction was chosen as the most suitable strategy because of the easier product purification and lower toxicity and inflammability. In these conditions, a maximum yield of 60% was obtained and the biocatalyst was stable for three consecutive cycles without a decrease in synthesis activity.

Besides esterification, transesterification reaction catalysed by ROL has also been employed for flavour esters synthesis, for example, Kumari et al. [224] reported isoamyl acetate ester synthesis—pleasant banana flavour—through isoamyl alcohol and vinyl acetate transesterification with immobilised proROL. Furthermore, as stated by these authors, the inhibitory effect of the acid [225] was avoided through the use of transesterification reaction with vinyl acetate ester instead of esterification reaction with the corresponding acid. Under optimal conditions, a conversion of 95% in 8 h of reaction was obtained including a great operational stability, after three reaction cycles no activity loss was detected. Garlapati et al. [226] described the use of covalently immobilised proROL onto activated silica to produce through transesterification reactions methyl butyrate and octyl acetate, flavour esters with pineapple and orange odours respectively. As a result of an optimisation process, authors reached high reaction yields in solvent-free system, 70.42% in 14 h and 92.35% in 12 h for methyl butyrate and octyl acetate respectively. Moreover, in both cases, the biocatalyst was reusable for five times retaining a relative activity of more than 95%. Transesterification reaction was as well employed for citronellol esters synthesis with immobilised proROL into HPMC–PVA polymer (hydroxypropyl methyl cellulose—polyvinyl alcohol) and in supercritical carbon dioxide reaction medium [227]. For the three studied flavour esters (citronellol acetate, citronellol butyrate and citronellol laurate) final yields over 90% were achieved indicating the suitability of this biocatalysts and the proposed system for these biotransformations.

4.4. Resolution of Racemic Mixtures

Enantiomerically pure compounds are very attractive for the preparation of a wide range of products, particularly in food and pharmaceutical industries where the desired organoleptic properties or effects might be only related to one of the isomers. Therefore, racemic resolution processes become relevant and arouse the interest in lipases considering the enantioselectivity and specificity of these enzymes [228][229].

Palomo et al. [230] employed proROL to carry out the enzymatic resolution of (*R*)-glycidyl butyrate because of its importance in linezolid synthesis. This product is already sold as a treatment for multidrug resistant Gram-positive infections. According to these authors, they followed the ‘conformational engineering’ strategy, that is, different techniques for proROL immobilisation were employed. This way, the enzyme structure would have different rigidity or the microenvironment surrounding the enzyme would alter the exact shape of the open form of the lipase influencing its catalytic performance. Amongst the three different biocatalysts formed, the best enantiomeric excess (ee) was obtained with proROL immobilised through adsorption on dextran sulphate-coated sepabeads, 99% ee with a 55% conversion.

Benzoin is a relevant α-hydroxy ketone that might act as building block in organic synthesis. Songür et al. [231] described its enantioselective production from benzoin acetate through the employment of *R. oryzae* cell homogenates. The objective of using cell homogenates was to combine the enantioselective hydrolysis of proROL with the racemisation process of the racemase of *R. oryzae* in order to increase the ee and conversion values. This way, a final conversion of (*S*)-benzoin close to the 100% and 96% ee was achieved.

Covalently immobilised proROL onto Lewatit-aldehyde support has been reported as an adequate biocatalyst for asymmetric hydrolysis of dimethyl 3-phenylglutarate [232]. Under the best conditions, it was possible to obtain the (*R*)-methyl-3-phenylglutarate with a 92% ee and an yield in monoester of 97%.

(S)-enantiomer of ibuprofen is 160 more active than its (R)-enantiomer, which can even cause side effects in the gastrointestinal tract. Therefore, obtaining the adequate enantiomer becomes crucial in this case. Yousefi et al. [233] reported the use of immobilised proROL onto octadecyl sepharose to carry out the enantioselective resolution of racemic ibuprofens esters.

The racemic resolution of (*R,S*)-1-phenylethanol to produce (*S*)-1-phenylethanol, a chiral building block, was carried out with proROL-displaying yeast whole cell biocatalyst, that is, a *S. cerevisiae* strain genetically modified to display proROL on the cell surface. After 36 h of reaction, significant results were obtained, 97.3% yield and 93.3% ee [234]. The same biocatalyst was employed to catalyse the optical resolution of the pharmaceutical precursor (R,S)-1-benzyloxy-3-chloro-2-propyl monosuccinate. In this case, the operational stability of the biocatalysts was assessed and it was stable after at least eight reaction cycles [235].

Abbreviations

28proROL-gene	Gene encoding a truncated prosequence of *Rhizopus oryzae* lipase 28 C-terminal amino acids fused to the N-terminal of the mature lipase region
2-MAG	2-monoacylglycerol
ALO	Alperujo oil
BR	Batch Reactor
C	TAG or FFA conversion (%)
CA	Capric acid
CBE	Cocoa butter equivalents
CI	Covalently immobilised or stabilised biocatalyst through crosslinking

CO	Canola oil
CRA	Caprylic acid
CRL	*Candida rugosa* lipase
DAG	Diacylglycerol
DoE	Design of experiments
EDTA	Ethylenediaminetetraacetic acid
ee	Enantiomeric excess
entire-proROL	*Rhizopus oryzae* lipase including the whole prosequence and mature sequence
EPAX 1050TG	TAG rich in omega-3 PUFAs
EtOH	Ethanol
FAME	Fatty acid methyl esters
FFA	Free fatty acid
HMFS	Human milk fat substitutes
IA	Immobilisation through adsorption
ICL	Isocitrate lyase

ID	Incorporation degree (%)
IE	Immobilisation through physical entrapment
JO	Jatropha oil
KO	Karanja oil
L	Long-chain fatty acid
M	Medium-chain fatty acid
MAG	Monoacylglycerol
MeOH	Methanol
MSFBR	Magnetically-stabilised fluidised bed reactor
Mut^+	Methanol utilisation plus phenotype
Mut^S	Methanol utilisation slow phenotype
MW	Molecular weight (kDa)
NBS	*N*-Bromosuccinimide
OA	Oleic acid
OO	Olive oil

OP	Olive pomace
OPO	TAG with oleic acid in *sn*-1,3 positions and palmitic acid in *sn*-2 position.
OS	Operational stability
PA	Palmitic acid
P_{AOX}	Inducible Alcohol oxidase promoter
PBR	Packed bed reactor
PFL	*Pseudomonas fluorescens* lipase
PMSF	Phenylmethylsulfonyl fluoride
proROL	*R. oryzae* lipase containing the N-terminal of mature sequence attached to 28 C-terminal amino acids of the prosequence
proROL-gene	Gene encoding the prosequence of 97 amino acids fused to the N-terminal of the mature lipase region of 269 amino acids
PUFA	Polyunsaturated fatty acids
PVA	Polyvinylalcohol
RO	Rapeseed oil
ROL	*Rhizopus oryzae* lipase
rROL	*Rhizopus oryzae* lipase containing mature sequence of *R. oryzae* lipase

rROL-gene	Gene encoding the mature lipase
S	Short-chain fatty acid
SA	Stearic acid
SCG	Spent coffee ground
SGLB	Solid gas liquid bioreactor
SL	structured lipid
SLLB	Solid liquid liquid bioreactor
SO	Sunflower oil
STR	Stirred tank reactor
SYO	Soybean oil
TAGs	Triacylglycerols
TGA40	commercial oil
TGA55E	Hydrolysed TGA40 oil
TGA58F	*Mortierella alpina* single-cell oil
TPB	Three phase bioreactor

UBC1	Ubiquitin-conjugating enzyme
UPR	5’ upstream region
WCB	Whole cells biocatalyst
WCO	Waste cooking oil
Y	Yield (%)

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