Understanding the Biocatalytic Potential of Lipase from *Rhizopus chinensis*

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**Abstract:** Lipases occupy the third position in the world market for the sale of the most industrially used enzymes, being one of the most versatile and promising classes, highlighting the genus *Rhizopus chinensis*, which is exceptionally useful as biocatalysts for short-chain fatty acid esterification reactions with ethanol, biodiesel, and n-heptane when used directly in the solvent-free system and in potential critical applications. In this context, the present work presents the first complete general review of the lipases of the genus *Rhizopus chinensis* focusing on their industrial applications, in addition to the different immobilization techniques and the main reactions as biocatalysts, since these are an excellent alternative due to their advantages in economic accessibility and respect for the environment. In addition, they have high specificity catalytic activity for esterification and transesterification reactions in the presence or absence of organic solvents; they can also have great thermophilicity and moderate pressure resistance, with their catalytic behavior modulated by changes in these conditions. This review showed that *Rhizopus chinensis* lipase has ample potential for many other biotechnological applications with the appropriate chemical modifications and immobilization strategy.

**Keywords:** lipases; *Rhizopus chinensis*; biocatalysis; industrial applications.

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1. Introduction

Biocatalysis is probably the future of fine chemical production. It will be available for most industrial and pharmaceutical processes, as it has advantages in economic accessibility and respect for the environment, making it an excellent alternative [1,2], since, with the aid of enzymes, biocatalysis provides all forms of existence, from the smallest microorganisms to man, through metabolic reactions and biotransformations [3]. Considering the natural origin, hydrolytic enzymes are a type of ubiquitous protein in the body and intrinsically biocompatible, capable of catalyzing chemical and biochemical reactions, cleavage by adding water to specific covalent bonds, such as CO, CN, OP, and CC, inside or outside cells [4,5]. They are the essential enzyme in the industrial market, responsible for more than 75% of sales [4,6,7].

For these reasons, enzymes are widely studied in order to enhance industrial processes, since they are the most significant and specific commercial natural catalysts for the type of
substrate, as they have advantages over chemicals [8–10], as a result of its wide availability, low cost, ability to participate in several biological processes, moderate reaction conditions, high specificity, low toxicity, and water solubility, besides operating under mild circumstances of pressure and pH, temperature and with high conversion rates [11–14].

In order for a successful industrial application, it is necessary to obtain enzymes with high catalytic efficiency, such as development in the production, concentration, purification, and immobilization of enzymes [15]. The most widely used methods of molecular modification are rational design, directed evolution, chemical modification, and immobilization, which comprise respectively methods of molecular biology and vitromodification [16–19]. The immobilization method is quite common for its low cost, reduced cycle, and simple production [20], becoming an essential step in the industrial planning of a biocatalyst enzyme [21], a desirable tool to improve many of the characteristic properties of enzymes, for example, stability, activity, pH, specificity and selectivity, reduction of inhibition and chemical reagents, in addition to favoring the recovery of the enzyme, the rapid completion, and repetition of enzymatic assays [22–24].

Therefore, the evolution of modern bioengineering techniques advances in computational, integration of rational enzyme design, and directed enzyme evolution instrumentation transformed the field of enzymatic engineering, allowing the investigation of almost all biodiversity in the search for enzymes with the appropriate properties [2,25–27].

Thus, enzymes play a notable role in industrial applications, especially proteases, lipases, and amylases [4,28]. Among these enzymes, lipases are one of the most versatile classes [29–31], used as biocatalysts in the scientific, biotechnological, and industrial areas, as they recognize a wide variety of substrates and can catalyze various reactions due to their characteristic mechanism of action [12], furthermore, the size of the global market for these enzymes is expected to exceed $ 797.7 million by 2025. It provides for a compound annual growth rate (CAGR) of 6.2% from 2017 to 2025 [27]. They feature a series of promising applications, including food, oils, pharmaceuticals, cosmetics, paper, leather, detergent, bioenergy, and fine chemicals [32–36]. According to Figure 1, its importance can be demonstrated by the significant increase in the number of scientific papers published with this material.

![Figure 1](https://biointerfaceresearch.com/)

**Figure 1.** Evolution of the number of papers retrieved by Scopus using as keyword “lipase.” The search on December 20, 2020, returned 81,536 documents.

Belonging to the EC 3.1.1.3 group (triacylglycerol acyl hydrolases), lipases have chemo, regional, stereoselective properties, molecular weight varying between 20 and 75 kDa, with an ideal pH performance in the 4-9 range and acting in the temperature range 25-70°C [37–39], high specificity for some substrates and excellent catalytic activity in esterification, interesterification,
hydrolysis or ester synthesis, reactions of alcoholism, acidolysis and aminolysis, among others [15,39–43].

Lipase producers, considerable interest has been given to the genus of *Rhizopus* lipases, which are especially attractive for academic and industrial applications, including lipid modification and biodiesel and chiral organic compound synthesis, especially for increased characteristics such as positional-1.3 specificity, enantioselectivity, and performance in non-aqueous media [39]. Fungal Lipase is an interesting enzyme that manifests its capacity in the application of lipolysis and the application of esterification, as it has been used in the production of various fermented foods for many years [44]. The genus *Rhizopus chinensis* stands out, which is exceptionally useful as biocatalysts for the esterification reactions of short-chain fatty acids with ethanol, biodiesel, and n-heptane when used directly in the solvent-free system and in critical potential applications [44–46] having the enzyme’s catalytic behavior easily modulated by temperature and pressure [47].

In this context, the present work is the first complete general review of lipases of the genus *Rhizopus chinensis*, focusing on their industrial applications since other reviews have been published in the last 10 years. However, they addressed the cloning and expression of *R. chinensis* in the lipase gene of *P. pastoris* and the characterization of enzymes, genetics, structure, heterologous expression, bioengineering, and other applications of *Rhizopus* lipases in general, highlighting the relevance of this genus [39,48]. Therefore, this review aims to better understand the growth potential in biocatalysis in industrial applications of lipases of the genus *Rhizopus chinensis*. A brief review of their properties will be presented, followed by how these enzymes are produced and purified. Special attention will be given to industrial applications, in addition to the different immobilization techniques mentioned. Finally, an overview of the main reactions of *Rhizopus chinensis* lipases as biocatalysts will be presented.

### 2. Properties of Lipase from *Rhizopus chinensis*

Lipases occupy the third position in the world market sales of enzymes most used industrially [4,28]. The leading producers of commercial lipases are fungi of the genera *Rhizopus, Aspergillus, Penicillium, Geotrichum*, and *Mucor* sp. [49,50].

Due to its relevance, several genus *Rhizopus* sp. have already been identified and characterized. One of them is isolated from moldy grain yeast, the fungal strain of *Rhizopus microsporus var. Chinensis*, [46,48], used as excellent sources of lipase, such as the whole-cell strain of *Rhizopus chinensis* CCTCC (China Center for Type Culture Collection) M201021, [51], which was cloned and expressed at a high level in *Pichia pastoris* as r27RCL, whose activity was about 580 times greater than that of primitive *R. chinensis* lipase [48]. They can produce several isolipases that can be found in different ways like pré-proRCL (42 kDa), proRCL (39 kDa), r27RCL (33 kDa), and forms of truncated "mature" mRCL (30 kDa) [52].

*Rhizopus chinensis* lipases (RCL) are versatile biocatalysts for various bioconversions [53]. They are preferably used in various applications in industries [39], food [54], with a high potential for use as a bakery additive [55], as well as for the production of biodiesel [46,56], chiral compounds [39], synthesis of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) [57], sorbitan oleate [58] and efficiency in catalyzing reactions of short-chain fatty acid ethyl esters with ethanol and biodiesel [44,46].

Investigation of the gene sequence encoding the RCL includes complete open identification without introns, which encodes 389 protein amino acids spanning a 26 amino acid signal sequence, 94 amino acid pro-sequence, and 269 amino acid mature lipase sequence [59].
They have significant hydrolytic properties that underlie their biological attributions [52], exhibit high stability in acidic conditions [60], can operate over a wide range of temperatures and pH values [61], presenting optimum pH and temperature at 8.5 and 40°C, respectively [48] besides other coveted characteristics for industrial applications such as high sn-1, 3 positional specificity, enantioselectivity, and non-aqueous media [39,62]. However, they have some limitations, and one of the items usually emphasized is their thermostability [16,53], which is affected significantly by a salt bridge, hydrogen bond, hydrophobic stacking, and disulfide bond [63].

Thus, one industrial interest is the search for thermostability to save excessive-high costs during the processes [54], since most *Rhizopus* lipases are mesophilic and express low thermostability [64].

Several scientific studies report attempts to improve the RCL thermostability. Initially, directed evolution was applied to design r27RCL, and a variant called S4-3 containing five mutations with a half-life at 60°C and 65°C were 46 and 23 times greater [53].

Posteriorly, Wang *et al.* (2020a), by rational design (FoldX) and molecular dynamics simulations, selected and combined four thermostable variants to generate an m31 variant, which exhibited thermostability with a half-life 41.7 times more at 60°C and an increase of 15.8°C where the enzyme remained at 50% of its activity after 30 min of heat treatment, compared to r27RCL expressed in *Pichia pastoris*, in order to expand its industrial applications [54]. Then, Wang *et al.* (2020b), through a buried disulfide bond and combinatorial mutagenesis, obtained another stable m32 term alkaline mutant, with a 74.7-fold increase in half-life at 60°C, stable at pH 9.0–10.0 [63].

The improvement in RCL activity can be attributed to the conformational modification of enzymes [65]. Thus, the enzymatic action is a delicate indicator of changes treated under high hydrostatic pressures [66].

Thus, Chen *et al.* (2017), through the molecular dynamic simulation, studied the movement of the LCR cap induced by high pressure and showed that it was partially opened (<200 MPa), moving away from the catalytic center, but became more closed in values (> 400 MPa). The interfacial activation changed little in the pressure below 400 MPa, but became marginal with the increase to 500 MPa. The lipase hydrolysis capacity by high-pressure treatment went through an initial increase and subsequent reduction, with maximum activity obtained at 200 MPa and 40°C [65].

Chen *et al.* (2018) investigated changes in the structure of the RCL lipase. With the results, they observed that there was an increase in catalytic capacity and stability after treatment with high hydrostatic pressure (HHP), in a moderate pressure range of 0.1– 400 Mpa, the latter being the pressure at which the unfolding started; however, after the application of higher pressures, the lipase became gradually deactivated, its activity dropped to 89% at 500 MPa and 600 MPa, the residual activity was 67% of the original value, indicating denaturation of the enzyme to a great extent [66].

Wang *et al.* (2021) used the RCL to explore the mechanism of interfacial activation, in addition to the critical micellar concentration (CMC), and found that the rotational key of Phe113 was the binding site for the activation of RCL by its dynamic inversion, in the transitions of the lid open and closed. The F113W mutant increased the lipase's catalytic efficiency (1.9 s⁻¹.μM⁻¹) to 280% at the ideal temperature of 40°C and pH 8.5 [67].
Therefore, knowing the properties of this enzyme is essential for the preparation of enzymatic biocatalysts, which makes lipase *R. chinensis* (Figure 2) an exciting candidate for future industrial applications.

![Figure 2](https://biointerfaceresearch.com/)

**Figure 2.** Representation of the 3D crystalline structure of the Lipase from *Rhizopus chinensis* (PDB code: 6a0w): (A) Closed conformation residues of the GLY109 - THR123 lid: (B) Residues of the catalytic triad SER172 (green), ASP231 (blue), HIS 284 (pink). The 3D structure was selected from the Protein Data Bank (PDB) using the pymol educational version.

### 3. Production of Lipases from *Rhizopus chinensis*

*Rhizopus* sp. strains, under different growing conditions, produce different isolipases, such as *R. chinensis* isolated from Daqu, a solid mixture of several microorganisms used to ferment traditional Chinese liquor [57]. It produces three forms of lipases (36 kDa, 62 kDa, and 33 kDa) through solid-state fermentation (SSF) and produces an identical 33 kDa lipase and two other forms (39 kDa and 59 kDa) by submerged fermentation (SMF) [39]. The cultivation of *R. chinensis* in SSF can allow it to return to its natural growth environment and recover its natural characteristic. Therefore, it appears as the most used strain for lipases production [60,68].

When produced in *Pichia pastoris*, the proRCL protein is cleaved by an endogenous Kex2-like protease in a Lys-Arg-Asp sequence, between the residual positions −29 and −28 pro sequence in a 27 amino acid r27RCL enzyme, the dominant form of the protein when expressed in *P. pastoris* [52,53,69,70].

*Pichia pastoris* is a methylotrophic yeast characterized as a system determined to produce heterologous proteins, especially biopharmaceuticals and industrial enzymes [71–73]. It has evolved significantly in recent years and has advantages in protein production, such as efficient high-density secretion in cell cultures, low concentrations of intrinsic proteins secreted from the host, and less extensive glycosylation than other yeasts [74,75].

### 4. Purification of Lipases from *Rhizopus chinensis*

The purpose of the enzymatic purification process is to improve their stability to play the role of isolating enzymes from contaminants, improving their catalytic activity, improving their stability, and extending their shelf life. After the purification step allows the enzyme to reach a high level of homogeneity, conformational and structural studies can be carried out. It also allows to successfully determine the primary amino acid sequence [76–78]. Knowing the three-dimensional structure of lipases is fundamental in the engineering and design development of lipases for specific purposes [79].

Through radiographic studies of the purified lipases, it is possible to establish the structure-function relationships and contribute to a better understanding of the thermodynamic
and kinetic mechanisms of the lipases for the synthesis reactions and group exchange of esters and hydrolysis of the substrate [79,80]. The use of lipases in commercial applications does not require a high level of homogeneity; only with a certain degree of purity is successful and efficient application possible [79]. The lipase from *Rhizopus chinensis* (RCL) has low hydrolytic activity in the aqueous phase and intense activity in the synthesis of ester in a non-aqueous and solvent-free medium when produced from the strain *Rhizopus chinensis* associated with a membrane [44,46,81]. The production of this lipase through this process is always low, and the purification of this enzyme is complex [45,82]. However, for it to be applied industrially, it is necessary to study its catalytic properties, and for that, it is necessary to carry out the purification stage [68,83,84].

In studies reported by Xiao-lan et al. (2005), the fibrinolytic lipase of *Rhizopus chinensis* 12 was purified by hydrophobic interaction, precipitation, and ammonium sulfate ion exchange, and gel filtration chromatography. The final yield after the purification protocol was 42.6%, and the enzyme was purified 893 times. The lipase had a molecular weight of 16.6 kDa, which was obtained by gel filtration chromatography, in which a monomeric form of the enzyme was revealed, and 18.0 kDa, which was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [85]. The isoelectric point was established by isoelectric focusing electrophoresis and found a value of 8.5. The activity of the final lipase preparation was estimated at 2,143.4 U/mg of protein [85].

In another study, the author's Sun et al. (2009) purified an intracellular lipase obtained from *Rizopus chinensis* grown in a solid-state. The lipase was purified to its homogeneity utilizing four purification steps: ammonium sulfate precipitation, hydrophobic interaction chromatography, anion exchange chromatography, and filtration gel. The lipase had a molecular weight of 36 kDa and was active in the pH range between 7.0 and 9.0 and the temperature range from 20 to 45 ºC. After purification, the enzyme showed essential properties such as stability and interfacial activation when exposed to the presence of some organic solvents and the ability to perform hydrolysis reactions on a wide variety of substrates, implying application in a wide range of industrial applications. In addition to the characteristics presented above, the enzyme had one particular ability to hydrolyze triolein, not in a specific way, but made it potentially applicable in the fish oil hydrolysis reaction [57].

*Rhizopus chinensis*, when grown under solid-state fermentation, produces two lipases that perform the synthesis of ester synthesis. A study developed by the author's Sun et al. (2009) reported the purification process called Lip2. The lipase was purified to its homogeneity by precipitation with ammonium sulfate, hydrophobic interaction chromatography, and gel filtration chromatography. It had a molecular weight of 32 kDa obtained from analytical gel permeation and 33 kDa estimated from SDS-PAGE, with a fold and synthetic activity of 138.3 and 96.8 U/mg, respectively. Using p-NPP as substrate, the maximum hydrolytic activity was obtained in the pH 8.0 - 8.5 range at 40 ºC. The purified enzyme showed maximum synthetic activity at pH 6.0 under a temperature of 30 ºC. The lipases showed an affinity for the solvents n-hexanol, n-propanol, and ethanol, with a conversion of 92%, 93%, and 92%, respectively, after being incubated with 20h [86].

Lipase from *Rhizopus chinensis* has excellent potential for application in reactions of hydrolysis and synthesis of esters, as it was possible to observe in the studies presented in the literature after the purification process. Although the RCL purification process is complicated, in which it always requires at least four steps, the results are satisfactory, and in all of them, it is possible to achieve its homogeneity, which allows more specific studies to be carried out.
5. Applications of the Free Lipases from *Rhizopus chinensis*

According to scientific literature, *Rhizopus chinensis* lipase has excellent catalytic activity in esterification and transesterification reactions [87], which is considered an enzyme with relevant industrial potential.

In this sense, Sun *et al.* (2009) analyzed the esterification potential of *Rhizopus chinensis* lipase (RCL) to synthesize flavor esters. In the experiments, the synthesis of ethyl esters catalyzed by RCL was carried out in n-heptane at 40°C; and it was tested considering several fatty acids to determine its specificity. The fatty acids used were: hexanoic acid, laurate acid, palmitic acid, butyric acid, capric acid, tetradecanoic acid, and caprylic acid. For ten hours, the reactions proceeded steadily and reached equilibrium after 30 h. Of all the fatty acids tested, the lowest conversion was obtained using butyric acid as a substrate (about 39.2%) and the highest using caprylic acid, with about 92%. Besides, caprylic acid was considered the most efficient acyl group donor among the tested saturated fatty acids. Therefore, the researchers concluded that the enzyme has a greater affinity for long-chain fatty acids [86].

In addition to the direct application of RCL, researchers have turned their efforts towards the production, characterization, optimization of this lipase and, later, application. This is justified by how the fermentation process with which the lipase will be produced changes the characteristics of the enzyme [39,45,54,57,86–88] purified and characterized the lipase of *Rhizopus chinensis* under solid-state fermentation and studied its potential to synthesize eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from fish oil. After the purification step, the lipases showed considerable stability and activity in the pH range between 7.0 and 9.0 and temperature between 20 and 45 °C after the purification step. Also, it was possible to notice an increase in enzymatic activity in the presence of Ca$^{2+}$ and Mg$^{2+}$. However, such activity was strongly inhibited by Hg$^{2+}$ and sodium dodecyl sulfate (SDS). According to the experimental data, the purified lipase showed more significant activity in medium-chain esters, emphasizing pNP-caprylate (C8). However, the lipase showed a non-specific behavior, considering that the macromolecule could cleave the 1,3-positioned and 2-positioned ester bonds in triolein. After characterization, high conversions of EPA (17.6%) and DHA (32.9%) were achieved using purified lipase for 10 h. Therefore, the purified lipase's activity, stability, and non-specificity suggest that the enzyme can be applied in various industrial applications, more specifically in producing EPA and DHA from fish oil with reasonable conversion rates [57].

Thus, the lipase from *Rhizopus chinensis*, for delivering high yields in both esterification and transesterification reactions, shows itself as a catalytic alternative to commercial demands, considering that it presents high activity solvent-free environments and non-aqueous media. Besides, based on scientific literature, the use of RCL in solvent-free media is noteworthy as it is configured in low-cost processes, easily accessible raw material, environmentally friendly, low need for large amounts of substrates, and need for mild conditions. Therefore, *Rhizopus chinensis* lipase appears as an enzyme with relevant potential for the oil-chemical and pharmaceutical industries [46,57,89].

6. Immobilization of Lipases from *Rhizopus chinensis*

6.1. Enzyme immobilization.

Enzyme immobilization involves isolating the enzyme on rigid support insoluble in organic solvents and aqueous media, is used alone or combined with other protein stabilization
methods [90,91]. Therefore, it is classified as the most efficient instrument to modify enzymes' selectivity, specificity, stability, and activity [92,93]. The methods of fixing enzymes on the carriers can be performed in different ways, such as encapsulation, confinement, physical adsorption or ion exchange resins, covalent bonding, and crosslinking, as illustrated in Figure 3 [94–96].

![Enzyme immobilization methods](https://doi.org/10.33263/BRIAC123.42304260)

**Figure 3.** Enzyme immobilization methods.

As highlighted in Figure 3, the enzymatic immobilization methods can be specific for each enzyme according to its applications and physical-chemical characteristics. Besides, each method has advantages and restrictions during the reaction [97,98]. Due to this, the best option of the immobilization procedure is based on parameters such as immobilization costs, operational stability, catalytic activity, recovery capacity, and toxicity of the reagents, among others [99–101]. Therefore, the immobilization protocols are different according to the type of support and efficiency. Thus, enzymatic immobilization is considered an innovative technique applied on a large industrial scale, being a breakthrough in the field of protein stabilization, evidenced by molecular biology and computer engineering [97, 102].

### 6.2. Adsorption.

The physical adsorption happens in a simple way in which the immobilized enzyme in the support is obtained through ionic bonds, hydrogen bonding, hydrophobic interactions, and Van der Waals forces [103–105]. The main media used in physical adsorption are inorganic, synthetic polymers, and natural polymers since these materials are exposed to surface treatments to boost retention capacity during the reaction [106,107]. So, the reactions can follow through the organic environment, in which the interactions are weak between support and the enzyme. Thus, the enzyme becomes insoluble in the nonpolar medium under appropriate conditions, making this technique usefully advantageous [108–110]. The use of immobilization by adsorption brings advantages in the low cost of not activating the support reusing and reusing the support in several cycles. The ease of application promotes low alteration in the enzyme's structural conformation in the reaction medium [23,111]. The disadvantages may occur due to the diversification of temperature, ionic strength, enzymatic leaching, and pH during the reaction [112]. However, methods have been developed and applied to reduce desorption, either by
chemical modification of the support or by the reticulation process inside the pores, resulting in improved enzymatic activity [113,114].

Considering the whole process of adsorption for its speed and simplicity, the efficiency of the reaction depends exclusively on optimal conditions of immobilization such as pH, nature of the solvent, ionic strength, and the relationship of concentration between enzyme and support and adsorbent [95,115]. Among the optimization parameters, lipases have adequate requirements for the immobilization mechanism in hydrophobic supports due to their enzymatic structure [21,116,117].

However, in the technique of immobilization by ionic force, there are ionic interactions between the enzyme and the matrix, in which the functional groups of the support will interact with the groups of the enzyme [118]. Therefore, the ionic interaction is more robust once the adsorption process occurs, which prevails in the reactional environment. It is necessary for an ionic solution with the appropriate buffering properties, with which enzyme will bind in a compatible way to the support, in which the greater the density of the matrix surface charge, the greater the interaction of the number of enzymes bound to this matrix [119,120]. Ionic interactions depend exclusively on pH, temperature, and enzyme concentration in the matrixes of synthetic polymers, inorganic materials, and polysaccharide derivatives [107,121,122]. Thus, this technique applied to immobilization by ionic interaction provides little change in the conformation of the enzyme, for example, the composition of biosensors in which the polyaniline becomes a polymer conducted positively [123-125].

6.3. Covalent attachment.

The process of enzymatic immobilization by covalent bonding happens through the generation of covalent bonds between the insoluble support and an enzyme binder group, immobilizing an enzyme through any of its reactive groups [90, 126]. This technique is applied in several types of bonding because of its more determinant in the thermal stabilization and operating mode of enzymes [96, 127–130]. The rules of covalent immobilization are essential to modify the support's surface through the activation reactions, by which the functional groups of the support are modified, producing reactive intermediaries [95]. Several methods are used to support function, such as glass bead signaling and, soon after, with glutaraldehyde [131–133]. The glutaraldehyde reagent is used in several reactions to support obtaining a stable and active enzymatic solution. Thus, by reacting glutaraldehyde with the enzyme and supporting covalent bonding, the amine groups bind to the aldehyde groups of the support, producing the Schiff bases [134,135].

It should be noted that the matrices used in this technique are natural or synthetic. Moreover, the matrix choice depends on the cost, the bonding capacity, the rigidity of the structure, and availability [118]. When the bonding power is high, the enzyme residues slow down, leaving the structure rigid, i.e., unchanged to several denaturing factors such as organic solvents, high pH, heat, and others [127,136].

The covalent immobilization process has several advantages in more incredible determination in physical and chemical arrangements. However, from time to time, changes can occur in structural properties and the catalytic activity's performance with the interaction between the enzyme and support [115,137]. However, the disadvantages include strict conditions in several reaction steps requiring more time. Since the entire immobilization process produces enzyme stability, only drastic changes can influence the covalent bonds [138,139].
The variety of supports provides the functional groups to perform covalent connections in several applications. This interaction process depends on the density of the reactive functional groups, the specificity of the active groups of the support, and the protonation state of the support and enzyme [140–142]. Other functional groups can also participate in the immobilization process, such as phenolic tyrosine groups, carboxylate groups of glutamate, and aspartate molecules [90,143,144].

6.4. Encapsulation.

The enzyme immobilization technique by encapsulation comprises the polymerization of the porous matrix produced by polymers crossed around the biocatalysts. In this case, the enzyme is absorbed in a reactive way to be polymerized, isolating its structure during the reaction process, thus reducing inactivation [118,145]. The central cross polymers are agarose, polyurethane resins, starch, and polyacrylamide [146–148].

This method allows the diffusion of the mixture of the substrates and products, blocking the enzyme path. However, if there is no link between substrate and enzyme, there is no damage to the catalytic activity due to inactivation [20,137,149]. Moreover, the immobilization by encapsulation presents advantageous characteristics because it protects the protein from the reactive environment in which inactivation can occur. This technique can be used in several enzymes concerning the degree of purification without favoring structural changes [20,91]. However, the disadvantages are associated with the high amount of enzymes ensuring encapsulation, control of the pore size of the substrate, the effects of diffusion of substrates within the pore matrix, and the process of desorption due to different pore sizes [114,150].

The microencapsulation method is determined by the categorization of enzymes around a microporous membrane. This immobilization type provides the inclusion of semipermeable, globular polymeric membranes with porous control [91,97,151]. Since these enzymes have extensive surface areas due to the catalytic activity, they also have cellulose nitrate, liquid surfactant, or polystyrene composition [152,153].

While the technique by semi-permeable fibrous membranes constitutes the enzyme's isolation through a semi-permeable membrane at a certain point of the solution [154,155], the protein process denaturation will not occur because no chemical components are used. Thus, the reaction factors are determined by the concentration of the reagents, composition of the mixture, the time spent, and the mode of agitation of the reaction, characterized by the membrane and the size of the microcapsules [156–158].

6.5. Crosslinked enzyme aggregates.

Immobilization by cross-linking is independent of support because biocatalysts are interconnected, building a complex three-dimensional structure. The cross-linking immobilization reaction can happen by physical or chemical means [90,159]. As the reaction occurs by chemical method, the covalent bonds between enzymes and matrices are formed employing the bi or multifunctional low molar mass reagents [160-162].

Highlighting the reagents in the cross-linking immobilization favors distinct means for the reaction to occur successfully, depending on the experimental conditions and proper selection of the enzyme and the reagent [91,163]. The most used reagents in cross-linking are glutaraldehyde due to its fast action with amine and carbodiimide groups because of its easy reaction with carboxylic groups of the enzyme [164,165]. Thus, the choice of the type of
bifunctional reagent and bond stability presupposes whether the reaction will be reversible or not during immobilization [166]. Moreover, the enzyme does not have an association with solid support, so it becomes insoluble due to the interconnection of the products of various enzymes producing catalytically active agglomerate [167,168].

The main advantage of this technique is the simplicity with which the reaction occurs. However, the loss of enzyme quantity may occur due to cross-link formation control [169]. Moreover, they can suffer conformational changes causing loss of catalytic activity, low stability, reproducibility, and limited diffusion, making the whole process of industrial applications demanding [170]. To contain these disadvantages, other alternatives were developed by creating cross-linking techniques using crystalline enzymes known as cross-linked crystals (CLEC) and physical aggregates called cross-linked enzyme aggregates (CLEAs). The use of CLEAs for their simplicity, stability, high catalytic activity, and enzymatic applicability [171,172].

The synthesis of CLEAs covers the precipitation of chemically cross-linked protein by using a bifunctional reagent transforming into insoluble enzymatic aggregates [173,174]. The enzymatic aggregates are formed by adding salts, organic solvents, acids, and polymers of a non-ionic nature, without interfering in the three-dimensional structural formation [175,176]. The cross-linking enzyme yield and stability of CLEAs come from the concentration of lysine and amino residues on the enzymatic surface. However, if the amino reactive amino group concentration, enzymatic desorption may occur after several cycles [97,177].

The process of coprecipitation of enzymes with ionic polymers brings advantages before the immobilization procedure by reticulation, such as polyethyleneimine, used for CLEAs formation because its structure has a high-density amino-terminal group favoring the cross-linking [178,179]. They are used to prepare various enzymes L-aminoacillase, β-galactosidase, and lipases, favoring stable biocatalysts or combi-CLEAs with high catalytic activity [180–183].

6.6. Other techniques.

The enzymatic immobilization processes have been intensified in recent years and associated with various technologies [95,97]. The biocromatographic technique consists of a high-efficiency liquid chromatography technique (HPLC), bringing stationary phase bioreactors for enzyme immobilization [98,184,185]. Using chromatographic media presents an excellent advantage in the immobilization process because it provides speed, stability concerning temperature, reuse, selectivity, high activity, reproducibility, specificity, and enzymatic sensitivity [21,186]. As the reaction occurs, the detection process's sensitivity is increased by IMER (Immobilized enzyme reactors) to immobilize or identify several active compounds [187–189].

Chitosan as support for enzymatic immobilization consists of a low-cost, renewable, biodegradable product and is found abundantly in nature in crustacean shells [190,191]. To increase the chitosan support's physicochemical stability, several possibilities are determined through chemical modification, that is, by reticulation in different activation agents [192,193]. Among the agents most used in chitosan immobilization processes is glutaraldehyde as a function of the amine groups that react in optimal conditions [194,195].

In its use in support of affinity, it is worth mentioning that chitosan aims to purify enzymes for large-scale industrial production [196–198]. Different strategies are employed to immobilize several classes of enzymes, such as hydrolases, immobilized through covalent bonding, adsorption, and encapsulation [90]. The different strategies used in purification and immobilization by the affinity of binders introduced in the chitosan and applied in different
media such as biotransformation, the transformation of oils and fats, extraction of polluters in wastewater, energy generation, among others [199–201].

6.7. Immobilization of lipases from Rhizopus chinensis.

The most varied methods have been used to improve the catalytic activity of lipases through the process of optimization through fermentation, the advancement of genetic engineering to improve several specific points of the enzyme, and enzymatic immobilization [54,202–204]. Lipases have a high cost for production, especially when used freely. To solve the problem, the most applied strategy is the immobilization of enzymes in various types of media considerably effective and economical [88,175,205].

The process of immobilization of whole cells in matrices reduces costs concerning the generation of biodiesel, providing the reuse of biocatalysts such as lipase Rhizopus chinensis used in several cycles [206–209]. It is worth mentioning that several porous materials (polyacrylamide gel and polyurethane foam) are very used to immobilize filamentous fungi, resulting in lipase generators for continuous application [39,210–212]. However, these polymeric media are not considered biodegradable after use, producing waste to the environment and increasing production costs [213,214].

Lipase Rhizopus chinensis is used as a biocatalyst due to its easy recovery and reuse [215] in several immobilization methods. It can be used to support macroporous and anionic resins, being an anionic exchange resin considered one of the best supports for industrial applications [216–218]. Therefore, the catalytic efficiency for immobilized enzymes is much higher than for whole cells, but whole cells have an advantage over immobilized lipase. They do not go through the purification process [56,219,220]. Besides, Rhizopus lipase can also be immobilized in magnetic chitosan microspheres for biodiesel production [39,221].

The immobilization technique by imprisonment with photo-crosslinking resins or porous silica polymers improves the enzyme's stability [96]. Although the immobilization method can also occur in inorganic material in compact bed reactors, most Rhizopus lipase biocatalysts require care in the purification and recovery process presenting disadvantages for industrial applications [45,222–224]. It is worth mentioning that the immobilization method from filamentous fungi-free culture, the lipase Rhizopus chinensis, using equal particles is favorable for industrial production. Moreover, the use of lipase Rhizopus chinensis through the support of porous biomass particles is used in several applications, mainly in microbial culture systems [89,225,226].

By highlighting the species Rhizopus and Aspergillus are filamentous fungi often used as biocatalysts in immobilization processes [227]. The application process of immobilized extracellular lipases proposes a specific strategy for enzyme purification [45]. The microbial cell system generates intracellular lipases used as whole-cell biocatalysts to have a satisfactory yield in triglyceride conversion at a lower cost [226].

The immobilization of Rhizopus chinensis lipase in dry mycelium through the fermentation process can synthesize short-chain ethyl esters in whole-cell lipases [82,87,228]. The lipase Rhizopus chinensis cultivated in lots and immobilized in biomass support particles of polyurethane foam composition have the intention to isolate the catalysts of the reaction and provide the reuse of the enzyme in several bioconversion processes. Thus, this enzyme's immobilization increases intracellular catalytic activity four to seven times compared to suspension cells [89,227,229].
The immobilization of the lipase *Rhizopus chinensis* on matrices with hydrophobic surfaces favors the activity and stability of the enzyme. Moreover, the immobilization of lipase *Rhizopus chinensis* by cross-linking - CLEAS in altered monocellular octylic foams using oxidized gum arabic provides thermal and mechanical balance, thus maintaining catalytic activity after several cycles of reaction [230,231].

7. Application of Lipases from *Rhizopus chinensis* as Biocatalysts

7.1. Hydrolysis reactions.

The hydrolysis reaction usually consists of a chemical reaction in which a water molecule breaks chemical bonds, generating several products, but mainly carboxylic acids and alcohols [232–236]. It is noteworthy that acids catalyze this reactional process, such as bases, metals, and enzymes [232,235,237]. In this perspective, enzymatic hydrolysis is the catalytic breakdown or decomposition of a chemical compound in the presence of enzymes after reacting with water [21,237–243]. Figure 4 presents the reactional equation of the hydrolysis process of an ester and esterification of carboxylic acid:

![Reactional scheme: 1) Hydrolysis of the ester; 2) Esterification of carboxylic acid.](https://biointerfaceresearch.com/)

![Figure 4. Reaction al- reactional scheme: 1) Hydrolysis of the ester; 2) Esterification of carboxylic acid.](https://biointerfaceresearch.com/)

The enzymatic hydrolysis process is widely applied in several fields, mainly in the food and renewable energy industry [244–247]. It is worth mentioning that the agricultural and food industry generates tons of waste and by-products that can be reused to produce various products, such as cellulosic ethanol, daily [247–252]. This promising biofuel is produced by hydrolysis of polysaccharides and can use sugarcane bagasse as an abundant source of polysaccharides [249,253–255]. It is noteworthy that bioethanol production is necessary to develop microorganisms capable of hydrolyzing cellulose, fermenting sugar, tolerating high concentrations of bioethanol, and producing bioethanol exclusively [256,257]. It is known that enzymes have unique characteristics, and in most processes, these esters, chemo, and regioselective enzymes [256,258,259].

Lipases are the most used enzymes in enzymatic catalysis of processes such as hydrolysis of long-chain triglycerides, esterification of compounds, and widely used enzymes for industrial applications [39,54]. Lipases are common enzymes, applying in several fields, studying for medical applications, biological treatment processes, and pharmaceutical synthesis [260]. It should be emphasized that lipases have a characteristic catalysis mechanism known as interfacial activation [21,228,244,245]. This characteristic allows these enzymes to act in the interfaces between the hydrophobic and aqueous environments that involve a polypeptide chain [253,261,262]. One strategy for the continuous and recyclable use of these enzymes is immobilization and stabilization in supports that allow easy separation, reuse, and preservation/improvement of enzymatic immobilization parameters [21,205,263].
Lipase *Rhizopus chinensis* (RCL) is a 33 kDa monomeric protein with extensive applications of industrial enzymes. This enzyme has stood out for its efficiency in esterification reactions of short-chain fatty acids with ethanol and biodiesel [215,264]. The thermostability of this enzyme is reduced. This prevents its application in several protocols [54,63]. However, some studies point out several strategies to enable its use in extreme conditions [63,65,215].

The main objective of using enzymes in reactional processes is to balance molecular stability, structural flexibility, and catalytic rate. It should be emphasized that the most flexible region of the lipase structure is "LID" because, in this part, interfacial activation occurs, which causes interaction with the proposed substrate. Yu *et al.* (2012) presented one of the first studies that analyzed the relationship between LID stiffness and catalyst activity of lipase *Rhizopus chinensis*. In this perspective, to improve the enzyme’s thermostability, a disulfide bond was introduced in the region of the lid hinge (RCLCYS). Besides, the objective was to improve catalytic activity in extreme environments. Therefore, pure RCL that has not undergone alteration and RCLCYS that has the addition of a disulfide bond were analyzed. According to the authors’ algorithm's prediction, disulfide bonding was introduced to lipase, entitled "Disulfide by Design." It is emphasized that pure RCL lipase showed significant loss of enzymatic activity at 60ºC, at almost 50% in 4 min, and after 10 min, enzymatic activity dropped to about 95% of the original [265].

In contrast, RCLCYS showed substantially improved enzymatic stability due to the new disulfide bond's formation, increasing half-life to 46 min at 60ºC. The disulfide variant demonstrated an improvement in the enzyme’s substantial thermostability, describing an increase of eleven times in the value of t1/2 to 60ºC and an increase of 7ºC of Tm, the parental enzyme probably contributed by stabilization of the geometric structure of the cap region. It is noteworthy that the rational design of experiments and enzyme engineering led the entire study and led to the development of a new strategy to improve the thermal stability of said lipase [265].

Many researchers have been developing studies related to RCL to improve its thermal and operational stability, envisioning possible industrial applications in various reactional [60,65,88]. We highlight the use of computational tools to perform dynamic simulations of the enzyme comportment, verify the regions with the highest activity, besides seeking to understand the impact of enzyme confirmation on its catalytic activity [54,59,60,88]. Chen *et al.* (2017) investigated changes in eyelid movement and catalysis behavior of *Rhizopus chinensis* lipase under treatment with high hydrostatic pressure (HHP) through a simulation of the molecular dynamics (SMD). It is noteworthy that the enzyme cap under pressure was partially opened below 200 MPa (MegaPascal) but was more closed at more than 400MPa. It is notelike that the effect of pressure on the reaction process catalyzed by RCL was investigated, with 4-nitrophenol palmitate being a substrate. The kinetic parameters of the reaction evolved significantly from the treatment with HHP. Thus, it was found by simulation of molecular dynamics and experimental methods that the movement of the lipase cap is causally related to the kinetic comportment of the RCL in the face of different reaction environments. Finally, it is noteworthy that the hydrolysis experiments with *p*-NPB found that the enzyme presents interfacial activation when exposed to moderate treatment with HHP, improving its catalytic parameters and operational stability [65].

Therefore, hydrolysis reactions using *Rhizopus chinensis* lipase are being widely studied to extract as many strategies and tools as possible to improve the is enzyme’s thermostability, either via immobilization or enzymatic stabilization design of experiments, dynamic simulation of bio comportment, among others. However, enzymatic engineering nuclei seek to combine all
these techniques to develop the best protocol for enzyme thermostabilization with a vast potential for industrial applications.

7.2. Esterification reaction.

The esterification of compounds commonly occurs when alcohol is treated with a carboxylic acid forming an ester [261,266–269]. Usually, the esterification reaction is reversible and has a low yield when there is no adequate treatment to optimize the reaction process [255,270–273]. Some researchers have long used acid catalysts (inorganic acids) to accelerate and improve process yield [269,274]. However, this reactional tool has become obsolete because it presents numerous environmental and human health [222,269,273–275]. However, several reactions could be optimized with the advent of enzymes, realizing that they had unique and potent properties regioselectivity, chemoselectivity, and enantioselectivity [268,274,276].

Specifically, lipases are superior to acid catalysts due to their vast library of substrate specificities [21,269,277]. These enzymes can recognize substrate structures, activated without cofactors, and functioning at room temperature and pressure [274,275,278]. On the other hand, separating these enzymes from the reagents of a reaction is complicated and costly. To solve this challenge, the process of immobilization and stabilization of enzymes in solid supports emerged with a potential tool to make processes viable from an industrial point of view [269,276,279]. Enzymatic immobilization provides these enzymes in extreme reaction conditions and would generally lose catalytic activity and soon denature and lose all their functions [269,273,273,279].

Lipase Rhizopus chinensis (RCL) is highly effective in short-chain fatty acid esterification reactions [222,278,280]. This lipase stands out as a potential enzyme for synthesizing aromatic esters, chiral resolution, and biofuel production, among other industrial products [65,215,269]. It is reported that the mutation in the hinge region of the cap of this enzyme (Met93 and Thr96) affects the specificity of the substrate and the thermostability of RCL, enabling the manipulation of these enzymes, expanding its versatility and stability [65,215].

The applicability of RCL has been improved with the evolution of research. It is noteworthy that the design of experiments, the dynamic simulation of bio comportment, and experimental planning are promising tools that have increased the is enzyme's versatility and improved some of its properties [65,88,215]. It is noteworthy that the ordered addition of surfactants in reactions catalyzed by lipases propitiate the improvement of catalytic activity [281–283]. This optimization is caused by the activation of the interface provided by the addition of surfactant [283–285]. It is noteworthy that surfactants generate micelles in an aqueous solution and lead to conformational changes of lipase molecules through the water-micelle interface [87,285,286]. Lyophilization is another process that generally increases the enzymatic activity in the organic phase of some enzymes, as it requires and ensures that the enzyme is in its active conformation for longer [287,288]. It should be emphasized that each lipase interacts synergistically better with a given surfactant, so the selection of the surfactant is essential for this optimization to occur [55,87,281–286].

In this sense, Yihan et al. (2018) reported a study that aimed to verify the potentialities of the RCL expressed by the source and the activation conditions and the effects of surfactants and their combined action with the inorganic sea in the synthesis of RCL esters. Thus, after analyzing several surfactants against RCL, it was identified that n-dodecyl- β-D- Maltoside (DDM) presented the best enzyme activation optimization. From the results obtained with the variation of surfactants, we also varied the inorganic leaves that could synergistically influence the activation of enzymes. The results obtained with the surfactants' variation in several inorganic
salts that could synergistically influence the enzymes' activation were also varied. The salts containing phosphate groups are combined, the catalytic activities are improved in the synthesis of esters catalyzed by RCL. Therefore, it is noteworthy that the catalytic rate in ester synthesis reactions in the non-aqueous phase is high when using activated RCL (surfactant + phosphate salts) in organic reaction environments [285].

7.3 Transesterification reaction.

Transesterification (Figure 5) is one of the most common reactions in oil modification, as in biodiesel synthesis, whether catalyzed by chemical or enzymatic catalysts [289–291]. This reaction has the main advantages: the shorter reaction time, high efficiency, and shorter steps than hydroesterification [277,292].

![Figure 5. Transesterification of triglycerides catalyzed by Rhizopus chinensis.](https://biointerfaceresearch.com/)

Whole-cell lipase of *Rhizopus chinensis* in its soluble form was compared with five commercial lipases (Lipase from *Candida antarctica* (Novozym 435), Lipase from *Pseudomonas cepacia*, Lipase from *Pseudomonas cepacia* immobilized on ceramic, Lipase from *Candida rugosa*, and Lipase from *porcine pancreas*) in transesterification of soybean oil to fatty acid methyl ester (biodiesel) in the solvent-free system [46]. *Rhizopus chinensis* showed high catalytic activity, and it was possible to obtain 86% methyl ester yield under optimal conditions. Furthermore, the *Rhizopus chinensis* proved to be an efficient and potential biocatalyst for biodiesel production from oleic acid and simulant high acid value oil in the solvent-free system [46].

He et al. (2016) studied the use of lipase from *Rhizopus chinensis* immobilized on loofah (Luffa cylindrica) sponges for biodiesel production via the transesterification of soybean oil. Under optimal condition (9.65 g soybean oil at 40 °C and 180 rpm using a 3:1 methanol-to-oil molar ratio were found to be 8% cell addition and 3-10% water content (depending on the oil’s weight), a 90% yield was achieved [56]. The operational stability study showed that *Rhizopus chinensis* was more stable than *Rhizopus oryzae* [56]. They were providing reuse of the enzyme, facilitating application on an industrial scale.

These outcomes suggest that using *Rhizopus chinensis* whole cells, vegetable oil's transesterification is a practical biodiesel production approach. In addition to the production of biodiesel, transesterification can be used to produce other biofuels (bio-jet, for example) [293], flavors [294], cosmetics [295], and lubricants [296].
8. Conclusions

Lipases still are important biocatalysts in the food, pharmaceutical, and chemical industries [202,204,210,297,298]. Lipase from *Rhizopus chinensis* shows high specificity catalytic activity to esterification and transesterification reactions in the presence or absence of organic solvents [44–46,57,215]. The enzyme can also have great *thermophilicity* and moderate pressure resistance, having its catalytic behavior modulated by changes in these conditions [47,54,60,63,88,215]. This review showed that with the appropriated chemical modifications and immobilization strategy, the lipase of *Rhizopus chinensis* has broad potential for many other biotechnological applications.

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**Conflicts of Interest**

The authors declare no conflict of interest.

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