*catalysts*

MDPI

*Review*

# Advances in Recombinant Lipases: Production, Engineering, Immobilization and Application in the Pharmaceutical Industry

**Fabiano Jares Contesini [1,*,†], Marcelo Gomes Davanço [2,†], Gustavo Pagotto Borin [3], Katherina Garcia Vanegas [1], João Pedro Gonçalves Cirino [2], Ricardo Rodrigues de Melo [4], Uffe Hasbro Mortensen [1], Kristiina Hildén [3], Daniel Rossi Campos [2] and Patricia de Oliveira Carvalho [2]**

1 Eukaryotic Molecular Cell Biology, Synthetic Biology Section, Department of Biotechnology and Biomedicine, Technical University of Denmark, Søltofts Plads, 2800 Kongens Lyngby, Denmark; katvanegas@gmail.com (K.G.V.); um@bio.dtu.dk (U.H.M.)

2 Laboratory of Multidisciplinary Research, São Francisco University, USF, São Francisco de Assis Avenue, 218, 12916900 Bragança Paulista, SP, Brazil; davanco.marcelo@gmail.com (M.G.D.); joao.cirino@usf.edu.br (J.P.G.C.); camposrossi@gmail.com (D.R.C.); patricia.carvalho@usf.edu.br (P.d.O.C.)

3 Department of Microbiology, Faculty of Agriculture and Forestry, University of Helsinki, Viikinkaari 9, 00790 Helsinki, Finland; gustavoborin01@gmail.com (G.P.B.); kristiina.s.hilden@helsinki.fi (K.H.)

4 Brazilian Biorenewables National Laboratory (LNBR), Brazilian Center for Research in Energy and Materials(CNPEM), Campinas, 13083100 São Paulo, Brazil; ricardorodriguesmelo@gmail.com

* Correspondence: fabiano.contesini@gmail.com

† These authors contributed equally to this work.

Received: 27 July 2020; Accepted: 3 September 2020; Published: 9 September 2020


**Abstract:** Lipases are one of the most used enzymes in the pharmaceutical industry due to their efficiency in organic syntheses, mainly in the production of enantiopure drugs. From an industrial viewpoint, the selection of an efficient expression system and host for recombinant lipase production is highly important. The most used hosts are *Escherichia coli* and *Komagataella phaffii* (previously known as *Pichia pastoris*) and less often reported *Bacillus* and *Aspergillus* strains. The use of efficient expression systems to overproduce homologous or heterologous lipases often require the use of strong promoters and the co-expression of chaperones. Protein engineering techniques, including rational design and directed evolution, are the most reported strategies for improving lipase characteristics. Additionally, lipases can be immobilized in different supports that enable improved properties and enzyme reuse. Here, we review approaches for strain and protein engineering, immobilization and the application of lipases in the pharmaceutical industry.

**Keywords:** biocatalysis; industrial applications; sustainable chemistry

## 1. Introduction

Lipases have a myriad of industrial and biotechnological applications that rely on the variety of reactions that they catalyze. Based on hydrolytic reactions these biocatalysts can be applied: in the food industry for lipid modification through complete or partial triacylglycerol degradation, resulting in food with improved sensorial properties [1,2]; as a digestive auxiliary [3]; being incorporated in detergents, facilitating the removal of fat stains from fabrics. Through synthetic reactions, lipases can be applied to biodiesel production since they catalyze the transesterification of vegetable oils and simple alcohols [4]. On the other hand, one of the most relevant lipase applications is in the pharmaceutical industry where, through hydrolytic or esterification reactions, lipases can discriminate enantiomers from

racemic substrates and produce specific enantiopure drugs, as in the case of profens and atenolol [5]. Additionally, in several of these applications, lipases can be immobilized in inert supports to improve their properties, including stability and selectivity, and enable reuse [6].

Lipases (EC 3.1.1.3, triacylglycerol lipase) are triacylglycerol hydrolases produced by various organisms with the natural function of hydrolyzing fats and lipids [7]. Although they hydrolyze triacylglycerol molecules into mono- and diacylglycerols, fatty acids and glycerol in aqueous solutions [8], lipolytic enzymes are the most used hydrolases in organic chemistry due to their capacity for catalyzing synthetic reactions in micro-aqueous conditions [9]. Among the different reactions they catalyze are hydrolysis, esterification, interesterification, aminolysis, transesterification, acidolysis and alcoholysis [10].

Lipases are ubiquitous enzymes produced by animals [11], plants [12] and microorganisms [13–15], with microorganisms being the most representative source of commercial lipases. Each lipase producing species has its own set of lipases with different levels of activity, stability and substrate selectivity, fitting its physiological and metabolic requirements. The challenge is to identify variants within the almost endless pool of natural lipases, which has the best potential as a catalyst in a desirable reaction. Elaborate and effective screening programs dedicated to searching for new lipases are constantly ongoing using microorganisms that can be cultured in the laboratory [16] or metagenomics libraries as a starting point [17]. Since new hits may be found in microorganisms that do not perform well in a production setup or just via a DNA sequence, heterologous production is key to exploiting lipase diversity. The predominant hosts used for recombinant lipase production are *Escherichia coli* and *Komagataella phaffii* (previously known as *Pichia pastoris*) [18–21], but other species with a more powerful secretory capacity are increasingly used. In this context, known potent natural secretor organisms, such as filamentous fungi, can serve as excellent high-yielding hosts for heterologous production [22]. For example, *Aspergillus* spp. and *Trichoderma reesei* are eukaryotic hosts that have highly efficient secretory pathways, achieving more than 30 g of protein per liter of media [23–25]. In addition, different *Bacillus* species are great alternatives for the recombinant production of prokaryotic enzymes, achieving levels of 20 g of protein per liter of media [26]. However, although some studies on the homologous or heterologous overproduction of lipases have been reported by these industrial workhorses [27–29], their ability to produce recombinant lipases is still a field that needs to be further explored.

Unlike esterases that preferentially hydrolyze "simple" esters and triglycerides composed by short chain fatty acids (shorter than C6), lipases are mostly active against water-insoluble substrates, such as triglycerides composed by long-chain fatty acids [30,31]. Consequently, lipases have specific kinetic properties, which are related to their catalytic site conformation conferred by a flexible subdomain lid or flap located over the active site. In the presence of insoluble substrates, this structure undergoes a structural change-called interfacial activation [32]-conferring an open conformation to the catalytic site and increasing the lipase activity. In a more aqueous condition, the lid assumes a closed structure, making the lipase inactive [33]. Lipases, in general, act in a wide range of pH conditions. Typically, bacterial lipolytic enzymes tend to act in alkaline pH conditions, while fungal lipases prefer acidic conditions [34]. Apart from those aspects, lipases can be investigated based on their substrate selectivity, including regio- and enantioselectivity, which makes these enzymes excellent choices for the production of enantiopure compounds with high added value [35].

Although lipases have tremendous potential industrial use, they often need to have their properties improved due to the biocatalytic bioprocess requirements. Therefore, using techniques, such as site directed mutagenesis based on rational design, different mutants can be constructed showing improved thermal stability, tolerance against organic solvents and optimized selectivity [36,37]. This is due to the necessity to achieve optimal performance in different industrial fields, and to effectively catalyze synthetic reactions in micro-aqueous conditions where organic solvents are required. In addition, directed evolution techniques can be applied to improve lipase properties [38,39].

In this review, we will focus on different aspects of recombinant lipases, addressing bottlenecks in enzyme production, engineering, immobilization and application in the pharmaceutical industry.

There is major focus on the limitations and strategies used to overcome drawbacks found in lipase use reported in recent investigations.

## 2. Production of Lipases

### *2.1. Microbial Sources of Lipases*

Lipases are found in microorganisms, including bacteria, yeasts and filamentous fungi. Compared to animal sources, including pancreatic lipases, and plant lipases, for instance from *Carica papaya* and seeds, microbial lipases have been more exploited due to the necessity of simple and inexpensive culture media, practical handling, possibility of scale-up cultivations, and availability of various tools for genetic and protein engineering. The existence of profound knowledge regarding their genetics and physiology (especially for model organisms such as *E. coli*, *K. phaffii* and *Saccharomyces cerevisiae*) makes them important candidates for lipase production [40,41].

Most lipases investigated are from bacterial sources, such as enzymes from the genera *Bacillus*, *Geobacillus*, *Pseudomonas*, *Streptomyces*, *Burkholderia*, *Chromobacterium*, *Achromobacter*, *Arthobacter* and *Alcaligenes* [41,42]. Among them, *Bacillus* lipases are the most explored enzymes, having stable activity at high temperatures in a broad range of pH conditions, besides their tolerance to organic solvents [41].

From an industrial perspective, however, lipases produced by yeasts and filamentous fungi are more attractive as they can be obtained in high concentrations and have unique properties regarding thermal stability and substrate specificity [34,40]. Lipases from *Candida*, *Geotrichum*, *Trichosporon*, *Yarrowia*, *Aspergillus*, *Penicillium*, *Rhizopus*, *Rhizomucor* and *Thermomyces* stand out as the most important enzymes of fungal origin [42,43]. Some of them have been optimized for better industrial properties and are available commercially, such as the lipase B (CALB) from *Candida antarctica* (Novozym® 435 and NS 88011), Lipozyme® TL IM from *Thermomyces lanuginosus* (Novozymes, Copenhagen, Denmark), Novozym® 40,086 from *Rhizomucor miehei*, and Lipase FE-01 from *A. oryzae* (ASA Spezialenzyme GmbH, Wolfenbüttel, Germany) [44].

Microorganisms that grow in environments with extreme pH, salinity and temperature conditions are also important sources of lipases with industrial potential. Unfortunately, they may not be easy to propagate at laboratory conditions and their lipases may therefore be more difficult to recruit for human use. Extremophile bacteria, such as thermophiles and psychrophiles, found in hot springs, deep sea sediments and extremely cold spots, such as Antarctica, can survive in those hostile environments and produce diverse lipases with unique tolerance properties. The lipases from extremophiles have been the subject of many studies [44–46], and, according to the Genomes OnLine Database, there are currently 1364 metagenomics studies of environmental samples covering more than 10,000 strains (https://gold.jgi.doe.gov/). Those data bring evidence of the great potential of the uncultured microbial communities as sources for prospecting new lipases with catalytic activity for a broad array of substrates, as well as being pH and temperature tolerant. We envision that codon optimized lipase genes from these sources will be systematically screened in heterologous hosts to deliver novel industrially important lipases in the future.

### *2.2. Production of Recombinant Lipases*

When the natural lipase producer can be efficiently propagated in a bioreactor, homologous expression is an option. In this case, the process is often more straight forward as the production of the lipase may already be naturally optimized. Higher lipase yields are often desirable, and this can be achieved by using stronger promoters to drive the lipase gene. Moreover, the host can be equipped with additional lipase gene copies via plasmids containing the lipase gene or by inserting lipase genes into the genome. To this end, there are a few studies on the production of recombinant lipases via homologous expression in the bacteria *Serratia marcescens* [47] and *Burkholderia cepacia* [48,49], and in the filamentous fungus *A. niger* [27].

However, native lipases may not be suitable for industrial applications, as they might not meet the technical requirements of the scale-up process, including fast growth, high levels of protein production, or optimal physiological properties required in bioreactors. Hence, heterologous expression is an alternative by using known efficient hosts. Technically heterologous expression systems comprise basically three steps: (i) cloning of the gene of interest in a vector having a selection marker, (ii) transformation of the host strain with the constructed plasmid, and (iii) expression of the gene of interest under the control of an inducible or constitutive promoter and a known terminator. The biological systems used for gene expression include prokaryotic and eukaryotic hosts, such as *E. coli* and *K. phaffi*, respectively [41,43,44]. Heterologous expression has also been the choice of many studies of recombinant lipases produced by uncultured microorganisms from extreme environmental conditions. Their lipases encoding genes can be isolated and expressed in heterologous expression systems by the construction of functional metagenomics libraries [50–52]. Functional metagenomics has the advantage of not requiring either individual genome sequencing or the cultivation of unknown producer microorganisms. For more metagenomics studies of lipases with biotechnological potential, Almeida and collaborators [52] provide an interesting review.

Regarding regulatory mechanisms of lipase biosynthesis, it has been observed that they broadly vary in different microorganisms. In the case of *Calvatia* [53], *Rhizopus* [54], *Aspergillus* [55], and *Rhodotorula* [56], apparently, lipase production is constitutive and independent of the addition of lipids, despite the fact that their presence enhanced the levels of lipase produced. Conversely, the literature suggests that long-chain fatty acids - for instance oleic acid - participate in lipase expression in *Geotrichum candidum*, by controlling induction in the transcription level [57]. Concerning carbon sources, in *Fusarium* sp., carbohydrates have been described as repressors of lipase production [58]. Interestingly, in terms of comparison, the expression levels of lipase genes in some organisms, including *K. phaffii* and *Aspergillus*, can be hundreds of times higher than that in the native host, resulting in levels even greater than grams per liter [59–61].

In addition to the construction of strains for overproduction of recombinant lipases, the fermentation conditions should be optimized in order to reach cost effective yields of productivity. The most relevant parameters are pH, temperature, nutrients (carbon and nitrogen sources and salts), oxygen and agitation. In recent decades, the use of multivariate analytic tools—i.e., response surface methodology (RSM), has allowed for the optimization of lipase production by investigating different parameters simultaneously, which allows higher yield levels compared to univariable studies [35]. A lipase from *Bacillus* sp. was heterologously produced in *E. coli* and the authors screened different media and observed that, when *E. coli* was cultivated in Nutrient Broth, maximum extracellular lipase was achieved. The influence of lipase was also improved by the addition of surfactants that might help the lipase to stay in soluble form even at high concentration. The combination of optimized host, vector, surfactant and media resulted in an 18 fold increase in lipase production (214 units/mL) [62]. Another study investigated the optimal conditions for an *Acinetobacter haemolyticus* lipase production in *E. coli*. The authors observed a 70% improvement using the following optimal conditions: $OD_{600}$ equal to 0.6 (before induction), IPTG (Isopropyl β-d-1-thiogalactopyranoside) equal to 0.5 mmol/L, post-induction temperature equal to 40 °C, and post induction time equal to 16 h [63].

The use of low-cost components, mainly agro-industrial wastes, for recombinant lipase production could be a good alternative to decrease lipase production costs. Nooh et al. [64] optimized the production of a lipase from *Geobacillus* sp. in *E. coli* after cultivation in low cost substrates using RSM. A maximum activity of 164.37 U/mL was achieved using 1.0 g/L of molasses, 2.29 g/L of fish waste, 3.46 g/L of NaCl, and 0.03 mM of IPTG.

Besides the nutrient composition of the cultivation media, the fermentation modes are also extremely important for scaling up recombinant lipase production. They include batch, fed-batch, and continuous cultivation systems. Robert et al. [65] assessed fed-batch operational modes for producing the recombinant lipase B from *Candida antarctica* under the constitutive promoter PGK in *K. phaffii*. More specifically, the continuous mode was proven to be more effective in the long run.

It was estimated that using continuous mode, CALB production was almost six times greater than with the fed-batch after six weeks of cultivation. Figure 1 illustrates sequential approaches for recombinant lipase production.

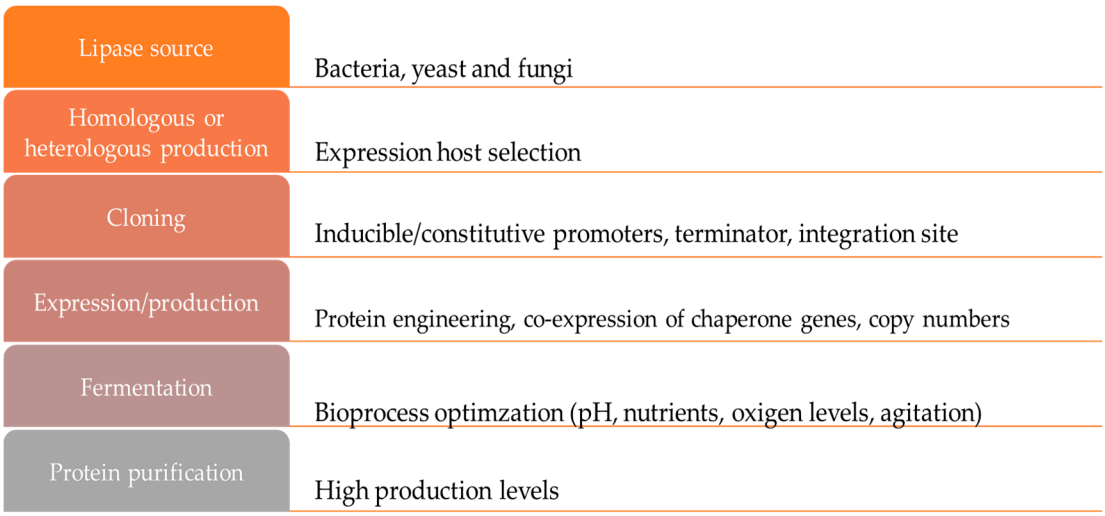


**Figure 1.** Sequential approach used for recombinant lipase production.

2.2.1. Prokaryotic Expression Systems

*E. coli* is by far the most used prokaryotic microorganism for recombinant production of lipases, followed by the Gram-positive soil bacterium *B. subtilis.* Hence, according to Borrelli and Trono [42], *E. coli* represents 50% of the total heterologous production of lipases by eukaryotic and prokaryotic hosts. This dominant position in the production hierarchy has been earned by the more than 40 years of intensive use as production host and the even longer record of serving as one of the most important biological model organisms. As a result, *E. coli* is likely the best understood organism on the planet with a superior genetic toolbox and profound insights into its genetics and physiology. Together, these advantages have been used to construct highly efficient *E. coli* based cell factories for heterologous protein production, and there are examples where the new protein constitutes 30% of the total intracellular content of proteins. Moreover, *E. coli* based processes are typically easy to manipulate and scale-up due to consolidated methodologies of fermentation, cloning and expression with different strains, protocols and studies available. Many strategies are available to optimize a production process. For example, in the context of recombinant lipases, purification can be simplified by fusing the lipase gene to sequences encoding signal peptides (PelB and OmpA) as they will direct the proteins to the periplasmic space. Moreover, yields of soluble protein may be increased by co-expression with chaperones (GroES, GroEL and ClpB). In the first case, the enzyme could be recovered by ultrasonication or other cell lysis processes. The co-expression with chaperones enables the correct folding of the lipase, increases its solubility and decreases protein aggregation. Molecular chaperones are included in a family of unrelated classes of protein that mediate the correct assembly of other polypeptides—i.e., correct folding [66].

The major limitations of using *E. coli* as an expression host include its inability to secrete large amounts of proteins or to perform post-translational modifications (PTMs)—i.e., protein glycosylation. However, *E. coli* is a suitable host organism for bacterial lipases, such as from the genera *Pseudomonas, Bacillus, Staphylococcus, Serratia*, *Burkholderia*, and from metagenomic libraries. Furthermore, *E. coli* has been explored in studies of the biochemical characterization of lipases produced by previously identified microorganisms [63,67] or uncultured strains discovered by metagenomics [68,69]. Table 1 shows different lipase sources, prokaryotic expression systems and lipase properties.

Commercial lipases are mostly obtained from mesophilic organisms that grow at temperatures between 20 and 45 °C. However, extremophile microorganisms—psychrophiles (<20 °C) and thermophiles (>40 °C)—have drawn attention as natural lipase sources due to the outstanding properties of their enzymes, especially related to thermal stability, pH and tolerance to high salt concentrations [44,70]. These lipases might be identified and characterized through the construction of metagenomics libraries, and heterologous expression, followed by screening. Activity assays are also conducted in the presence of detergents, organic solvents and enzyme inhibitors to evaluate their industrial potential as biocatalysts. Sahoo and collaborators [68], for example, have investigated the metagenomics of samples harvested from hot springs (Table 1). After the selection of the best clones, the lipase RK-lip479 was cloned and expressed in the strains *E. coli* DH5α and *E. coli* BL21 (DE3), respectively. The lipase was expressed intracellularly, had maximum activity at 65 °C and pH 8.0, and maintained 89, 92 and 60% of its activity after 6 h at 55, 65 and 75 °C [68]. Similarly, the thermostable lipase BaG7Lip from *B. amyloliquefaciens* G7 was isolated from hot spring samples, cloned and expressed in *E. coli* BL21-Star (DE3) (Table 1) [71]. In that study, the lipase gene without its signal peptide sequence was fused to a His-tag, purified from the intracellular environment, and showed optimal temperature at 50 °C and pH 8.0. An activity improvement of 15% was observed with 30% acetone and glycerol [71].

Salwoom and collaborators [72] conducted a study of expression and biochemical characterization of a recombinant lipase from a previously isolated *Pseudomonas* sp. strain from Antarctica (Table 1). The lipase LSK25 was cloned and expressed in *E. coli* BL21 (DE3). The enzyme was stable in a range of 5–30 °C and pH 6–8. Among the several metal ions evaluated, only $Ca^{2+}$ ions increased the lipase activity. Supplementation of 1 and 3–5 mM $Ca^{2+}$ enhanced the lipase activity in 50 and 150%, respectively, confirming the activation of this lipase by $Ca^{2+}$ ions, as happens with other cold-adapted lipases as well [72]. The lipase LSK25 activity was further boosted up to three times in organic solvents, namely toluene, xylene, n-hexane, n-heptane and n-hexadecane. Higher activities were shown for long chain fatty acids contained in coconut oil and rice bran oil, and also for pNP-C12 synthetic ester. The authors proposed the application of the lipase LSK25 in the food, agrochemical and pharmaceutical industries [72].

The co-expression of the lipase gene with chaperones could be a reliable tool for obtaining the overproduction of recombinant lipases. Alnoch and collaborators [73], for example, co-expressed the lipase *LipBC* and its foldase *LifBC* genes from the Gram-negative bacterium *Burkholderia contaminans* LTEB11 (Table 1). *E. coli* BL21 (DE3) strain was used as expression host and the best lipolytic activity was verified with the co-expression of the N-terminal truncated lipase gene with the full-length foldase gene. This combination resulted in an activity of 127 U/mL against olive oil. Interestingly, no lipolytic activity was found when the *lifBC* gene was not expressed, showing that this foldase plays a key role in lipase conformation and function [73]. Although only the *lipBC* gene was fused to a His-tag, both proteins were purified in a single affinity chromatography step, which could be attractive from an industrial perspective. The foldase LifBC must have formed a complex with the lipase LipBC, which was bound to the column, through a hydrophobic interaction between them. The complex formed, rLipBC, had maximum specific activity of 1426 U/mg towards tributyrin, and maintained a high conversion rate (>80%) of ethyl-oleate in n-hexane over five reaction cycles of 6 h at 45 °C, when it was immobilized on a hydrophobic support (Sepabeads™ FP-BU) [73].

**Table 1.** Prokaryotic systems used for heterologous and homologous production of lipases. The main characteristics of recombinant lipases are also depicted.

| Enzyme | Enzyme Source | Expression Vector (Expression Host) | Cloning Vector (Recipient Strain) | Expression Vector | Molecular Mass (kDa) | Optimal Temperature and pH | Thermal Stability | Additional Remarks | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| Lipase KV1 | *Acinetobacter haemolyticus* | *E. coli* BL21 (DE3) | pGEM-T Easy (*E. coli* JM109) | pET-30a (+) | 39 | 40 °C, 8.0 | $T_{1/2}$ −40 °C, 24 h | Optimization of production conditions using response surface methodology (RSM) | [63] |
| Lipase BaG7Lip | *Bacillus amyloliquefaciens* G7 | *E. coli* BL21-Star (DE3) | p15TV-L (*E. coli* BL21-Star (DE3)) | p15TV-L | 26 | 50 °C, 8.0 | Remaining activity of ~85% at 50 °C for 250 min | Construction of metagenomics libraries + prediction of the best producing conditions using a Boolean network analysis. Activity enhancement with acetone, glycerol and $K^+$ ions. | [71] |
| Lipase LipBC (LipA) + foldase LifBC (LipB) | *Burkholderia contaminans* LTEB11 | *E. coli* BL21 (DE3) | - | pET28a(+) and pT7-7 for LipA and LipB, respectively | 36 (LipA), 37 (LipB) and 66 (complex LipA–LipB) | 25–45 °C, 6.5–10.0 | $T_{1/2}$ −50 °C, 1.5 h | Co-expression of lipase and chaperone genes. Specific activity of 1426 U/mg (tributyrin); > 80% conversion of ethyl-oleate in n-hexane over five reaction cycles of 6 h at 45 °C. | [73] |
| Cholesterol esterase + foldase | *Burkholderia stabilis* FERMP-21014 | *E. coli* BL21 (DE3), *E. coli* Rosetta (DE3) and *B. stabilis* | *E. coli* DH5α and JM109 | pET26b(+), pET39b(+), pET40b(+), pBBR122 | - | - | - | Promoters screening by RNA-Seq + co-expression of lipase and foldase + heterologous and homologous expression. Recombinant activity was 243 fold higher than the WT without oleic acid; *B. stabilis* system was more efficient to produce esterase. | [67] |
| Lipase LipBT and foldase LifBT | *Burkholderia territorii* GP3 | *E. coli* DH5α, *E. coli* DH10β, *E. coli* BL21 (DE3) pLysS, *E. coli* Origami B, *E. coli* Shuffle B, and *E. coli* SHuffle K | pGEM-T Easy (*E. coli* DH5α), pET15b (*E. coli* DH10β) | pGEM-T Easy and pET15b | 30 | 80 °C, 11.0 | $T_{1/2}$ −70 °C, 30 min, pH 8.0 | Metagenomics for screening and identification of lipolytic strains + evaluation of the best expression systems. Higher lipase activity in *E. coli* BL21 (DE3) pLysS (pET15b) (6.73 ± 0.24 U/mg); optimum substrate pNP-C10; activity enhancement in n-hexane, Triton X100, and $Ca^{2+}$ and $Mg^{2+}$ ions. | [74] |
| Lipase Ca-Est | *Clostridium acetobutylicum* (ATCC 824) | *E. coli* BL21 (DE3) | pMCSG7 (*E. coli* DH5α) | pMCSG7 | 29 | 60 °C, 7.0 | Remaining activity of ~70% at 30 °C for 300 min | Rational design + docking analysis + site-directed mutagensis. Activity enhancement with methanol; residues Ser89 and His224 are crucial for catalysis. | [75] |
| Lipase Lip3 | *Drosophila melanogaster* | *E. coli* BL21 (DE3) | - | pETMCSIII | 43 | - | $T_{1/2}$ −37.3 °C (WT) and 52.9 °C (R7_47D) after 45 min | Directed evolution + error-prone PCR + construction of variant libraries. R7_59A mutant activity was higher than the WT towards tributyrin, glyceryl trioctanoate, coconut oil, glyceryl trioleate and pNP (pNP-C3, pNP-C8, pNP-C16, pNP-C18) substrates; 57_59A activity enhancement of 228 fold compared to the WT using pNP-C8 substrate. | [76] |

**Table 1.** *Cont.*

| Enzyme | Enzyme Source | Expression Vector (Expression Host) | Cloning Vector (Recipient Strain) | Expression Vector | Molecular Mass (kDa) | Optimal Temperature and pH | Thermal Stability | Additional Remarks | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| Lipase HT1-5M | *Geobacillus zalihae* | *E. coli* BL21 (DE3)pLysS | pUC57 and pGEX-4T1 (*E. coli* TOP10) | pLysS | 44 | 70 °C, 9.0 | Stable at 30–60 °C for 30 min | Rational design + molecular dynamics (MD) + site-directed mutagenesis. Activity enhancement with $Ca^{2+}$ ions; more stable in DMSO, n-hexane, and n-heptane with $Ca^{2+}$ ions. | [77] |
| Lipase GnMgl | *Glaciecola nitratireducens* FR1064$^{T}$ | *E. coli* BL21 (DE3) | - | pET22b | 39 | 30 °C, 9.0 | $T_{1/2}$ −35 °C, 30 min; lipase retained 30% activity at 0 °C | Site-directed mutagenesis. Higher activity towards monoacylglycerols C12:0 and C14:0, pNP-C6 and pNP-C8, and tributyrin; tolerance to 3.5 M NaCl; improved activity with detergents and organic solvents; mutation of residues Ser156, Asp290, or His318 fully disrupted the lipase activity. | [78] |
| Lipase LipPN1 | *Proteus* sp. NH 2-2 | *E. coli* BL21 (DE3) | - | pET-28a(+) | 31 | 40 °C, 9.0 | Remaining activity of 61.75% (40 °C), 58.30% (50 °C) and 19.63% (60 °C) after 30 min | Site-directed mutagenesis. Highest activity towards pNP-butyrate (pH 9.0, 40 °C); activity enhancement with acetone and ions $Ca^{2+}$, $Mn^{2+}$ and $Mg^{2+}$; rLipPN1 and rLipPN1_C85S reached 1662 U/L and 1436 U/L, respectively; 91.5% of soybean oil was converted into biodiesel. | [79] |
| G55Y, T52Y and AMS8 recombinant lipases | *Pseudomonas fluorescens* AMS8 | *E. coli* BL21 (DE3) | - | pET32b | - | - | $T_{1/2}$ −25 °C, 5 h + 37 °C, 5 h (AMS8); 25 °C, > 10 h + 37 °C, > 9 h (T52Y); 25 °C, 2.5 h + 37 °C, 1.5 h (G55Y) | Rational design + site-directed mutagenesis. G55Y and T52Y lipases had lower affinity by pNP-palmitate, laurate and caprylate substrates compared to WT AMS8 lipase; efficiency of G55Y lipase was higher than T52Y for pNPL and pNPP substrates. | [80] |
| Lipase LSK25 | *Pseudomonas* sp. LSK25 | *E. coli* BL21 (DE3) | pGEMT Easy (-) | pET32b(+) | 65 | 30 °C, 6.0 | Stable at 5–30 °C and at pH 6–8 | Activity enhancement with $Ca^{2+}$ ions; activity boosted in toluene, xylene, n-hexane, n-heptane and n-hexadecane; higher activities towards long chain fatty acids contained in coconut oil and rice brain oil, and pNP-C12. | [72] |
| Lipase RK-lip479 | Uncultured microorganism isolated from hot springs | *E. coli* BL21 (DE3) | pUC19 (*E. coli* DH5α) | pETite C-His Kan | 42 | 65 °C, 8.0 | Remaining activity of 89, 92 and 60% after 6 h at 55, 65 and 75 °C, respectively | Construction of metagenomics libraries. Remaining activity of 50% after 72 h in 25% (*v*/*v*) methanol; maximum activity towards pNP-C12; activity enhancement with DMSO and DMF; yield of biodiesel production was 40–76%. | [68] |

**Table 1.** *Cont.*

| Enzyme | Enzyme Source | Expression Vector (Expression Host) | Cloning Vector (Recipient Strain) | Expression Vector | Molecular Mass (kDa) | Optimal Temperature and pH | Thermal Stability | Additional Remarks | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| Lipase RPK01 | Uncultured microorganism isolated from hot springs | *E. coli* BL21 (DE3) | pGEM-T Easy (*E. coli* DH5α) | pET23(+) | 24 | 40 °C, 8.0 | Stable at 30 °C after 3h; remaining activity of ~50% at 50 and 60 °C up to 3 h | MD simulations. Preference for pNP-decanoate substrate, specific activity 6.2 ± 0.065 U/mg, activity improvement with $Ca^{2+}$ ions, Tween 20 and Triton X-100; tolerance to 1% methanol and n-hexane. | [69] |
| Lipase CALB | *Candida antarctica* | *Corynebacterium glutamicum* MB001 | pEC-CALB and pEC-H36-CALB (*E. coli* DH5α) | pEC-CALB and pEC-H36-CALB | 33 | 40 °C, 9.0 | Stable at 30–50; lipase activity was reduced to 60% at ≥ 60 °C | Activity inhibited by 31% with 10 mM $MgSO_4$. | [81] |

#### 2.2.2. Eukaryotic Expression Systems

The main studied and used eukaryotic expression systems are the yeasts *K. phaffii*, *S. cerevisiae*, *Y. lipolytica*, and the species of filamentous fungus *Aspergillus* that represent nearly 35, 8, 1 and 3% of all heterologously produced lipases, respectively [42]. These microorganisms are not only efficient producers, but some have the status of being generally recognized as safe (GRAS) (*S. cerevisiae*, *Y. lipolytica*, *A. niger* and *A. oryzae*). Proteins produced in eukaryotic cells can undergo a series of PTMs that can affect their chemical and physical properties [82]. Such modifications can increase protein solubility, stability and secretion rate. In industry, fungal lipases have gained attention because of their stability under different conditions and substrate specificity. Moreover, extraction procedures are simpler in comparison to bacterial lipases due to their extracellular character [34]. Table 2 summarizes different eukaryotic sources of lipases, expression systems and properties.

**Table 2.** Eukaryote systems used for heterologous production of lipases. The main characteristics of recombinant lipases are also depicted.

| Enzyme | Enzyme Source | Expression Vector (Expression Host) | Cloning Vector (Recipient Strain) | Expression Vector | Molecular Mass (kDa) | Optimal Temperature and pH | Thermal Stability | Additional Remarks | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| Lipase MAS1 | Marine *Streptomyces sp.* strain W007 | *K. phaffii* X-33 | pPICZαA (*E. coli* DH5α) | pPICZαA | 30 | 25 °C, 8.0 | - | PDI co-expression gives 1.7 fold increase in lipase expression. The highest lipase production was achieved at pH 6.0 and at 24 °C with an activity of 440 U/mL. | [83] |
| r27RCL | *Rhizopus chinensis* | *K. phaffii* GS115 | pPIC9K | pPIC9K | 37 | 37 °C, 8.0 | - | PDI co-expression gives 1 fold increase. The highest lipase activity reached 355 U/mL with one copy of PDI and five copies of r27RCL gene. | [84] |
| CALB | *Candida antarctica* | *K. phaffii* GS115 | pPICZαB (*E. coli* TOP10F′) | pPICZαB | 33 | 40 °C, 7.0 | - | Combinatorial overexpression of Ydj1p-Ssa1p resulted in the highest fold increase, 2.5. Individual overexpression of Ydj1p, Ssa1p and Sec63p increased CALB expression level by 1.6, 1.4 and 1.4 fold, respectively. Co-expression of Ydjlp-Sec63p Kar2p-Ssalp, Kar2p-Sec63p, resulted in 1.5, 1.5 and 1.5 fold increase, respectively. Kar2p-Ydj1p combination resulted in decreased CALB secretion. | [85] |
| CALB | *Candida antarctica* | *K. phaffii* X-33 and M12 *(leu2)* | *E. coli* Stellar™ and XL10-Gold® | pBluescript II SK *pPGKΔ3PRO_LIPB* | 33 | 40 °C, 7.0 | Remaining activity of 15% at 70 °C after 20 min | Strain with three copies achieved 48.760 U/L enzyme yield, 2.3 fold higher than the one-copy strain. | [65] |
| ROL | *Rhizopus oryzae* | *K. phaffii* GS115 | pPICZα and pAO815 (*E.coli* Top10 cells) | pPICZα-ROL | 35 | 35 °C, 8.0 | - | Strain with five copies resulted in 8 fold increase in ROL expression. Co-expression of both genes *Ubc1* and *Hrd1* resulted in.54.2% higher ROL activity, 4750 U/mL. | [86] |
| ROL | *Rhizopus oryzae* | *K. phaffii* X-33 | | pPICZFLDαROL | 35 | 30 °C, 7.25 | - | pFLD gave a 1.9 fold increase compared to pAOX1. The best ROL production strategy with the PFLD-based system is a fed-batch induction phase with a constant sorbitol excess. | [87] |

**Table 2.** *Cont.*

| Enzyme | Enzyme Source | Expression Vector (Expression Host) | Cloning Vector (Recipient Strain) | Expression Vector | Molecular Mass (kDa) | Optimal Temperature and pH | Thermal Stability | Additional Remarks | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| FSL | *Fusarium solani* | *K. phaffii* X-33 | pPICZαA and pGAPZαA (*E. coli* DH5α) | pPICZαA-FSL and pGAPZαA-FSL | 30 | 35 °C, 7.0 | Remaining activity of 96% at 20 °C and 81% at 25 °C after 30 min | Strain expressing pGAPZαA-FSL produced the highest specific lipase activity, 18.81 ± 1.98 U/mg, in 3 days of cultivation time. Lipase activity was enhanced by $Mn^{2+}$, $Ba^{2+}$, $Li^{+}$, $Ca^{2+}$, $Ni^{2+}$, CHAPS and Triton X-100 but was inhibited by $Hg^{2+}$, $Ag^{+}$ and SDS. | [88] |
| CALB | *Candida antarctica* | *K. phaffi* GS115 and *Y. lipolytica* RIY368 | *E. coli* | *K. phaffii* (pIB4, promoter pAOX1, HIS4 marker) and *Y. lipolytica* (JMP4266, promoter pEYK1-A3B, URA3 marker) | 33 | 40 °C, 7.0 | - | The lipase activity after 72 h cultivation was for *Y. lipolytica* strain RIY368 5540 U/mg dcw and for *K. phaffii* strain RIY311 1066 U/mg dcw. For *Y. lipolytica*, the lipase activity increased during the growth phase, whereas for *P. pastoris* it increased both during growth and stationary phase. | [89] |
| Lipase with accession no. AF054513 | *Thermomyces lanuginosus* | *A. niger* NW297 | - | pBOEL960-24 | 30 | 30 °C, 7.5 | - | During chemostat cultivations, the maximal lipase production was observed during sporulation. The heterologous TAKA amylase promoter from *Aspergillus oryzae* was demonstrated to express high levels of lipase in *A. niger*. | [90] |
| Lipase lip | *A. niger* | *T. reesei* Tu6 strain | pBluescript II SK(+) and pMD18-T Simple (*E. coli* DH5α) | pSKpyr4 and pSK-lip | 30 | 45 °C, 7.5 | - | All cph1 gene silencing transformants expressed higher levels of lipase and less cbh 1 transcript than the reference strain, approximately lower than 2%. The RNAi mediated gene silencing of cbh 1 did not negatively affect the lipase transcript abundance. | [91] |
| TDL | *Thermomyces dupontii* | *K. phaffii* X33 | pPICZαA (*E. coli* Top 10) | PICZαA–*tdl*-opt | 30 and 38 (N-glycosylation) | 60 °C, 9.5 | - | Of 15 methanol-inducible promoters, the highest TDL activity was achieved with pFLD1 (27.076 U/mL), whereas of nine constitutive promoters, pGCW14 gave the highest activity (17.353 U/mL). | [92] |

*K. phaffii* is a methylotrophic yeast, which is able to express and produce lipases under the control of methanol-responsive alcohol oxidase promoters. It naturally secretes low amounts of proteins in the extracellular medium, but high levels of secreted recombinant lipase can be achieved using methanol as an inducer. This special characteristic makes the lipase purification step easier and cost-effective. In addition, *K. phaffii* has a number of advantages in comparison to higher eukaryotes. It is a single-celled microorganism, which is easy to genome engineer and it only requires simple culture conditions. It performs many PTM modifications typical for higher eukaryotes, such as disulfide bond formation, glycosylation, proteolytic processing and appropriate folding. Consequently, it can result in a higher degree of biological active lipases in comparison to bacteria [93]. Compared to *S. cerevisiae*, by using *K. phaffii* as the production host, the enzyme can be produced in the intracellular space, secreted to the extracellular broth or displayed at cell surface with a reduced hyperglycosylation level [42,43,50]. Moreover, in comparison to *S. cerevisiae*, *K. phaffii* can be produced in higher cell densities than *S. cerevisiae*, resulting in a higher protein productivity. *K. phaffii* also has low nutritional requirements because it is adapted to catabolize raw carbon and nitrogen sources. Those properties can potentially reduce production costs since these nutrient sources can be found in many industrial by-products. There are several reported strategies to increase the production and secretion of different lipases in *K. phaffii*. Examples and descriptions of some of the most applied strategies are presented in the paragraphs below.

Promoters play an important role in the first step of setting up a cell factory for effective production of a desired protein. Different types of promoters, such as inducible and constitutively active ones, are available for heterologous gene expression in *K. phaffi* [94]. One of the most commonly used promoters is the inducible alcohol oxidase 1 gene promoter (pAOX1). The enzyme expressed by the pAOX1 is responsible for the conversion of methanol to formaldehyde, which is the first reaction in the metabolism of methanol. The promoter is tightly regulated by the presence of repressing carbon sources, such as glucose, glycerol and ethanol. To become fully activated, it requires the presence of methanol [95]. The pAOX1-based protein expression system can be easily applied to control bioreactor cultivations. The tight regulation by the repressing carbon source can enable cell growth to high densities prior to heterologous protein production. However, on an industrial scale this system is hazardous because methanol is a flammable chemical. For that reason, there have been attempts to identify or create promoters that achieve equal or even better protein yields [94,96]. Another inducible promoter that has shown promising results is the formaldehyde dehydrogenase 1 gene promoter (pFLD). The formaldehyde dehydrogenase 1 enzyme is involved in both methanol metabolism and in the assimilation of some amines (ex. methylamine) as a nitrogen source. pFLD can be activated by methanol or methylamine and, similar to pAOX1, be repressed by some carbon sources. Cultivations using sorbitol as carbon source and methylamine as nitrogen source demonstrated that sorbitol did not repress the expression of enzymes involved in methanol metabolism, as formaldehyde dehydrogenase 1 [87].

It should be considered that inducible promoters require longer fermentation runs due to the induction step. Therefore, constitutive promoters may serve as a tool to create more cost-effective fermentation cultivations. One promoter that has been applied successfully to lipase production is the glyceraldehyde-3-phosphate dehydrogenase promoter (pGAP). Since it is a strong constitutive promoter, no induction step is required, resulting in simpler and shorter fermentation cultivations. The expression of *Fusarium solani* NAN103 lipase (FSL) under pGAP or pAOX1 showed that pGAP-FSL produced the highest specific lipase activity and, in addition, there was a two-day reduction in the cultivation time [88]. Many promoters with different strengths and properties are available for expression in *K. phaffii*. Testing different types of promoters for constructing a specific protein cell factory is a good starting point for the further development and optimization of the heterologous expression system. With respect to lipase production, a promoter that gives good yields for one enzyme will not necessarily give the best results for another [92].

In most cases, increasing the copy number of a desired gene enhances the expression of the recombinant protein. In *K. phaffi*, the production of the lipase CALB was improved 2.3 fold by increasing the copy number to three. In this experiment, a defective marker approach was applied based on the leucine marker *leu2-d*, in which multiple copies were necessary to reach a state of prototrophy [65]. However, in some cases a high gene dosage can negatively affect the production because the secretory pathway may become overwhelmed by the effects of high levels of recombinant protein that need to be correctly folded [97]. When *R. oryzae* ROL was expressed in up to eight copies, the best results were achieved with the strain harboring five copies, with a fold increase of eight. Since strains harboring more copies produced lower levels of ROL, these strains may have potentially produced unfolded proteins that lead to ER stress and the activation of the unfolded protein response (UPR) [86]. Therefore, it is important to adjust the copy number by taking into account the protein folding capacity of the host.

Even though *K. phaffii* has the potential to fold lipases properly, the native system's secretory pathway may reach its full capacity. This might result in the accumulation of unfolded proteins in the endoplasmic reticulum (ER) that will trigger the activation of UPR. In this case, the UPR functions by reducing the translation of proteins, degrading misfolded proteins and increasing the production of molecular chaperones involved in protein folding. There are many different types of chaperones with various refolding and/or stabilization properties [98]. As an example, protein disulfide isomerase (PDI) catalyzes the formation and breakage of disulfide bonds between cysteine residues, permitting proteins to achieve their correct folding. Other chaperones, such as Kar2p, also known as binding immunoglobulin protein (BIP) in mammals, play key roles in protein translocation through the ER. BIP/Kar2p stabilizes immature proteins by binding to their hydrophobic amino acids, allowing the protein to achieve its correct folding. Moreover, BIP/Kar2p prevents unfolded protein aggregation by assisting in ER-associated degradation (ERAD).

The overexpression and co-overexpression of chaperones in *K. phaffii* have been demonstrated to improve protein secretion and production yield [99]. The co-expression of PDI increased the production of the MAS1 lipase from marine *Streptomyces* 1.7 fold. MAS1 was expected to have two pairs of disulfide bonds and the higher levels of PDI were predicted to assist their formation [83]. Interestingly, increasing the copy number of PDI does not always automatically increase the protein production and secretion. *Rhizopus chinensis* lipase r27RCL was co-expressed with different copy numbers of PDI in *K. phaffii*. PDI was expected to ease bottlenecks in the ER, since the crystal structure of the lipase r27RCL revealed that it contains three disulfide bonds. One copy number of PDI resulted in a one fold increase in enzyme activity, whereas two, three and four copies resulted in lower activity. The higher copy number of PDI may reduce the availability of transcription and translation machinery for the expression of the lipase gene [84].

The overexpression of different combinations of chaperones has also resulted in an increase in lipase production. The production of the lipase CALB from *C. antarctica* was tested with the individual overexpression of different chaperones (Ydj1p, Ssa1p, Sec63p, and Kar2) and with a combination of them. The chaperones Ssa1p and Ydj1p stabilize the nascent peptide chain and help its translocation into the ER. Sec63p binds to the translocation peptide and prevents it from moving backwards through the ER channel. The best results were achieved with the combination of Ydj1p-Ssa1p, increasing the production by 2.5 fold. This suggests that these chaperones interact synergistically by enhancing protein folding, secretion and preventing protein aggregation. In contrast, a decrease in CALB was observed with the expression of Kar2p. This could indicate that the excessive presence of Kar2p shifted the ER balance towards the ERAD pathway, resulting in decreased CALB secretion [85].

### Alternative Eukaryotic Hosts

Different hosts can provide a range of diverse properties, which may be better suited to a specific cell factory design. For that reason, a lot of effort has been made in recent years to find other eukaryotic hosts for lipase production. The yeast *Y. lipolytica* is considered a suitable production host because of its expanding genetic tools for heterologous expression, and its ability to metabolize raw substrate and

secrete protein in large amounts [100]. The comparison of CALB production by *Y. lipolytica* and *K. phaffii* was performed by using well-characterized integration targets for both strains - the *leu2* locus in *Y. lipolytica* and the *his4* locus in *K. phaffii*. CALB was expressed in *Y. lipolytica* under the control of erythritol inducible promoter pEYK3AB and in *K. phaffii* under the methanol inducible pAOX1. The results showed that, in *Y. lipolytica*, growth rate and detected CALB activity were higher than those achieved in *K. phaffii*. The difference in growth rate has been suggested to be due to the loss of some of the carbon source methanol, which can be converted directly to $CO_2$ instead of biomass production in *K. phaffii*. Interestingly, even though enzyme activity was higher in *Y. lipolytica*, the mRNA expression levels of CALB encoding gene in *K. phaffii* were higher than in *Y. lipolytica*. Higher CALB stability with suitable secretion levels may explain the better performance of *Y. lipolytica* [89].

The filamentous fungus *A. niger* represents an interesting alternative host because of its high production yield of extracellular enzymes, non-toxicity, robustness and GRAS status [101]. However, there are indications that lipase might not be secreted properly by *A. niger*. As an example, the production of a lipase from *T. lanuginosus* (accession no. AF054513) resulted in only 1% of *A. niger* total cellular protein because most of the lipase produced was attached to the mycelium [90].

Another promising fungal host for lipase production is Trichoderma reesei, with potential for high protein secretion and a well-established fermentation platform. It also has a well-characterized genetic toolbox and transformation can be achieved using selection markers and by stable integration into the genome [102]. A drawback encountered with T. reesei as a lipase production host is that it produces a high level of cellobiohydrolase I (CBHI) that represents approximately 50% of all proteins secreted. The reduction in the unnecessary production of CBHI could result in improved levels of heterologous expression. The RNAi mediated gene silencing of cbhI improved the production of the lipase lip from A. niger in T. reesei, with an increase up to 3.2 fold. The reduction in CBHI enhanced the availability of the protein folding and secretion machinery. Moreover, this strategy allowed the host to still have CBHI present, thereby keeping the strain in a more natural state [91].

## 3. Lipase Characteristics and Engineering

### *3.1. Structural Characteristics of Lipases*

Knowing and understanding the structure of an enzyme is of key importance not only to improve enzyme production, but also mainly to provide information for protein engineering through site directed mutagenesis. Regarding lipase structural features, they consist of a catalytically active core domain (the α/β-hydrolase fold), similar to other hydrolases, containing the catalytic triad and the oxyanion hole. They might also contain additional structural modules, such as a lid or flap-like structure. Most of the lipases possess an active core domain predominantly composed of eight parallel β strands forming a super-helically twisted central β sheet surrounded by a varying number of α helices [103,104]. However, the number, as well as the organization of β strands, may still diversify. For instance, *Bacillus subtilis* lipase lacked the β1 and β2 strands in the canonical fold [105]. In addition, the first X-ray structure of a triglyceride lipase (*Mucor miehei*) was reported by Brady et al. [106]. Figure 2 illustrates the three-dimensional structure of two microbial lipases with the lid in open and closed conformation.

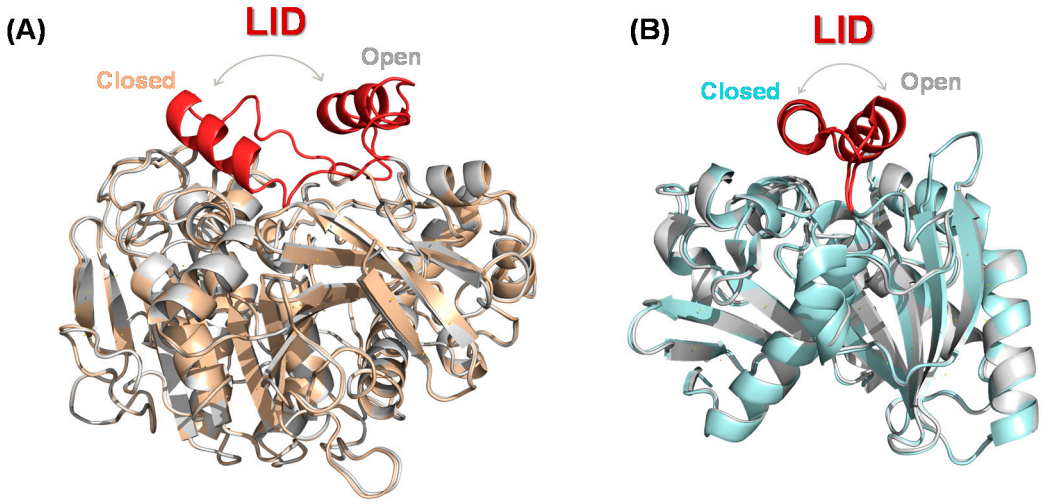


**Figure 2.** Overview of the three-dimensional structure of two microbial lipases. (**A**) Crystallographic structure of *Candida rugosa* lipase with its α/β-hydrolase fold in gray exemplifies the lid in its open conformation (PDB access number 1CRL) and its α/β-hydrolase in a wheat tint exemplifies the lid in its closed conformation (PDB access number 1TRH). (**B**) Crystallographic structure of *Rhizomucor miehei* lipase with its α/β-hydrolase fold in gray exemplifies the lid in its open conformation (PDB access number 4TGL) and its α/β-hydrolase fold in cyan exemplifies the lid in its closed conformation (PDB access number 3TGL). The lids in both structures are in red. All figures were prepared using molecular visualization software, PYMOL.

The catalytic mechanism of lipases is based on a catalytic triad made up of a nucleophile amino acid (Ser), an acid (Asp or Glu), and a His. The catalytic triad containing Asp is known in enzymes, such as proteases. Interestingly, however, different from the other lipases and serine proteases, the catalytic triad of a *G. candidum* lipase is Ser-His-Giu, with glutamic acid replacing the common aspartate [107]. The central nucleophile residue is located within a conserved Glu-X-Ser-X-Glu motif [108]. The oxyanion hole is another important component involved in the catalytic efficiency of this enzyme, since it helps in stabilizing the transition state in the catalysis. During the catalytic process, a negatively charged tetrahedral intermediate is generated, and the oxygen ion thus formed is stabilized by the oxyanion hole amino acids. The oxyanion hole residues play an essential role in stabilizing this oxygen by hydrogen bonding. The catalytic region of lipases has one of the oxyanion hole's residues positioned adjacent to the nucleophile amino acid serine, while the second residue is located between the β3 strand and α helix [104]. Lipases also possess a lid or flap-like structure, which is composed of one or more α helices of variable length. The binding pocket of lipases is present on the central β sheet, which can be a hydrophobic, crevice-like binding site located near the protein surface or funnel-like or tunnel-like binding sites. The lid domain involves specific interactions with the substrate and controls the inactive/active-form enzyme equilibrium [33].

### 3.2. Lipase Engineering

Protein engineering is the process of customizing new enzymes with improved features by altering their primary amino acid sequences. Given the considerable diversity of possible alterations, this procedure has already produced remarkable results in the design of optimized lipases used in important industrial areas. Protein engineering strategies, such as rational design and directed evolution have led to interesting results in the improvement of different lipases.

Engineering a protein by rational design uses prior knowledge of protein structure and in-depth computational modeling approaches to deliberately design new biocatalysts [109]. In part, the number of lipase structures deposited in the Protein Data Bank (PDB, https://www.rcsb.org/) and lipase sequences information in various databases has substantially facilitated the rational design

of these proteins. Furthermore, many different types of modeling software have been created, which makes this methodology easier to use and enhances the success rate of modeling predictions. Usually, the information derived from computer modeling identifies certain amino acids (hot-spots) that should be altered to lead to a change in the lipases' properties. By using computational predictions, Mohammadi et al. [110] constructed four mutants of *S. marcescens* lipase. The MutG2P and MutG59P mutants showed higher catalytic efficiency and thermal stability than the wild-type (WT) enzyme. Furthermore, other computational approaches have been developed to predict the effects of mutations on lipases. Li et al. [37] used a strategy denoted as RIF that consists of three computational methods—i.e., Rosetta ddg monomer, I Mutant 3.0, and FoldX—to rationally design and optimize the thermal stability of *R. miehei* lipase. The most stable mutant, harboring the mutations T18K/T22I/E230I/S56C-N63C/V189C-D238C, exhibited a 14.3 °C higher melting temperature and a 12.5 fold increase in half-life at 70 °C. Kim et al. [111] selected different mutants of CALB based on the B-factor value of amino acid residues and computational modeling by RosettaDesign. The melting temperature of the mutant R249L was increased by 2.3 °C compared to that of WT CALB. Moreover, the rational strategy was applied to block the primary acyl-binding tunnel of CALA lipase to create variants with high specificity for medium-chain-length fatty acids [112]. Likewise, rational design was used to engineer CALA, aiming to develop a highly selective variant for *trans*-fatty acids [113,114].

The dynamic behavior of lipases in different conditions has also been investigated using computational approaches, such as molecular dynamics (MD) simulations. The MD simulations have made progress in this direction since they provide atomistic information about the dynamic molecular interactions, which determine the protein stability and function [115]. Haque and Prabhu [116] carried out MD simulations of a mutant porcine pancreatic lipase in open and closed conformations using different solvents to explain the dynamics of lid opening. At higher temperatures, it was possible to observe the lid opening by the mutants Asp250Val and Glu254Leu, suggesting the important role of these residues in holding the lid in closed conformation, which can affect the activity of this enzyme. A computational design scheme based on MD simulations was also applied to improve the thermal stability of the lipase LIP2 from *Y. lipolytica* [117]. Based on the four structural parameters RMSD, Rg, SASA and the number of internal hydrogen bonds, it was found that the V213P mutant would have higher thermal stability than its WT parent. V213P also has an optimal temperature of 42 °C, which is 5.0 °C higher than that of the wild type.

Directed evolution (also called molecular evolution) does not require that the amino acid sequence and three-dimensional structure should be previously available, as random mutagenesis generates libraries with random mutations in the gene of interest. Those random mutations can be induced by error-prone PCR, chemicals, irradiation, DNA shuffling or staggered extension process (StEP). Owing to the high number of possible clones and enzyme variants, it is necessary to conduct high-throughput and time-consuming screening for the selection of the best enzyme variants created. Besides rational design and directed evolution, there is a third approach for engineering an enzyme to acquire better properties. Semi rational design is the combination of the former two approaches. It can be summarized as the generation of libraries of mutant genes via the saturation mutagenesis method. In this technique, one or a few amino acid residues are replaced by the other 19 amino acid possibilities through degenerate codons. Semi rational design has been proven to be a rapid laboratory method for engineering proteins, once a relatively smaller size of libraries are generated, facilitating the further screening and selection step [50,118,119].

Curiously, Alfaro-Chávez and collaborators [76] scrutinized how many changes are necessary to transform a non-attractive lipase into an enzyme with better properties and potential for use in industrial applications. Lip3-encoding gene from *Drosophila melanogaster* was cloned into the vector pETMCSIII and expressed in the *E. coli* BL21 (DE3) system. Concomitantly, error-prone PCR was used to generate libraries of mutant variants harboring random mutations along the lipase gene. After seven rounds of directed evolution, screening and selection, only five mutant lipases stood out from tens of hundreds of lipase variants. The best variant, R7_59A, had eight substitutions in its sequence

compared to the WT lipase. The lipase R7_59A showed an activity 4, 5, 6 and 12 fold higher than WT towards coconut oil, tributyrin, glyceryl trioctanoate, and glyceryl trioleate substrates, respectively. Using crude lysate, there was an improvement of 228 times in the 57_59A activity against pNP-C8 compared to the WT. Furthermore, the 57_59A mutant had a 6.3, 1.5, 7.8 and 2.6 fold increment in the activity against pNP-C3, pNP-C8, pNP-C16 and pNP-C18 substrates compared to WT. The induced mutations in the best five variants were present in amino acid residues related to the cap domain stability, residues near to the catalytic triad and in residues of the hinge points of the cap domain. According to the study, those lipase variants could be targeted in further evolutions aiming to use them in detergent formulation [76].

## 4. Relevant Immobilization Methods for Recombinant Lipases

The use of enzymes in various industrial areas has significantly increased due to several associated benefits. However, some factors, such as low enzymatic stability, may limit the use of lipases and other industrial enzymes [120,121]. One of the most important and widely used techniques to improve enzyme properties is their immobilization, in which the biocatalysts are attached to an inert support. The immobilization of enzymes provides several benefits, such as (1) the production of efficient and stable biocatalysts; (2) possibility of recovery and reusability of enzymes [122,123]; (3) easy purification of the products when compared to the free enzymes; (4) increasing the efficiency; (5) permitting their applications in continuous fixed-bed operation [122].

The immobilization methods can be separated into two classes—namely, the physical and chemical methods. Physical methods include support and enzyme interactions of different types, including hydrogen bonds and hydrophobic interactions. In the latter method, the formation of covalent bonds can be achieved through amide, ether, thio-ether or carbamate bonds between the support and the enzyme [124–126]. The physical methods specifically include entrapment and adsorption, while chemical methods include cross-linking and covalent binding. Different enzyme immobilization methods are grouped and listed in Figure 3.

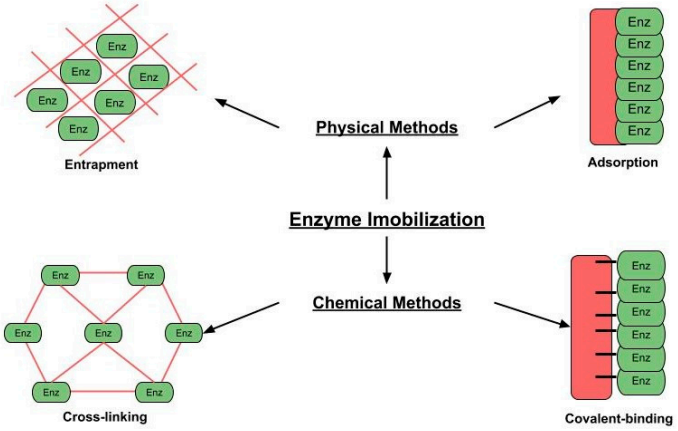


**Figure 3.** Different methods of enzyme immobilization.

The immobilization of an enzyme by adsorption is a simple technique with high commercial value due to its simplicity, regular use in large-scale processes, low cost, retention of high enzyme activity, and relatively chemical-free enzyme binding [127]. Supports with different degrees of hydrophobicity were previously used such as butyl Sepabeads and octadecyl Sepabeads [126], decaoctil-Sepabeads [128], macroporous resin HPD826 [129], polypropylene powder [124,130,131] and pore-expanded mesoporous silica (SBA-15) [132]. The use of hydrophobic supports is of particular interest, because these supports mimic the enzymes' natural media and can often promote hyperactivation, highly selective adsorption, purification, increased enantioselectivity, and strong but reversible immobilization, making support reuse possible after the enzyme has been deactivated [133,134].

Hydrophobic core-shell polymeric supports, including Accurel, PMMA/PMMA (poly(methyl methacrylate) on core and shell) and PMMA-co-DVB/PMMA-coDVB (methyl methacrylate copolymerized with divinylbenzene on core and shell) obtained by a combined suspension and emulsion polymerization process have demonstrated great potential in the immobilization of a recombinant *C. antarctica* lipase [135]. This technique involves the lipase interfacial activation versus the hydrophobic surface of the support, which is the main cause of the lipase improved activity and stabilization [135,136].

One example of commercial lipase immobilized on a hydrophobic support is Novozym 435 (N435), supplied by Novozymes. The enzyme is expressed in *A. niger* and produced by interfacial activation of lipase B from *C. antarctica* on the resin, Lewatit VP OC 1600. This resin is a macroporous support formed by poly(methyl methacrylate) crosslinked with divinylbenzene. N435 is considered one of the most widely used commercial biocatalysts in both academy and industry [137].

In physical adsorption, enzyme leaching is a critical problem that limits the use of the immobilized enzyme in different reaction conditions [138]. To solve this problem, entrapment is employed to restrict the enzyme in the polymer frameworks [139]. In the entrapment method, the enzyme is locked inside the lattice of a polymer matrix or membrane so that it is retained while allowing the penetration of the substrate. That can be achieved with a variety of materials, including polymers, sol-gels, polymer/sol-gel composites and other inorganic materials.

It has been observed that the entrapment of lipases in silica sol-gels increases thermal stability and enhances reaction rates by several orders of magnitude [140]. The entrapment of CALB was successfully achieved with poly 1-vinyl-3-ethylimidazolium bromide (VEImBr) [139], and the immobilization of the whole cells or cell lysates of *E. coli*, producing a thermostable lipase from *T. lanuginosus* (r*E.coli*/lip), was also reported using silica xerogel and nanocarbon in silica composites [141].

For chemical methods, the enzyme is firmly immobilized on the chemically modified support through covalent binding and cross-linking, effectively preventing enzyme leakage. Cross-linking is an improvement on the covalent attachment because the enzyme is cross-linked to the support with the help of a cross-linker. The most commonly used cross-linking reagent is glutaraldehyde, as it is economical and easily obtainable in large quantities [142].

CALB was immobilized by covalent binding on strongly hydrophobic polystyrene microspheres (poly glycidyl methacrylate) activated by epoxy groups, and this immobilization has been proven to play a key role in improving the properties of the lipase [143]. The carrier hollow silica microspheres (MAT540TM) activated by six bisepoxides enclosing different spacers was applied for the immobilization of CALB. The immobilized preparation was used in the kinetic resolution of racemic 1-phenyethanol and five racemic amines using shaken flasks and continuous-flow packed-bed microreactors with good yields and high enantiomeric excess (ee > 99%, for all) [144]. Furthermore, the recombinant CALB expressed in *K. phaffii* was immobilized by cross-linked enzyme aggregate (CLEA) technology and tested for the synthesis of olvanil [145].

A recombinant lipase from *Thermus thermophilus* WL expressed in *E. coli* was immobilized onto the surface, being rich in the amine functional groups ($-NH_2$), of the 3-APTES-modified $Fe_3O_4$@SiO2 nanoparticles [125]. The covalent bond on the aldehyde activated-agarose in the presence of DTT (glyoxyl-DTT-agarose) at pH 7.0 and on glyoxyl-agarose at pH 10.2 was applied for the immobilization of the lipase from Archaeon *Pyrococcus furiosus* (Pf2001) produced in *E. coli* B21 [126].

In the last decade, applications of new materials for the immobilization of lipases have drawn special interest. These new material groups include magnetized nano-sized materials [146,147], which provide large surface areas for the attachment of the enzyme and increase the probability of its efficient activity. By using this technique, the improvement of the downstream processing is clear as it facilitates the immobilized enzyme separation from the reaction mixture. Abd El-Aziz et al. [147] immobilized a recombinant *Pseudomonas aeruginosa* lipase through surface conjugation with gold nanoparticles and observed higher lipolytic activity in the bioconjugate compared to the free enzyme. Dandavate et al. [148] and Yilmaz et al. [149] immobilized a *Candida rugosa* lipase onto the silica

nanoparticles surface and glutaraldehyde-activated aminopropyl glass beads, resulting in easy recovery, and enzyme reuse for ester synthesis. In this context, when *Candida antarctica* lipase was immobilized on chitosan-coated magnetic nanoparticles of iron activated with glutaraldehyde for the production of the biolubricant ester, the biocatalyst gave half of the initial conversion after seven cycles of esterification [150].

Another special area of interest has been the applications of hybrid and composite materials for lipase immobilization. The hybrid origin materials are synthesized from combinations of precursors of inorganic, organic and mixed inorganic and organic origin and offer high stability, good affinity for enzymes and the presence of many chemical functional groups compatible with the chemical moieties present in the protein structure [133]. An interesting example of a composite material used for enzyme immobilization is the combination of graphene oxide (GO) decorated with ZnO nanoparticles (GO/ZnO). Zhang et al. [151] used this support for the adsorption immobilization of a *C. rugosa* lipase (CRL) and it exhibited excellent performance in terms of thermal stability and reusability.

A general combined purification and immobilization method, based on modified controlled porosity glass (GPGs) and polymer-coated versions thereof (HybCPGs), which can bind protein affinity tags, named EziG™, was used to immobilize *C. antarctica* and could be obtained in a significantly shorter time compared to the corresponding Accurel®-based preparations [152]. Affinity tags are exogenous amino acid residues that selectively bind to a biological or chemical ligand placed on a solid support (e.g., His-tags connected to immobilized Ni) or an immobilized protein partner. Among all the tags, the His-tags are the most used because they are small, less disruptive than other tags and non-immunogenic [153]. The combination of nanotechnology with fusion strategy seems a promising approach and can be devised by using several nanomaterials combined with self-cleaving peptides for one-step, economical, high-yield, and large-scale purification and the immobilization of recombinant proteins [154].

## 5. Application of Recombinant Lipases

The versatility of lipases in catalyzing the hydrolysis of esters and reactions of esterification, trans-esterification and inter-esterification, considerably expands the commercial and technological applications of these enzymes. For this reason, they can be applied in the food, oleochemical, pharmaceutical, fine chemical, cosmetics, pulp and paper, leather and biosensor industries and in the treatment of effluents rich in oils and fats [155–157]. In addition, they have been used for the synthesis of different oleochemical products, such as free fatty acids, glycerin and its derivatives, biodiesel, biolubricants and different alkyl esters [158].

Despite the vast application potential of the lipases, their application spectrum is limited due to an absence of the process-suitable characteristics necessary to endure harsh processing conditions during industrial applications operating at relatively high temperature and pH conditions. Hence, the lipases envisioned for industrially applicable processes must possess the necessary characteristics, such as stability and high yield, in order to prove their worth. The use of lipases as industrial catalysts depends heavily on the production of recombinant enzymes with biochemical and catalytic characteristics improved by protein engineering. The evolution of strategies to apply these techniques on an industrial scale has enabled a significant reduction in lipase cost, consequently stimulating the development of other industrial applications.

Lipases are commonly applied in the dairy industry for the selective hydrolysis of milk triacylglycerols during cheese ripening. Particularly, a lipase from *R. miehei* expressed in *A. oryzae* is commercially available with the trade name Palatase M (Novozymes) and used in the dairy industry to improve cheese properties. Lipase (Est_p6) isolated from a metagenomic library from marine sediments was produced in *E. coli* and applied to efficiently hydrolyze milk fat and impart a desirable and distinctive flavor to milk products [159]. A *Penicillium cyclopium* lipase produced in *K. phaffii* [160] has been used for the synthesis of mono- and diacylglycerols and showed its potential

for food emulsifier preparations. The commercial enzyme Novozym 435, an immobilized lipase from *C. antarctica* expressed in *A. niger*, has been successfully used to produce sugar esters [161].

Specifically, in the pharmaceutical industry, recombinant enzymes are mainly used for applications as biocatalysts in the synthesis of chiral intermediates for the development of drugs, and as therapeutic drugs for the treatment of various diseases. Lipases offer several advantages compared to other enzymes and several chemical catalysts, such as capacity for the resolution of racemic mixtures by the synthesis of a simple enantiomer being currently exploited for the production of medicines by the pharmaceutical industry, mainly for the manufacturing of antidepressant, antihypertensive and vasodilator drugs [156,162].

Single-enantiomer intermediate synthesis for the preparation of drugs is important in the pharmaceutical industry. Single enantiomers can be produced either by chemical or biocatalytic routes. Biocatalysis is emerging as an efficient alternative to traditional chemical methods for the production of pharmaceuticals. The advantages of the enzyme-catalyzed reactions compared to chemical processes include enantioselectivity and the use of mild reaction conditions which may reduce energy consumption and waste generation.

The following reported examples show that recombinant lipases have been an important tool in improving pharmaceutical processes for chiral drug intermediates and vitamin synthesis.

*Pharmaceutical Applications of Recombinant Lipases*

Profens (2-arylpropionic acids) are drugs commonly used in the treatment of pain and inflammation associated with tissue injury. The anti-inflammatory activity of ketoprofen, flurbiprofen, ibuprofen, and naproxen is mainly related to their (S)-enantiomer. For these drugs, (R)-enantiomers contribute weakly to cyclooxygenase (COX) inhibition and have been related to side effects—e.g., gastrointestinal pain [163,164].

In recent years, the kinetic resolution of 2-arylpropionic acids by enantioselective esterification has been widely studied. Lipases have been applied in this process and their relevant characteristics demonstrated in many papers; the promising results point to the future applications of these enzymes [5,6,164,165]. For instance, lipases from *C. antarctica*, *C. rugosa* [164,166] and *A. niger* [6] have been applied in the kinetic resolution of profens, making it possible to obtain drugs with high enantiomeric purity—e.g., (R)-flurbiprofen, $ee_p$ = 96.3%; E = 90.5%; C = 35.7%), therefore, demonstrating their great potential as biocatalysts in pharmaceutical synthesis. Figure 4 illustrates the kinetic resolution of racemic profens using lipases.

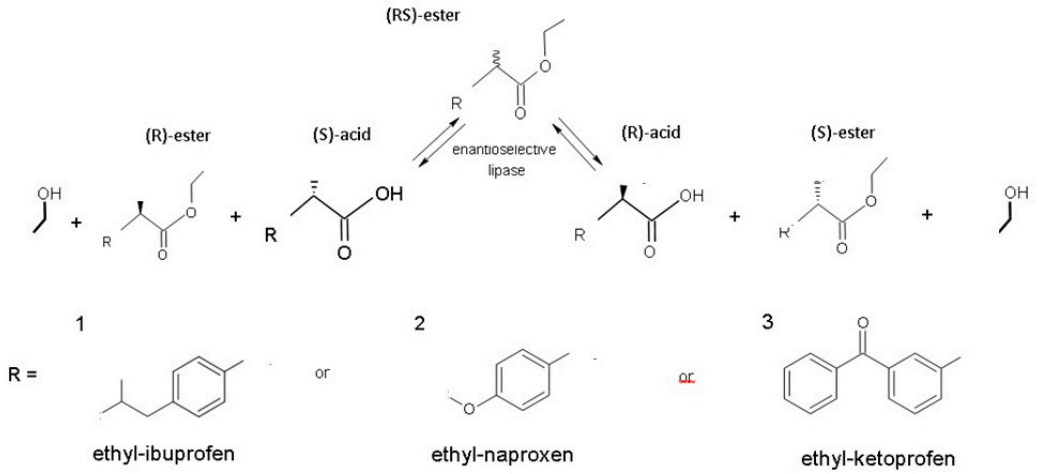


**Figure 4.** Lipase-catalyzed scheme of (RS)-ethyl ester racetame of naproxen, ketoprofen and ibuprofen by an enantioselective lipase (adapted from Gérard et al. [167]). R (Radical) represents the different ethyl profens (ethyl-ibuprofen, ethyl-naproxen and ethyl-ketoprofen).

A lipase from the *S. marcescens* ECU1010 strain produced in *E. coli* catalyzed the hydrolysis of racemic ketoprofen esters into (S)-ketoprofen, with the highest ee (enantiomeric excess) (91.6%) and yield (48.2%) [168]. A lipase from *C. rugosa* produced in *K. phaffii* was used for the kinetic resolution of racemic ibuprofen by means of enantioselective esterification with 74% ee [130].

Commercial lipase from *C. antarctica* produced in *A. niger* and immobilized onto polymethylmethacrylate (Novozym 435) has been efficiently used for the enantioselective esterification of (R,S)-flurbiprofen to obtain the (R)- flurbiprofen methyl ester in 96% ee [166]. A lipase gene from *S. marcescens* ES-2 was reinserted in the same strain for lipase homologous production and was applied in the enantioselective hydrolysis of (R,S)-flurbiprofen ethyl ester for the production of the optically active form (S)-flurbiprofen [169].

Taking into consideration that the lipase Lip2p from *Y. lipolytica* has higher catalytic activity than *C. rugosa* lipases to hydrolyze profen ethyl esters, though its selectivity is not sufficient, Gérard et al. [167] improved Lip2p enantioselectivity by site-directed mutagenesis. The authors identified two amino acid residues (232 and 235) in the lipase hydrophobic substrate binding site as crucial for enantiomer discrimination and enzyme activity. Their results demonstrated the high potential of rational engineering to create biocatalysts suitable for industrial applications.

Chemical synthesis is the common process for obtaining vitamin A derivatives. However, the process presents some issues, such as synthesis steps, which could destroy raw materials (use of acid and alkali), create difficulties in the separation of the final product due to the instability of intermediate products, as well as considerable energy consumption and pollution. Accordingly, a green and environmentally friendly manufacturing method has become the current trend in obtaining vitamin A derivatives. To conveniently prepare the immobilized enzyme for the green synthesis of vitamin A palmitate, *T. lanuginosus* lipase (TLL) was assembled with apatite derived from calcium phosphate in solution to form TLL@apatite hybrid nanoflowers (hNFs) by mimetic biomineralization. Under the optimized conditions, when vitamin A acetate and palmitic acid were applied as substrates, the yield of the product was up to 90.4% [170].

Recombinant CALB produced by *K. phaffii* was immobilized and used to synthesize vitamin A palmitate by transesterification of vitamin A acetate and palmitic acid in organic solvent with a conversion ability of 54.3% after 15 cycles [129]. As represented in Figure 5, vitamin A palmitate synthesis could be catalyzed by *C. antarctica* lipase B.

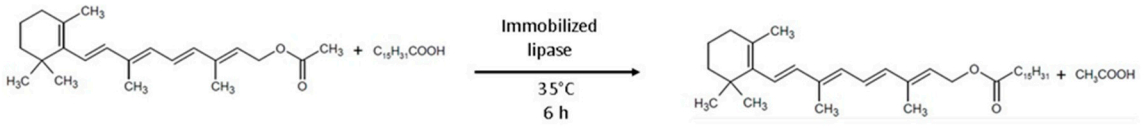


**Figure 5.** Immobilized lipase-catalyzed scheme of vitamin A palmitate (adapted from Yao et al. [129]).

Pregabalin, a GABA (gamma-aminobutyric acid) analogue, is a drug developed for treatment of several central nervous system disorders, involving neuropathic pain, fibromyalgia, anxiety, social phobia and epilepsy. The synthesis of pregabalin involves 2-carboxyethyl-3-cyano-5-methylhexanoic acid ethyl ester, an intermediate substance with chiral characteristic. The (S)-2-carboxyethyl-3-cyano-5-methylhexanoic acid enantiomer is the key chiral intermediate for pregabalin synthesis. Considering the significant increase in the use of pregabalin in recent years, different routes have been explored using chemical and enzymatic approaches [171,172].

Lipozyme TL IM® and Lipolase® (Novozymes) are commercially available lipases for the kinetic resolution of (S)-2-Carboxyethyl-3-cyano-5-methylhexanoic acid [172]. In particular, high resolution yields (45%) and enantioselectivity (98% enantiomeric excess, ee) were achieved by using commercial Lipolase, a recombinant *T. lanuginosus* lipase expressed in *A. oryzae* [173]. Figure 6 illustrates an optimized route of pregabalin synthesis by enzymatic resolution and decarboxylation steps, considering the work of Martinez et al. [173].

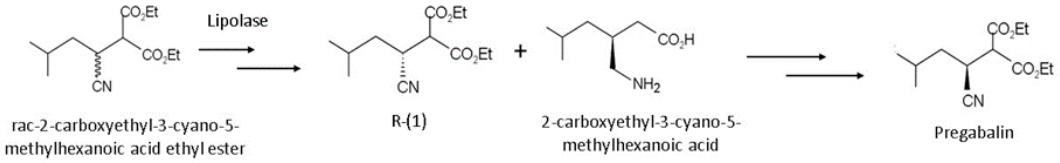


**Figure 6.** Optimized chemoenzymatic pregabalin synthesis (adapted from Martinez et al. [173]).

A mutant L206F/P207F/L259F of *T. thermophilus* lipase was efficiently immobilized onto epoxy resin D5730 to enable the reuse (using n-heptane/water biphasic system) of the enzyme and to improve the conversion at a high concentration of 2-carboxyethyl3-cyano-5-methylhexanoic acid ethyl ester with 49.7% conversion and 95% $ee_p$ (enantiomeric excess of the product) under the optimized conditions [172].

Diltiazem hydrochloride is a typical calcium channel blocker clinically used as an antianginal and antihypertensive agent [174]. *Trans*-3-(4′-methoxyphenyl)glycidic acid methyl ester ((2R, 3S)-MPGM or (−)-MPGM) is a key chiral intermediate for its synthesis. Some approaches involving lipases have been applied in the production of this diltiazem intermediate, such as the application of the *S. marcescens* lipase, a well-known and important lipase for industrial applications.

Lipase from *S. marcescens* ECU1010 catalyzed production of (−)-MPGM by the enzymatic resolution of *trans*-3-(4′-methoxyphenyl)glycidic acid methyl ester ((±)-MPGM) with chitosan in an isopropyl ether water biphasic system (Zhao et al., 2010). Overexpression of *S. marcescens* lipase in *E. coli* often promotes inclusion bodies, which is one of the major problems in industrial recombinant protein production [174,175]. A simple and efficient method for refolding with simultaneous purification of *S. marcescens* lipase in *E. coli* in the production of (−)-MPGM has been described with an overall yield of 41.5% and *ee* of 99% [175]. Figure 7 shows a lipase-catalyzed scheme of the synthetic process of diltiazem.

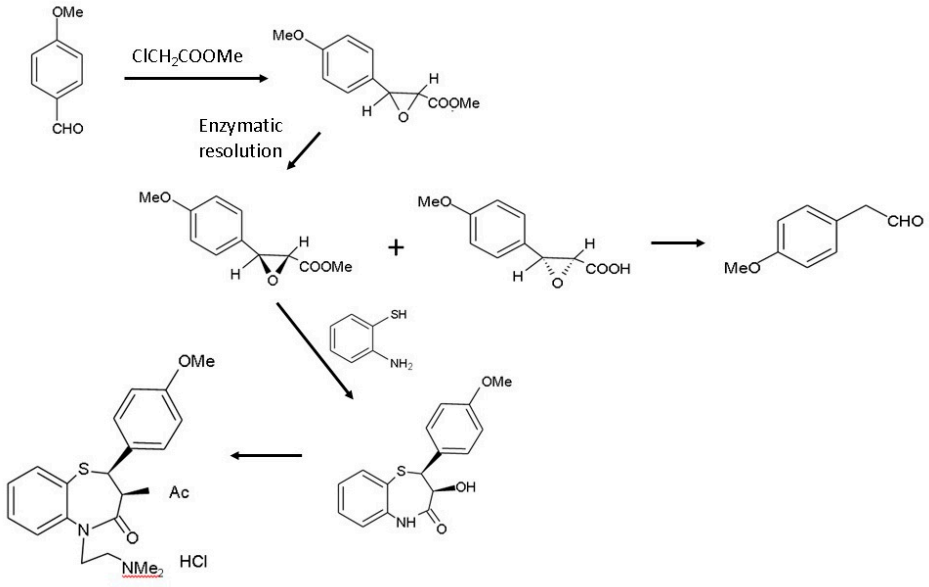


**Figure 7.** Lipase-catalyzed scheme of the synthetic process for diltiazem (adapted from Shibatani et al. [174]).

Beta-blockers belong to an important therapeutic class, which is used to treat several cardiovascular conditions [176]. Chirality is a key factor to consider for the efficacy of this class of drugs. Atenolol is a synthetic, beta1-selective (cardioselective) adrenoreceptor blocking agent and it is used in the treatment of hypertension, angina pectoris due to coronary atherosclerosis, as well as acute myocardial infarction angina [177]. It is known that its b1-blocking activity is attributed to its (S)-enantiomer [178]. In a

screening with commercially available lipases, *Candida antarctica* lipase-A was considered adequate in a new chemoenzymatic route for the (R)- and (S)-atenolol synthesis based on an enzymatic kinetic resolution approach. Compared to the other published methods, the synthesis of the intermediates in an aqueous medium and enzymatic kinetic resolution in an ionic liquid improved overall yield and made the process greener [179].

Lipase B from *Candida antarctica* (CALB) is one of the most used lipases for pharmaceuticals manufacturing, mainly to obtain key enantioselective intermediates, as well as final enantiomer drug products. There are some characteristics that make this enzyme widely used in pharmaceutical processes, such as good stability, the production of optically pure products, esterification reactions and regioselective modifications of multifunctional substrates. Novozym 435 (Novozymes) is a commercially available immobilized preparation of CALB, being the most used lipase preparation described in the literature. The large-scale production involves the expression of CALB gene in *Aspergillus niger* and the immobilization of the enzyme on an hydrophobic carrier (acrylic resin) [137,180].

Many pharmaceutical industries and academic research groups have published patents that applied CALB to catalyze reactions in order to obtain pharmaceutical intermediates, as well as "final" active pharmaceutical ingredients [180]. Table 3 shows some examples of granted patents involving enzymatic synthesis of drugs and/or intermediates using CALB.

**Table 3.** Examples of granted patents applying CALB for enzymatic synthesis of pharmaceuticals.

| Substance | Reaction Analyzed | Application | Patent Number | Sponsor | Reference |
|---|---|---|---|---|---|
| Valsartan | Stereoselective hydrolysis | Enantioselective hydrolysis of racemic esters of valsartan | CN105420338A | Tiantai Yisheng Biochemical Technology Co. Ltd. | [181] |
| Levetiracetam | Stereoselective hydrolysis | Kinetic resolution of a racemic 2-haloester | CNA2009100263523A | Zhejiang Changming Pharmaceutical Co. Ltd. | [182] |
| Ticagrelor | Stereoselective hydrolysis | Kinetic resolution of an alcohol used for the drug preparation | CN104164469A | Beijing University of Chemical Technology | [183] |
| Sofosbuvir | Site-selective acyl-transfer | Mono-deacetylation of a precursor for sofosbuvir preparation | WO2017144423A1 | HC-Pharma AG | [184] |
| Posaconazole | Stereoselective acyl-transfer | Stereoselective monoacylation of prochiral intermediate with isopropanoic anhydride. | CN105753693A | Ningbo Xinkai Biotechnology Co. Ltd. | [185] |
| L-carnitine | Stereoselective acyl-transfer | Kinetic resolution of racemic hidroxynitrile | CN106748843A | Wuxi Fortune Pharmaceutical Co. Ltd. | [186] |
| Carbamate prodrug | Stereoselective hydrolysis | Kinetic resolution of an intermediate | WO2016208709A1 | Daiichi Sankyo Co. Ltd. | [187] |

## 6. Conclusions

Lipases are one of the most versatile industrial biocatalysts and the most used enzyme in organic synthesis. For their industrial use, they should be highly stable in specific conditions, active and frequently selective for the substrate. Obviously, most of native lipases are far from being ready for industrial use. In this context, promising lipase candidates from various sources can be optimized through procedures that include strain engineering for enhanced lipase production, lipase engineering with improved properties and lipase immobilization to be reused for long time periods in batches or packed bed reactors. However, these techniques should be applied in combination with bioprocess optimization. The past and recent advances in genetics and computational modelling have incited a revolution in recombinant DNA technologies in addition to complementary techniques, such as molecular dynamics (MD) simulations fields. A great number of new genomes, vectors, genetically modified strains, genetic toolbox, and immobilization and chromatographic matrices are currently available, and their will be an increasing trend in the coming years, accompanying industrial demand. These new technologies have been substantially used in academia and by companies to explore and improve the features of lipases, with the final goal of making them commercial biocatalysts.

**Author Contributions:** F.J.C., G.P.B., R.R.d.M., M.G.D., K.G.V., J.P.G.C, D.R.C., P.d.O.C., U.H.M. and K.H. wrote the manuscript. J.P.G.C., M.G.D., R.R.d.M., G.P.B. and F.J.C. designed the figures. F.J.C., P.d.O.C., U.H.M. and K.H. revised and corrected the manuscript. All authors have read and agreed to the published version of the manuscript.

**Funding:** We would like to thank FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo), process numbers 2006/013932 and 2018/13317-6. We would like to acknowledge the EU/RIA Falcon project (720918) and Finnish Academy consortium project AromaFung (2988892) and the scholarship provided by FAPESP to R.R.d.M. (17/14253-9).

**Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the study, in the writing of the manuscript, or in the decision to publish it.

## References

1. Gerits, L.R.; Pareyt, B.; Decamps, K.; Delcour, J.A. Lipases and their functionality in the production of wheat-based food systems. *Compr. Rev. Food Sci. Food Saf.* **2014**, *13*, 978–989. [CrossRef]
2. Rani, S.; Jagtap, S. Acceleration of Swiss cheese ripening by microbial lipase without affecting its quality characteristics. *J. Food Sci. Technol.* **2019**, *56*, 497–506. [CrossRef]
3. Antonini, F.; Crippa, S.; Falconi, M.; Macarri, G.; Pezzilli, R. Pancreatic enzyme replacement therapy after gastric resection: An update. *Dig. Liver Dis.* **2018**, *50*, 1–5. [CrossRef]
4. Amini, Z.; Ong, H.C.; Harrison, M.D.; Kusumo, F.; Mazaheri, H.; Ilham, Z. Biodiesel production by lipase-catalyzed transesterification of *Ocimum basilicum* L. (sweet basil) seed oil. *Energy Convers. Manag.* **2017**, *132*, 82–90. [CrossRef]
5. Carvalho, P.D.O.; Contesini, F.J.; Ikegaki, M. Enzymatic resolution of (R, S)-ibuprofen and (R, S)-ketoprofen by microbial lipases from native and commercial sources. *Braz. J. Microbiol.* **2006**, *37*, 329–337. [CrossRef]
6. Da Silva, V.C.F.; Contesini, F.J.; de Oliveira Carvalho, P. Enantioselective behavior of lipases from *Aspergillus niger* immobilized in different supports. *J. Ind. Microbiol. Biotechnol.* **2009**, *36*, 949–954. [CrossRef]
7. Svendsen, A. Lipase protein engineering. *Biochim. Biophys. Acta (BBA)/Protein Struct. Mol. Enzym.* **2000**, *1543*, 223–238. [CrossRef]
8. Naik, S.; Basu, A.; Saikia, R.; Madan, B.; Paul, P.; Chaterjee, R.; Brask, J.; Svendsen, A. Lipases for use in industrial biocatalysis: Specificity of selected structural groups of lipases. *J. Mol. Catal. B Enzym.* **2010**, *65*, 18–23. [CrossRef]
9. Saxena, R. Microbial lipases: Potential biocatalysts for the future industry. *Curr. Sci.* **1999**, *77*, 101–115.
10. Haki, G.; Rakshit, S. Developments in industrially important thermostable enzymes: A review. *Bioresour. Technol.* **2003**, *89*, 17–34. [CrossRef]
11. Rueda-López, S.; Martínez-Montaño, E.; Viana, M.T. Biochemical characterization and comparison of pancreatic lipases from the Pacific bluefin tuna, Thunnus orientalis; totoaba, Totoaba macdonaldi; and striped bass, Morone saxatilis. *J. World Aquac. Soc.* **2017**, *48*, 156–165. [CrossRef]
12. Rivera, I.; Gutiérrez-Ortega, A.; Sandoval, G. Functional Expression of Plant Lipases: The Case of CpLip1 from Carica papaya. In *Lipases and Phospholipases*; Humana Press: New York, NY, USA, 2018; pp. 169–178.
13. Li, M.; Yang, L.-R.; Xu, G.; Wu, J.-P. Cloning and characterization of a novel lipase from Stenotrophomonas maltophilia GS11: The first member of a new bacterial lipase family XVI. *J. Biotechnol.* **2016**, *228*, 30–36. [CrossRef] [PubMed]
14. Carvalho, T.; Finotelli, P.V.; Bonomo, R.C.; Franco, M.; Amaral, P.F. Evaluating aqueous two-phase systems for *Yarrowia lipolytica* extracellular lipase purification. *Process Biochem.* **2017**, *53*, 259–266. [CrossRef]
15. Rehman, S.; Wang, P.; Bhatti, H.N.; Bilal, M.; Asgher, M. Improved catalytic properties of Penicillium notatum lipase immobilized in nanoscale silicone polymeric films. *Int. J. Biol. Macromol.* **2017**, *97*, 279–286. [CrossRef]
16. Lanka, S.; Latha, J.N.L. A short review on various screening methods to isolate potential lipase producers: Lipases-the present and future enzymes of biotech industry. *Int. J. Biol. Chem.* **2015**, *9*, 207–219. [CrossRef]
17. Almeida, J.M.; Martini, V.P.; Iulek, J.; Alnoch, R.C.; Moure, V.R.; Müller-Santos, M.; Souza, E.M.; Mitchell, D.A.; Krieger, N. Biochemical characterization and application of a new lipase and its cognate foldase obtained from a metagenomic library derived from fat-contaminated soil. *Int. J. Biol. Macromol.* **2019**, *137*, 442–454. [CrossRef]
18. Dartois, V.; Baulard, A.; Schanck, K.; Colson, C. Cloning, nucleotide sequence and expression in *Escherichia coli* of a lipase gene from *Bacillus subtilis* 168. *Biochim. Biophys. Acta (BBA)-Gene Struct. Expr.* **1992**, *1131*, 253–260. [CrossRef]
19. Van Tassel, L.; Moilanen, A.; Ruddock, L.W. Efficient production of wild-type lipase B from Candida antarctica in the cytoplasm of Escherichia coli. *Protein Expr. Purif.* **2020**, *165*, 105498. [CrossRef]

20. Minning, S.; Serrano, A.; Ferrer, P.; Solá, C.; Schmid, R.D.; Valero, F. Optimization of the high-level production of Rhizopus oryzae lipase in Pichia pastoris. *J. Biotechnol.* **2001**, *86*, 59–70. [CrossRef]
21. Huang, J.; Zhao, Q.; Chen, L.; Zhang, C.; Bu, W.; Zhang, X.; Zhang, K.; Yang, Z. Improved production of recombinant Rhizomucor miehei lipase by coexpressing protein folding chaperones in Pichia pastoris, which triggered ER stress. *Bioengineered* **2020**, *11*, 375–385. [CrossRef]
22. Kaieda, M.; Nagayoshi, M.; Hama, S.; Kondo, A.; Fukuda, H. Enantioselective transesterification using immobilized Aspergillus oryzae overexpressing lipase. *Appl. Microbiol. Biotechnol.* **2004**, *65*, 301–305. [CrossRef] [PubMed]
23. Finkelstein, D.B. Improvement of enzyme production in Aspergillus. *Antonie Van Leeuwenhoek* **1987**, *53*, 349–352. [CrossRef] [PubMed]
24. Cherry, J.R.; Fidantsef, A.L. Directed evolution of industrial enzymes: An update. *Curr. Opin. Biotechnol.* **2003**, *14*, 438–443. [CrossRef]
25. Bischof, R.H.; Ramoni, J.; Seiboth, B. Cellulases and beyond: The first 70 years of the enzyme producer *Trichoderma Reesei*. *Microb. Cell Fact.* **2016**, *15*, 106. [CrossRef]
26. Harwood, C.R.; Cranenburgh, R. Bacillus protein secretion: An unfolding story. *Trends Microbiol.* **2008**, *16*, 73–79. [CrossRef]
27. Zhu, S.-Y.; Xu, Y.; Yu, X.-W. Improved Homologous Expression of the Acidic Lipase from Aspergillus niger. *J. Microbiol. Biotechnol.* **2020**, *30*, 196–205.
28. Ma, R.J.; Wang, Y.H.; Liu, L.; Bai, L.L.; Ban, R. Production enhancement of the extracellular lipase LipA in Bacillus subtilis: Effects of expression system and Sec pathway components. *Protein Expr. Purif.* **2018**, *142*, 81–87. [CrossRef]
29. Rantasalo, A.; Vitikainen, M.; Paasikallio, T.; Jäntti, J.; Landowski, C.P.; Mojzita, D. Novel genetic tools that enable highly pure protein production in Trichoderma reesei. *Sci. Rep.* **2019**, *9*, 5032. [CrossRef]
30. Helisto, P.; Korpela, T. Effects of detergents on activity of microbial lipases as measured by the paranitrophenyl alkanoate esters method. *Enzym. Microb. Technol.* **1998**, *23*, 113–117. [CrossRef]
31. Kulkarni, N.; Gadre, R.V. Production and properties of alkaline, thermophilic lipase from Pseudomonas fluorenscens NS2W. *J. Ind. Microbiol. Biotechnol.* **2002**, *28*, 344–348. [CrossRef]
32. Verger, R. 'Interfacial activation'of lipases: Facts and artifacts. *Trends Biotechnol.* **1997**, *15*, 32–38. [CrossRef]
33. Khan, F.I.; Lan, D.; Durrani, R.; Huan, W.; Zhao, Z.; Wang, Y. The lid domain in lipases: Structural and functional determinant of enzymatic properties. *Front. Bioeng. Biotechnol.* **2017**, *5*, 16. [CrossRef] [PubMed]
34. Mehta, A.; Bodh, U.; Gupta, R. Fungal lipases: A review. *J. Biotech Res.* **2017**, *8*, 58–77.
35. Contesini, F.J.; da Silva, V.C.F.; Maciel, R.F.; de Lima, R.J.; Barros, F.F.C.; de Oliveira Carvalho, P. Response surface analysis for the production of an enantioselective lipase from *Aspergillus niger* by solid-state fermentation. *J. Microbiol.* **2009**, *47*, 563–571. [CrossRef]
36. Skjold-Jørgensen, J.; Vind, J.; Svendsen, A.; Bjerrum, M.J. Understanding the activation mechanism of Thermomyces lanuginosus lipase using rational design and tryptophan-induced fluorescence quenching. *Eur. J. Lipid Sci. Technol.* **2016**, *118*, 1644–1660. [CrossRef]
37. Li, G.; Fang, X.; Su, F.; Chen, Y.; Xu, L.; Yan, Y. Enhancing the thermostability of Rhizomucor miehei lipase with a limited screening library by rational-design point mutations and disulfide bonds. *Appl. Environ. Microbiol.* **2018**, *84*. [CrossRef]
38. Markel, U.; Zhu, L.; Frauenkron-Machedjou, V.J.; Zhao, J.; Bocola, M.; Davari, M.D.; Jaeger, K.-E.; Schwaneberg, U. Are directed evolution approaches efficient in exploring nature's potential to stabilize a lipase in organic cosolvents? *Catalysts* **2017**, *7*, 142. [CrossRef]
39. Cen, Y.; Singh, W.; Arkin, M.; Moody, T.S.; Huang, M.; Zhou, J.; Wu, Q.; Reetz, M.T. Artificial cysteine-lipases with high activity and altered catalytic mechanism created by laboratory evolution. *Nat. Commun.* **2019**, *10*, 3198. [CrossRef]
40. Sarmah, N.; Revathi, D.; Sheelu, G.; Yamuna Rani, K.; Sridhar, S.; Mehtab, V.; Sumana, C. Recent advances on sources and industrial applications of lipases. *Biotechnol. Prog.* **2018**, *34*, 5–28. [CrossRef]
41. Samoylova, Y.V.; Sorokina, K.; Piligaev, A.; Parmon, V. Application of Bacterial Thermostable Lipolytic Enzymes in the Modern Biotechnological Processes: A Review. *Catal. Ind.* **2019**, *11*, 168–178. [CrossRef]
42. Borrelli, G.M.; Trono, D. Recombinant lipases and phospholipases and their use as biocatalysts for industrial applications. *Int. J. Mol. Sci.* **2015**, *16*, 20774–20840. [CrossRef] [PubMed]

43. Ghasemian, A.; Moradpour, Z. Production of recombinant microbial thermostable lipases. In *New and Future Developments in Microbial Biotechnology and Bioengineering*; Elsevier: Amsterdam, The Netherlands, 2019; pp. 133–150.
44. Atalah, J.; Cáceres-Moreno, P.; Espina, G.; Blamey, J.M. Thermophiles and the applications of their enzymes as new biocatalysts. *Bioresour. Technol.* **2019**, *280*, 478–488. [CrossRef] [PubMed]
45. Memarpoor-Yazdi, M.; Karbalaei-Heidari, H.R.; Khajeh, K. Production of the renewable extremophile lipase: Valuable biocatalyst with potential usage in food industry. *Food Bioprod. Process.* **2017**, *102*, 153–166. [CrossRef]
46. Samoylova, Y.V.; Sorokina, K.N.; Romanenko, M.V.; Parmon, V.N. Cloning, expression and characterization of the esterase estUT1 from Ureibacillus thermosphaericus which belongs to a new lipase family XVIII. *Extremophiles* **2018**, *22*, 271–285. [CrossRef]
47. Chen, K.-C.; Zheng, M.-M.; Pan, J.; Li, C.-X.; Xu, J.-H. Protein engineering and homologous expression of *Serratia marcescens* lipase for efficient synthesis of a pharmaceutically relevant chiral epoxyester. *Appl. Biochem. Biotechnol.* **2017**, *183*, 543–554. [CrossRef]
48. Wang, X.; Yu, X.; Xu, Y. Homologous expression, purification and characterization of a novel high-alkaline and thermal stable lipase from Burkholderia cepacia ATCC 25416. *Enzym. Microb. Technol.* **2009**, *45*, 94–102. [CrossRef]
49. Yang, J.; Guo, D.; Yan, Y. Cloning, expression and characterization of a novel thermal stable and short-chain alcohol tolerant lipase from Burkholderia cepacia strain G63. *J. Mol. Catal. B Enzym.* **2007**, *45*, 91–96. [CrossRef]
50. Yang, H.; Li, J.; Du, G.; Liu, L. Microbial production and molecular engineering of industrial enzymes: Challenges and strategies. In *Biotechnology of Microbial Enzymes*; Elsevier: Amsterdam, The Netherlands, 2017; pp. 151–165.
51. Tang, W.; Lan, D.; Zhao, Z.; Li, S.; Li, X.; Wang, Y. A thermostable monoacylglycerol lipase from marine Geobacillus sp. 12AMOR1: Biochemical characterization and mutagenesis study. *Int. J. Mol. Sci.* **2019**, *20*, 780. [CrossRef]
52. Almeida, J.M.; Alnoch, R.C.; Souza, E.M.; Mitchell, D.A.; Krieger, N. Metagenomics: Is it a powerful tool to obtain lipases for application in biocatalysis? *Biochim. Biophys. Acta (BBA)-Proteins Proteom.* **2020**, *1868*, 140320. [CrossRef]
53. Cristakopoulos, P.; Tzia, C.; Kekos, D.; Macris, B.J. Production and characterization of extracellular lipase from *Calvatia gigantean*. *Appl. Microbiol. Biotechnol.* **1992**, *38*, 194–197.
54. Salleh, A.B.; Musani, R.; Basri, M.; Ampon, K.; Yunus, W.M.Z.; Razac, C.N.A. Extra- and intra-cellular lipases from a thermophilic Rhizopus oryzae and factors affecting their production. *Can. J. Microbiol.* **1993**, *39*, 978–981. [CrossRef]
55. Pokorny, D.; Friedrich, J.; Cimerman, A. Effect of nutritional factors on lipase biosynthesis by *Aspergillus Niger*. *Biotechnol. Lett.* **1994**, *16*, 363–366. [CrossRef]
56. Papaparaskevas, D.; Christakopoulos, P.; Kekos, D.; Macris, B.J. Optimizing production of extracellular lipase from *Rhodotorula glutinis*. *Biotechnol. Lett.* **1992**, *14*, 397–402. [CrossRef]
57. Shimada, Y.; Sugihara, A.; Nagao, T.; Tominaga, Y. Induction of Geotrichum candidum lipase by long-chain fatty acids. *J. Ferment. Bioeng.* **1992**, *74*, 77–80. [CrossRef]
58. Rapp, P. Production, regulation, and some properties of lipase activity from *Fusarium oxysporum* f. sp. Vasinfectum. *Enzym. Microb. Technol.* **1995**, *17*, 832–838. [CrossRef]
59. Brunel, L.; Neugnot, V.; Landucci, L.; Boze, H.; Moulin, G.; Bigey, F.; Dubreucq, E. High-level expression of Candida parapsilosislipase/acyltransferase in *Pichia pastoris*. *J. Biotechnol.* **2004**, *111*, 41–50. [CrossRef]
60. Jahic, M.; Wallberg, F.; Bollok, M.; Garcia, P.; Enfors, S.O. Temperature limited fed-batch technique for control of proteolysis in Pichia pastoris bioreactor cultures Microb. *Cell Fact.* **2003**, *2*, 6. [CrossRef]
61. Yamaguchi, S.; Takeuchi, K.; Mase, T.; Matsuura, A. Efficient Expression of Mono- and Diacylglycerol Lipase Gene from Penicillium camembertii U-150 in Aspergillus oryzae under the Control of Its Own Promoter. *Biosci. Biotechnol. Biochem.* **1997**, *61*, 800–805. [CrossRef]
62. Khuarana, J.; Pratibha, C.S.; Kaur, J. Studies on recombinant lipase production by E. coli: Effect of media and bacterial expression system optimization. *Int. J. Mol. Biol.* **2017**, *2*, 17–23.
63. Batumalaie, K.; Khalili, E.; Mahat, N.A.; Huyop, F.Z.; Wahab, R.A. A statistical approach for optimizing the protocol for overexpressing lipase KV1 in *Escherichia coli*: Purification and characterization. *Biotechnol. Biotechnol. Equip.* **2018**, *32*, 69–87. [CrossRef]

64. Nooh, H.M.; Masomian, M.; Salleh, A.B.; Mohamad, R.; Ali, M.S.M.; Rahman, R.N.Z.R.A. Production of Thermostable T1 Lipase Using Agroindustrial Waste Medium Formulation. *Catalysts* **2018**, *8*, 485. [CrossRef]
65. Robert, J.M.; Betancur, M.O.; Machado, A.C.O.; Arruda, A.; Reis, V.C.B.; Almeida, R.V.; Torres, F.A.G.; Alegre, P.F.; Valero, F.; Freire, D.M.G. Increase of Candida antarctica lipase B production under PGK promoter in Pichia pastoris: Effect of multicopies. *Braz. J. Microbiol.* **2019**, *50*, 405–413. [CrossRef] [PubMed]
66. Ellis, R.J.; Hemmingsen, S.M. Molecular chaperones: Proteins essential for the biogenesis of some macromolecular structures. *Trends Biochem. Sci.* **1989**, *14*, 339–342. [CrossRef]
67. Yoshida, K.; Konishi, K.; Magana-Mora, A.; Rougny, A.; Yasutake, Y.; Muramatsu, S.; Murata, S.; Kumagai, T.; Aburatani, S.; Sakasegawa, S.-I. Production of recombinant extracellular cholesterol esterase using consistently active promoters in Burkholderia stabilis. *Biosci. Biotechnol. Biochem.* **2019**, *83*, 1974–1984. [CrossRef]
68. Sahoo, R.K.; Kumar, M.; Sukla, L.B.; Subudhi, E. Bioprospecting hot spring metagenome: Lipase for the production of biodiesel. *Environ. Sci. Pollut. Res.* **2017**, *24*, 3802–3809. [CrossRef]
69. Kaur, R.; Kumar, R.; Verma, S.; Kumar, A.; Rajesh, C.; Sharma, P.K. Structural and functional insights about unique extremophilic bacterial lipolytic enzyme from metagenome source. *Int. J. Biol. Macromol.* **2020**, *152*, 593–604. [CrossRef]
70. Kavitha, M. Cold active lipases–An update. *Front. Life Sci.* **2016**, *9*, 226–238. [CrossRef]
71. Khan, M.T.; Kaushik, A.C.; ul ain Rana, Q.; Malik, S.I.; Khan, A.S.; Wei, D.-Q.; Sajjad, W.; Ahmad, S.; Ali, S.; Irfan, M. Characterization and synthetic biology of lipase from Bacillus amyloliquefaciens strain. *Arch. Microbiol.* **2020**, *202*, 1497–1506. [CrossRef]
72. Salwoom, L.; Salleh, A.B.; Convey, P.; Mohamad Ali, M.S. New recombinant cold-adapted and organic solvent tolerant lipase from psychrophilic Pseudomonas sp. LSK25, isolated from Signy Island Antarctica. *Int. J. Mol. Sci.* **2019**, *20*, 1264. [CrossRef]
73. Alnoch, R.C.; Stefanello, A.A.; Martini, V.P.; Richter, J.L.; Mateo, C.; de Souza, E.M.; Mitchell, D.A.; Muller-Santos, M.; Krieger, N. Co-expression, purification and characterization of the lipase and foldase of *Burkholderia contaminans* LTEB11. *Int. J. Biol. Macromol.* **2018**, *116*, 1222–1231. [CrossRef]
74. Putra, L.; Natadiputri, G.H.; Meryandini, A.; Suwanto, A. Isolation, Cloning and Co-Expression of Lipase and Foldase Genes of Burkholderia territorii GP3 from Mount Papandayan Soil. *J. Microbiol. Biotechnol.* **2019**, *29*, 944–951. [CrossRef] [PubMed]
75. Nagaroor, V.; Gummadi, S.N. Biochemical characterization of an esterase from Clostridium acetobutylicum with novel GYSMG pentapeptide motif at the catalytic domain. *J. Ind. Microbiol. Biotechnol.* **2020**, *47*, 169–181. [CrossRef] [PubMed]
76. Alfaro-Chávez, A.L.; Liu, J.-W.; Porter, J.L.; Goldman, A.; Ollis, D.L. Improving on nature's shortcomings: Evolving a lipase for increased lipolytic activity, expression and thermostability. *Protein Eng. Des. Sel.* **2019**, *32*, 13–24. [CrossRef] [PubMed]
77. Ishak, S.N.H.; Masomian, M.; Kamarudin, N.H.A.; Ali, M.S.M.; Leow, T.C.; Rahman, R.N.Z.R.A. Changes of thermostability, organic solvent, and pH stability in Geobacillus zalihae HT1 and its mutant by calcium ion. *Int. J. Mol. Sci.* **2019**, *20*, 2561. [CrossRef]
78. Li, P.-Y.; Zhang, Y.-Q.; Zhang, Y.; Jiang, W.-X.; Wang, Y.-J.; Zhang, Y.-S.; Sun, Z.-Z.; Li, C.-Y.; Zhang, Y.-Z.; Shi, M. Study on a Novel Cold-Active and Halotolerant Monoacylglycerol Lipase Widespread in Marine Bacteria Reveals a New Group of Bacterial Monoacylglycerol Lipases Containing Unusual C (A/S) HSMG Catalytic Motifs. *Front. Microbiol.* **2020**, *11*, 9. [CrossRef]
79. Shao, H.; Hu, X.; Sun, L.; Zhou, W. Gene cloning, expression in *E. coli*, and in vitro refolding of a lipase from Proteus sp. NH 2-2 and its application for biodiesel production. *Biotechnol. Lett.* **2019**, *41*, 159–169. [CrossRef]
80. Yaacob, N.; Kamarudin, N.H.A.; Leow, A.T.C.; Salleh, A.B.; Abd Rahman, R.N.Z.R.; Ali, M.S.M. Effects of Lid 1 Mutagenesis on Lid Displacement, Catalytic Performances and Thermostability of Cold-active Pseudomonas AMS8 Lipase in Toluene. *Comput. Struct. Biotechnol. J.* **2019**, *17*, 215–228. [CrossRef]
81. Gonzalez, T.; M'Barek, H.N.; Gomaa, A.E.; Hajjaj, H.; Zhen, C.; Dehua, L. Molecular Cloning and Expression of Candida antarctica lipase B in Corynebacterium genus. *Microbiol. Biotechnol. Lett.* **2019**, *47*, 546–554.
82. Robinson, P.J.; Pringle, M.A.; Woolhead, C.A.; Bulleid, N.J. Folding of a single domain protein entering the endoplasmic reticulum precedes disulfide formation. *J. Biol. Chem.* **2017**, *292*, 6978–6986. [CrossRef]
83. Lan, D.; Qu, M.; Yang, B.; Wang, Y. Enhancing production of lipase MAS1 from marine Streptomyces sp. strain in Pichia pastoris by chaperones co-expression. *Electron. J. Biotechnol.* **2016**, *22*, 62–67. [CrossRef]

84. Sha, C.; Yu, X.-W.; Lin, N.-X.; Zhang, M.; Xu, Y. Enhancement of lipase r27RCL production in Pichia pastoris by regulating gene dosage and co-expression with chaperone protein disulfide isomerase. *Enzym. Microb. Technol.* **2013**, *53*, 438–443. [CrossRef] [PubMed]
85. Samuel, P.; Vadhana, A.K.P.; Kamatchi, R.; Antony, A.; Meenakshisundaram, S. Effect of molecular chaperones on the expression of Candida antarctica lipase B in Pichia pastoris. *Microbiol. Res.* **2013**, *168*, 615–620. [CrossRef] [PubMed]
86. Jiao, L.; Zhou, Q.; Su, Z.; Xu, L.; Yan, Y. High-level extracellular production of Rhizopus oryzae lipase in Pichia pastoris via a strategy combining optimization of gene-copy number with co-expression of ERAD-related proteins. *Protein Expr. Purif.* **2018**, *147*, 1–12. [CrossRef] [PubMed]
87. Resina, D.; Cos, O.; Ferrer, P.; Valero, F. Developing high cell density fed-batch cultivation strategies for heterologous protein production in Pichia pastoris using the nitrogen source-regulated FLD1 Promoter. *Biotechnol. Bioeng.* **2005**, *91*, 760–767. [CrossRef]
88. Wongwatanapaiboon, J.; Malilas, W.; Ruangchainikom, C.; Thummadetsak, G.; Chulalaksananukul, S.; Marty, A.; Chulalaksananukul, W. Overexpression of Fusarium solani lipase in Pichia pastoris and its application in lipid degradation. *Biotechnol. Biotechnol. Equip.* **2016**, *30*, 885–893. [CrossRef]
89. Theron, C.W.; Vandermies, M.; Telek, S.; Steels, S.; Fickers, P. Comprehensive comparison of Yarrowia lipolytica and Pichia pastoris for production of Candida antarctica lipase B. *Sci. Rep.* **2020**, *10*, 1741. [CrossRef]
90. Prathumpai, W.; Flitter, S.J.; McIntyre, M.; Nielsen, J. Lipase production by recombinant strains of Aspergillus niger expressing a lipase-encoding gene from Thermomyces lanuginosus. *Appl. Microbiol. Biotechnol.* **2004**, *65*, 714–719. [CrossRef]
91. Qin, L.-N.; Cai, F.-R.; Dong, X.-R.; Huang, Z.-B.; Tao, Y.; Huang, J.-Z.; Dong, Z.-Y. Improved production of heterologous lipase in Trichoderma reesei by RNAi mediated gene silencing of an endogenic highly expressed gene. *Bioresour. Technol.* **2012**, *109*, 116–122. [CrossRef] [PubMed]
92. Wang, J.; Wu, Z.; Zhang, T.; Wang, Y.; Yang, B. High-level expression of Thermomyces dupontii thermophilic lipase in Pichia pastoris via combined strategies. *3 Biotech* **2019**, *9*, 62. [CrossRef]
93. Cregg, J.M.; Cereghino, J.L.; Shi, J.; Higgins, D.R. Recombinant protein expression in *Pichia pastoris*. *Mol. Biotechnol.* **2000**, *16*, 23–52. [CrossRef]
94. Vogl, T.; Glieder, A. Regulation of Pichia pastoris promoters and its consequences for protein production. *New Biotechnol.* **2013**, *30*, 385–404. [CrossRef] [PubMed]
95. Hartner, F.S.; Glieder, A. Regulation of methanol utilisation pathway genes in yeasts. *Microb. Cell Fact.* **2006**, *5*, 39. [CrossRef] [PubMed]
96. Xu, L.; Yu, J.; Wang, A.; Zuo, C.; Li, H.; Chen, X.; Pei, X.; Zhang, P. Efficient synthesis of vitamin A palmitate in nonaqueous medium using self-assembled lipase TLL@ apatite hybrid nanoflowers by mimetic biomineralization. *Green Chem. Lett. Rev.* **2018**, *11*, 476–483. [CrossRef]
97. Hohenblum, H.; Gasser, B.; Maurer, M.; Borth, N.; Mattanovich, D. Effects of gene dosage, promoters, and substrates on unfolded protein stress of recombinant Pichia pastoris. *Biotechnol. Bioeng.* **2004**, *85*, 367–375. [CrossRef]
98. Craig, E.A.; Gambill, B.D.; Nelson, R.J. Heat shock proteins: Molecular chaperones of protein biogenesis. *Microbiol. Mol. Biol. Rev.* **1993**, *57*, 402–414. [CrossRef]
99. Zhang, W.; Zhao, H.L.; Xue, C.; Xiong, X.H.; Yao, X.Q.; Li, X.Y.; Chen, H.P.; Liu, Z.M. Enhanced secretion of heterologous proteins in Pichia pastoris following overexpression of Saccharomyces cerevisiae chaperone proteins. *Biotechnol. Prog.* **2006**, *22*, 1090–1095. [CrossRef]
100. Park, Y.-K.; Vandermies, M.; Soudier, P.; Telek, S.; Thomas, S.; Nicaud, J.-M.; Fickers, P. Efficient expression vectors and host strain for the production of recombinant proteins by Yarrowia lipolytica in process conditions. *Microb. Cell Factories* **2019**, *18*, 167. [CrossRef]
101. Fleißner, A.; Dersch, P. Expression and export: Recombinant protein production systems for Aspergillus. *Appl. Microbiol. Biotechnol.* **2010**, *87*, 1255–1270. [CrossRef]
102. Keränen, S.; Penttilä, M. Production of recombinant proteins in the filamentous fungus Trichoderma reesei. *Curr. Opin. Biotechnol.* **1995**, *6*, 534–537. [CrossRef]
103. Schrag, J.D.; Cygler, M. Lipases and αβ hydrolase fold. In *Methods in Enzymology*; Elsevier: Amsterdam, The Netherlands, 1997; Volume 284, pp. 85–107.
104. Gupta, R.; Kumari, A.; Syal, P.; Singh, Y. Molecular and functional diversity of yeast and fungal lipases: Their role in biotechnology and cellular physiology. *Prog. Lipid Res.* **2015**, *57*, 40–54. [CrossRef]

105. Eggert, T.; van Pouderoyen, G.; Dijkstra, B.W.; Jaeger, K.-E. Lipolytic enzymes LipA and LipB from Bacillus subtilis differ in regulation of gene expression, biochemical properties, and three-dimensional structure. *FEBS Lett.* **2001**, *502*, 89–92. [CrossRef]
106. Brady, L.; Brzozowski, A.M.; Derewendat, Z.S.; Dodson, E.; Dodson, G.; Tolley, S.; Turkenburg, J.P.; Christiansent, L.; Huge-Jensent, B.; Norskovt, L.; et al. A serine protease triad forms the catalytic centre of a triacylglycerol lipase. *Nature* **1990**, *343*, 767–770. [CrossRef] [PubMed]
107. Schrag, J.D.; Li, Y.; Wu, S.; Cygler, M. Ser-His-Glu triad forms the catalytic site of the lipase from Geotrichum candidum. *Nature* **1991**, *351*, 761–764. [CrossRef] [PubMed]
108. Mala, J.G.S.; Takeuchi, S. Understanding Structural Features of Microbial Lipases—An Overview. *Anal. Chem. Insights* **2008**, *3*, ACI.S551. [CrossRef] [PubMed]
109. Tiwari, M.K.; Singh, R.; Singh, R.K.; Kim, I.-W.; Lee, J.-K. Computational approaches for rational design of proteins with novel functionalities. *Comput. Struct. Biotechnol. J.* **2012**, *2*, e201204002. [CrossRef]
110. Garmroodi, M.; Mohammadi, M.; Ramazani, A.; Ashjari, M.; Mohammadi, J.; Sabour, B.; Yousefi, M. International Journal of Biological Macromolecules Covalent Binding of Hyper-Activated Rhizomucor miehei Lipase (RML) on Hetero-Functionalized Siliceous Supports. *Int. J. Biol. Macromol.* **2016**, *86*, 208–215. [CrossRef]
111. Kim, H.S.; Le, Q.A.T.; Kim, Y.H. Development of thermostable lipase B from Candida antarctica (CalB) through in silico design employing B-factor and RosettaDesign. *Enzym. Microb. Technol.* **2010**, *47*, 1–5. [CrossRef]
112. Bornscheuer, U.T. Enzymes in lipid modification: Past achievements and current trends. *Eur. J. Lipid Sci. Technol.* **2014**, *116*, 1322–1331. [CrossRef]
113. Brundiek, H.B.; Evitt, A.S.; Kourist, R.; Bornscheuer, U.T. Creation of a lipase highly selective for trans fatty acids by protein engineering. *Angew. Chem. Int. Ed.* **2012**, *51*, 412–414. [CrossRef]
114. Warwel, S.; Borgdorf, R. Substrate Selectivity of Lipases in the Esterification of Cistrans-Isomers and Positional Isomers of Conjugated Linoleic Acid (CLA). *Biotechnol. Lett.* **2000**, *22*, 1151–1155. [CrossRef]
115. Childers, M.C.; Daggett, V. Insights from molecular dynamics simulations for computational protein design. *Mol. Syst. Des. Eng.* **2017**, *2*, 9–33. [CrossRef] [PubMed]
116. Haque, N.; Prabhu, N.P. Lid dynamics of porcine pancreatic lipase in non-aqueous solvents. *Biochim. Biophys. Acta (BBA)-Gen. Subj.* **2016**, *1860*, 2326–2334. [CrossRef] [PubMed]
117. Zhang, H.; Sang, J.; Zhang, Y.; Sun, T.; Liu, H.; Yue, R.; Zhang, J.; Wang, H.; Dai, Y.; Lu, F. Rational design of a Yarrowia lipolytica derived lipase for improved thermostability. *Int. J. Biol. Macromol.* **2019**, *137*, 1190–1198. [CrossRef] [PubMed]
118. Sayous, V.; Lubrano, P.; Li, Y.; Acevedo-Rocha, C.G. Unbiased libraries in protein directed evolution. *Biochim. Biophys. Acta (BBA)-Proteins Proteom.* **2020**, *1868*, 140321. [CrossRef]
119. Tripathi, A.; Varadarajan, R. Residue specific contributions to stability and activity inferred from saturation mutagenesis and deep sequencing. *Curr. Opin. Struct. Biol.* **2014**, *24*, 63–71. [CrossRef]
120. Guisan, J.M. Immobilization of enzymes as the 21st century begins. In *Immobilization of Enzymes and Cells*; Humana Press: Totowa, NJ, USA, 2006; pp. 1–13.
121. Hernandez, K.; Fernandez-Lafuente, R. Control of protein immobilization: Coupling immobilization and site-directed mutagenesis to improve biocatalyst or biosensor performance. *Enzym. Microb. Technol.* **2011**, *48*, 107–122. [CrossRef]
122. Sheldon, R.A.; Sorgedrager, M.; Janssen, M.H. Use of cross-linked enzyme aggregates (CLEAs) for performing biotransformations. *Chim. Oggi* **2007**, *25*, 62.
123. Saifuddin, N.; Raziah, A.; Junizah, A. Carbon nanotubes: A review on structure and their interaction with proteins. *J. Chem.* **2013**, *2013*, 676815. [CrossRef]
124. Guillén, M.; Benaiges, M.D.; Valero, F. Immobilization and stability of a Rhizopus oryzae lipase expressed in Pichia pastoris: Comparison between native and recombinant variants. *Biotechnol. Prog.* **2011**, *27*, 1232–1241. [CrossRef]
125. Song, C.; Sheng, L.; Zhang, X. Immobilization and characterization of a thermostable lipase. *Mar. Biotechnol.* **2013**, *15*, 659–667. [CrossRef]
126. Branco, R.V.; Gutarra, M.L.; Guisan, J.M.; Freire, D.M.; Almeida, R.V.; Palomo, J.M. Improving the thermostability and optimal temperature of a lipase from the hyperthermophilic archaeon *Pyrococcus furiosus* by covalent immobilization. *Biomed Res. Int.* **2015**, *2015*, 250532. [CrossRef] [PubMed]

127. Chronopoulou, L.; Kamel, G.; Sparago, C.; Bordi, F.; Lupi, S.; Diociaiuti, M.; Palocci, C. Structure–activity relationships of *Candida rugosa* lipase immobilized on polylactic acid nanoparticles. *Soft Matter* **2011**, *7*, 2653–2662. [CrossRef]
128. Bavaro, T.; Ubiali, D.; Brocca, S.; Rocchietti, S.; Nieto, I.; Pregnolato, M.; Lotti, M.; Terreni, M. Recombinant lipase from *Candida rugosa* for regioselective hydrolysis of peracetylated nucleosides. A comparison with commercial non-recombinant lipases. *Biocatal. Biotransform.* **2010**, *28*, 108–116. [CrossRef]
129. Yao, C.; Lin, W.; Yue, K.; Ling, X.; Jing, K.; Lu, Y.; Tang, S.; Fan, E. Biocatalytic synthesis of vitamin A palmitate using immobilized lipase produced by recombinant Pichia pastoris. *Eng. Life Sci.* **2017**, *17*, 768–774. [CrossRef] [PubMed]
130. Benaiges, M.D.; Alarcón, M.; Fuciños, P.; Ferrer, P.; Rua, M.; Valero, F. Recombinant *Candida rugosa* lipase 2 from *Pichia pastoris*: Immobilization and use as biocatalyst in a stereoselective reaction. *Biotechnol. Prog.* **2010**, *26*, 1252–1258. [CrossRef] [PubMed]
131. Badillo-Zeferino, G.L.; Ruiz-López, I.I.; Oliart-Ros, R.; Sánchez-Otero, M.G. Improved expression and immobilization of *Geobacillus thermoleovorans* CCR11 thermostable recombinant lipase. *Biotechnol. Appl. Biochem.* **2017**, *64*, 62–69. [CrossRef]
132. Rios, N.S.; Pinheiro, B.B.; Pinheiro, M.P.; Bezerra, R.M.; dos Santos, J.C.S.; Gonçalves, L.R.B. Biotechnological potential of lipases from Pseudomonas: Sources, properties and applications. *Process Biochem.* **2018**, *75*, 99–120. [CrossRef]
133. Zdarta, J.; Meyer, A.S.; Jesionowski, T.; Pinelo, M. A general overview of support materials for enzyme immobilization: Characteristics, properties, practical utility. *Catalysts* **2018**, *8*, 92. [CrossRef]
134. Ghiaci, M.; Aghaei, H.; Soleimanian, S.; Sedaghat, M. Enzyme immobilization: Part 1. Modified bentonite as a new and efficient support for immobilization of Candida rugosa lipase. *Appl. Clay Sci.* **2009**, *43*, 289–295. [CrossRef]
135. Manoel, E.A.; Robert, J.M.; Pinto, M.C.; Machado, A.C.; Besteti, M.D.; Coelho, M.A.Z.; Simas, A.B.; Fernandez-Lafuente, R.; Pinto, J.C.; Freire, D.M. Evaluation of the performance of differently immobilized recombinant lipase B from Candida antarctica preparations for the synthesis of pharmacological derivatives in organic media. *RSC Adv.* **2016**, *6*, 4043–4052. [CrossRef]
136. Manoel, E.A.; Ribeiro, M.F.; Dos Santos, J.C.; Coelho, M.A.Z.; Simas, A.B.; Fernandez-Lafuente, R.; Freire, D.M. Accurel MP 1000 as a support for the immobilization of lipase from Burkholderia cepacia: Application to the kinetic resolution of myo-inositol derivatives. *Process Biochem.* **2015**, *50*, 1557–1564. [CrossRef]
137. Ortiz, C.; Ferreira, M.L.; Barbosa, O.; dos Santos, J.C.; Rodrigues, R.C.; Berenguer-Murcia, Á.; Briand, L.E.; Fernandez-Lafuente, R. Novozym 435: The "perfect" lipase immobilized biocatalyst? *Catal. Sci. Technol.* **2019**, *9*, 2380–2420. [CrossRef]
138. George, R.; Sugunan, S. Kinetic and thermodynamic parameters of immobilized glucoamylase on different mesoporous silica for starch hydrolysis: A comparative study. *J. Mol. Catal. B Enzym.* **2014**, *106*, 81–89. [CrossRef]
139. Grollmisch, A.; Kragl, U.; Großeheilmann, J. Enzyme immobilization in polymerized ionic liquids-based hydrogels for active and reusable biocatalysts. *SynOpen* **2018**, *2*, 0192–0199. [CrossRef]
140. Reetz, M.T. Practical protocols for lipase immobilization via sol–gel techniques. In *Immobilization of Enzymes and Cells*; Humana Press: Totowa, NJ, USA, 2013; pp. 241–254.
141. Kovalenko, G.; Beklemishev, A.; Perminova, L.; Chuenko, T.; Mamaev, A.; Ivanov, I.; Moseenkov, S.; Kuznetsov, V. Recombinant strain producing thermostable lipase from Thermomyces lanuginosus immobilized into nanocarbon-in-silica matrices and properties of the prepared biocatalysts. *Appl. Biochem. Microbiol.* **2013**, *49*, 296–305. [CrossRef]
142. Lee, D.H.; Park, C.H.; Yeo, J.M.; Kim, S.W. Lipase immobilization on silica gel using a cross-linking method. *J. Ind. Eng. Chem.* **2006**, *12*, 777–782.
143. Wang, W.; Zhou, W.; Li, J.; Hao, D.; Su, Z.; Ma, G. Comparison of covalent and physical immobilization of lipase in gigaporous polymeric microspheres. *Bioprocess Biosyst. Eng.* **2015**, *38*, 2107–2115. [CrossRef]
144. Oláh, M.; Suba, S.; Boros, Z.; Kovács, P.; Gosselin, M.; Gaudreault, C.; Hornyánszky, G. Lipase B from Candida antarctica Immobilized on Epoxy-functionalized Hollow Silica Microspheres: Efficient Biocatalysts for Enantiomer Selective Acylation of Alcohols and Amines. *Period. Polytech. Chem. Eng.* **2018**, *62*, 519–532. [CrossRef]
145. Diaz-Vidal, T.; Armenta-Perez, V.P.; Rosales-Rivera, L.C.; Mateos-Díaz, J.C.; Rodríguez, J.A. Cross-linked enzyme aggregates of recombinant *Candida antarctica* lipase B for the efficient synthesis of olvanil, a nonpungent capsaicin analogue. *Biotechnol. Prog.* **2019**, *35*, e2807. [CrossRef]

146. Zlateski, V.; Fuhrer, R.; Koehler, F.M.; Wharry, S.; Zeltner, M.; Stark, W.J.; Moody, T.S.; Grass, R.N. Efficient magnetic recycling of covalently attached enzymes on carbon-coated metallic nanomagnets. *Bioconjugate Chem.* **2014**, *25*, 677–684. [CrossRef]
147. El-Aziz, A.M.A.; Shaker, M.A.; Shaaban, M.I. Enhanced Biocatalytic Activity of Recombinant Lipase Immobilized on Gold Nanoparticles. *Curr. Pharm. Biotechnol.* **2019**, *20*, 497–505. [CrossRef]
148. Dandavate, V.; Keharia, H.; Madamwar, D. Ethyl isovalerate synthesis using *Candida rugosa* lipase immobilized on silica nanoparticles prepared in nonionic reverse micelles. *Process Biochem.* **2009**, *44*, 349–352. [CrossRef]
149. Yilmaz, E.; Can, K.; Sezgin, M.; Yilmaz, M. Immobilization of Candida rugosa lipase on glass beads for enantioselective hydrolysis of racemic Naproxen methyl ester. *Bioresour. Technol.* **2011**, *102*, 499–506. [CrossRef] [PubMed]
150. Monteiro, R.R.C.; Lima, P.J.M.; Pinheiro, B.P.; Freire, T.M.; Dutra, L.U.; Fechine, P.B.A.; Gonçalves, L.R.B.; de Souza, M.C.M.; dos Santos, J.C.S.; Lafuente, R.F. Immobilization of Lipase A from Candida antarctica onto Chitosan-Coated Magnetic Nanoparticles. *Int. J. Mol. Sci.* **2019**, *20*, 4018. [CrossRef] [PubMed]
151. Zhang, S.; Shi, J.; Deng, Q.; Zheng, M.; Wan, C.; Zheng, C.; Li, Y.; Huang, F. Preparation of carriers based on ZnO nanoparticles decorated on graphene oxide (GO) nanosheets for efficient immobilization of lipase from Candida rugosa. *Molecules* **2017**, *22*, 1205. [CrossRef] [PubMed]
152. Cassimjee, K.E.; Kadow, M.; Wikmark, Y.; Humble, M.S.; Rothstein, M.; Rothstein, D.; Bäckvall, J.-E. A general protein purification and immobilization method on controlled porosity glass: Biocatalytic applications. *Chem. Commun.* **2014**, *50*, 9134–9137. [CrossRef] [PubMed]
153. Yadav, D.K.; Yadav, N.; Yadav, S.; Haque, S.; Tuteja, N. An insight into fusion technology aiding efficient recombinant protein production for functional proteomics. *Arch. Biochem. Biophys.* **2016**, *612*, 57–77. [CrossRef] [PubMed]
154. Mahmoodi, S.; Pourhassan-Moghaddam, M.; Wood, D.W.; Majdi, H.; Zarghami, N. Current affinity approaches for purification of recombinant proteins. *Cogent Biol.* **2019**, *5*, 1665406. [CrossRef]
155. Couto, R.S.; Sanromán, M. Effect of Two Wastes Fromgroundnut Processing on Laccase Production and Dye Decolouriza-Tion Ability. *J. Food Eng.* **2006**, *73*, 388–393. [CrossRef]
156. Carvalho, A.C.L.D.M.; Fonseca, T.D.S.; Mattos, M.C.D.; Oliveira, M.D.C.F.D.; Lemos, T.L.G.D.; Molinari, F.; Romano, D.; Serra, I. Recent advances in lipase-mediated preparation of pharmaceuticals and their intermediates. *Int. J. Mol. Sci.* **2015**, *16*, 29682–29716. [CrossRef]
157. Gandhi, N.N. Applications of lipase. *J. Am. Oil Chem. Soc.* **1997**, *74*, 621–634. [CrossRef]
158. Abdelmoez, W.; Mustafa, A. Oleochemical industry future through biotechnology. *J. Oleo Sci.* **2014**, *63*, 545–554. [CrossRef] [PubMed]
159. Peng, Q.; Wang, X.; Shang, M.; Huang, J.; Guan, G.; Li, Y.; Shi, B. Isolation of a novel alkaline-stable lipase from a metagenomic library and its specific application for milkfat flavor production. *Microb. Cell Factories* **2014**, *13*, 1–9. [CrossRef]
160. Huang, J.; Yang, Z.; Guan, F.; Zhang, S.; Cui, D.; Guan, G.; Li, Y. A novel mono-and diacylglycerol lipase highly expressed in Pichia pastoris and its application for food emulsifier preparation. *Process Biochem.* **2013**, *48*, 1899–1904. [CrossRef]
161. Neta, N.D.A.S.; dos Santos, J.C.S.; de Oliveira Sancho, S.; Rodrigues, S.; Gonçalves, L.R.B.; Rodrigues, L.R.; Teixeira, J.A. Enzymatic synthesis of sugar esters and their potential as surface-active stabilizers of coconut milk emulsions. *Food Hydrocoll.* **2012**, *27*, 324–331. [CrossRef]
162. Patel, R.N. Biocatalytic synthesis of intermediates for the synthesis of chiral drug substances. *Curr. Opin. Biotechnol.* **2001**, *12*, 587–604. [CrossRef]
163. Kim, M.G.; Lee, E.G.; Chung, B.H. Improved enantioselectivity of Candida rugosa lipase towards ketoprofen ethyl ester by a simple two-step treatment. *Process Biochem.* **2000**, *35*, 977–982. [CrossRef]
164. Sikora, A.; Siódmiak, T.; Marszałł, M.P. Kinetic resolution of profens by enantioselective esterification catalyzed by Candida antarctica and Candida rugosa lipases. *Chirality* **2014**, *26*, 663–669. [CrossRef]
165. Ceynowa, J.; Rauchfleisz, M. High enantioselective resolution of racemic 2-arylpropionic acids in an enzyme membrane reactor. *J. Mol. Catal. B Enzym.* **2003**, *23*, 43–51. [CrossRef]
166. Siódmiak, T.; Mangelings, D.; Vander Heyden, Y.; Ziegler-Borowska, M.; Marszałł, M.P. High enantioselective novozym 435-catalyzed esterification of (R, S)-flurbiprofen monitored with a chiral stationary phase. *Appl. Biochem. Biotechnol.* **2015**, *175*, 2769–2785. [CrossRef]

167. Gerard, D.; Gueroult, M.; Casas-Godoy, L.; Condoret, J.-S.; Andre, I.; Marty, A.; Duquesne, S. Efficient resolution of profen ethyl ester racemates by engineered Yarrowia lipolytica Lip2p lipase. *Tetrahedron Asymmetry* **2017**, *28*, 433–441. [CrossRef]
168. Long, Z.-D.; Xu, J.-H.; Zhao, L.-L.; Pan, J.; Yang, S.; Hua, L. Overexpression of Serratia marcescens lipase in Escherichia coli for efficient bioresolution of racemic ketoprofen. *J. Mol. Catal. B Enzym.* **2007**, *47*, 105–110. [CrossRef]
169. Lee, K.-W.; Bae, H.-A.; Lee, Y.-H. Molecular cloning and functional expression of esf gene encoding enantioselective lipase from Serratia marcescens ES-2 for kinetic resolution of optically active (S)-flurbiprofen. *J. Microbiol. Biotechnol.* **2007**, *17*, 74–80. [PubMed]
170. Xu, N.; Zhu, J.; Zhu, Q.; Xing, Y.; Cai, M.; Jiang, T.; Zhou, M.; Zhang, Y. Identification and characterization of novel promoters for recombinant protein production in yeast Pichia pastoris. *Yeast* **2018**, *35*, 379–385. [CrossRef] [PubMed]
171. Li, X.-J.; Zheng, R.-C.; Ma, H.-Y.; Zheng, Y.-G. Engineering of Thermomyces lanuginosus lipase Lip: Creation of novel biocatalyst for efficient biosynthesis of chiral intermediate of Pregabalin. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 2473–2483. [CrossRef]
172. Ding, X.; Tang, X.-L.; Zheng, R.-C.; Zheng, Y.-G. Efficient biocatalytic synthesis of chiral intermediate of Pregabalin using immobilized *Talaromyces thermophilus* lipase. *BioMed Res. Int.* **2018**, *2018*, 6192059. [CrossRef]
173. Martinez, C.A.; Hu, S.; Dumond, Y.; Tao, J.; Kelleher, P.; Tully, L. Development of a chemoenzymatic manufacturing process for pregabalin. *Org. Process Res. Dev.* **2008**, *12*, 392–398. [CrossRef]
174. Shibatani, T.; Omori, K.; Akatsuka, H.; Kawai, E.; Matsumae, H. Enzymatic resolution of diltiazem intermediate by Serratia marcescens lipase: Molecular mechanism of lipase secretion and its industrial application. *J. Mol. Catal. B Enzym.* **2000**, *10*, 141–149. [CrossRef]
175. Yin, Y.-C.; Li, H.-Q.; Wu, X.-S. Refolding with Simultaneous Purification of Recombinant Serratia marcescens Lipase by One-Step Ultrasonication Process. *Appl. Biochem. Biotechnol.* **2020**, *191*, 1670–1683. [CrossRef]
176. Dézsi, C.A.; Szentes, V. The Real Role of β-Blockers in Daily Cardiovascular Therapy. *Am. J. Cardiovasc. Drugs* **2017**, *17*, 361–373. [CrossRef]
177. Kuyper, L.M.; Khan, N.A. Atenolol vs. nonatenolol β-blockers for the treatment of hypertension: A meta-analysis. *Can. J. Cardiol.* **2014**, *30*, 47–53. [CrossRef] [PubMed]
178. Nguyen, L.A.; He, H.; Pham-Huy, C. Chiral drugs: An overview. *Int. J. Biomed Sci.* **2006**, *2*, 85–100. [PubMed]
179. Dwivedee, B.P.; Ghosh, S.; Bhaumik, J.; Banoth, L.; Banerjee, U.C. Lipase-catalyzed green synthesis of enantiopure atenolol. *RSC Adv.* **2015**, *5*, 15850–15860. [CrossRef]
180. María, P.D.; de Gonzalo, G.; Alcántara, A.R. Biocatalysis as Useful Tool in Asymmetric Synthesis: An Assessment of Recently Granted Patents. *Catalysts* **2019**, *9*, 802. [CrossRef]
181. Xia, J.; Chen, D. Method for Preparing L-Valsartan by Separating DL-Valsartan Ester with Lipase. CN Patent 105420338A, 23 March 2016.
182. Ji, Y.; Chen, B.; Quian, F.; He, Y.; Gao, X.; Hong, X. A Method for Preparing Levetiracetam. CN Patent 105063120B, 18 October 2015.
183. Zheng, G.; Chen, Q. Method for Producing Ticagrelor Chiral Drug Intermediates by Using Candida Antarctica Lipase B. CN Patent 104164469A, 26 November 2014.
184. Gaboardi, M.; Pallanza, G.; Baratella, M.; Castaldi, G.; Castaldi, M. Chemoenzymic Preparation of Sofosbuvir Involving a Lipase Catalyzed Regioselective Deacetylation. WO Patent 2017144423A1, 31 August 2017.
185. Ku, L. Process for Synthesis of Posaconazole Intermediate 2-Methylpropionic Acid 4-(2,4-Difluorophenyl)-2-(2S)-hydroxymethyl-4-pentenyl Ester. CN Patent 105753693A, 13 July 2016.
186. Wang, Q.; Wang, T.; Sun, Y.; Wang, B.; You, B.; Pu, J.; Li, X.; Jiang, Y. Preparation of Levocarnitine. CN Patent 106748843A, 31 May 2017.
187. Ueda, T.; Abe, Y. Novel Method for Producing 1-(acyloxy)alkyl Carbamate Derivatives. WO Patent 2016208709A1, 29 December 2016.