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Review

Aspergillus sp. lipase: Potential biocatalyst for industrial use

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ABSTRACT

The lipases obtained from the genus *Aspergillus* present remarkable importance in biotechnological applications, and numerous studies have reported the importance of the fermentation parameters, such as nutrients, temperature and fermentation time. Moreover, many *Aspergillus* spp. lipases present several properties of immense industrial importance, such as their pH and temperature stability and excellent enantioselectivity. Different strategies have been used in order to immobilize crude or purified *Aspergillus* spp. lipases. Hence, *Aspergillus* spp. lipases have been studied for different industrial applications such as in the food and detergent industries, and also in the kinetic resolution of pharmaceuticals and chiral intermediates. This review highlights the production, purification, characterization, applications and immobilization of lipases from *Aspergillus* spp.

Contents

1. Introduction

Nowadays, biocatalysts are commonly used in many industrial applications. The main reason is that chemical catalysts show several disadvantages as well as producing a large range of byproducts and toxic effluents. The use of cells or enzymes has gradually substituted many chemical catalysts due to their great selectivity for

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the substrate. In addition, they are biodegradable and act under environmentally friendly conditions.

Lipases (triacylglycerol acyl hydrolases, EC 3.1.1.3) are natural catalysts of the hydrolysis of triacylglycerols into di- and monoacylglycerols, fatty acids and glycerol at an oil–water interface, a phenomenon known as interfacial activation [1]. However, under certain conditions, they are also able to catalyze synthetic reactions [2]. The most reported of the reactions carried out by these enzymes are hydrolysis, acidolysis, alcoholysis, aminolysis, esterification and inter-esterification [3]. Currently, lipases are a popular choice as a biocatalyst because they can be applied to chemo-, regio- and enantioselective hydrolyses and also in the syntheses of a broad range of compounds [4]. These enzymes are considered to have great potential as biocatalysts in numerous industrial processes, such as the synthesis of food ingredients [5], their use as additives to detergents [6] and to obtain enantiopure drugs and other refined products [7].

Lipases occur in animals [8,9], plants [10,11] and microorganisms [12,13]. Microbial lipases show a broad spectrum of industrial application due to their greater stability, substrate specificity and lower production costs when compared to other sources. In addition, the immense biodiversity of microorganisms improves their biotechnological importance and justifies the search for new lipases. Filamentous fungi are recognized as the best lipase producers and are currently the preferred sources since they produce extracellular lipases, facilitating the extraction from fermentation media [14]. The most reported species belong to the genera *Rhizopus* sp., *Mucor* sp., *Geotrichum* sp., *Penicillium* sp. and *Aspergillus* sp. [15,16]. Furthermore, the use of directed evolution can be very helpful to optimize existing lipases with respect to desired properties [17].

The *Aspergilli* are a ubiquitous group of filamentous fungi spanning over 200 million years of evolution. They have an impact on human health and society and there are more than 180 officially recognized species, including 20 human pathogens as well as beneficial species used to produce foodstuffs and industrial enzymes [18]. This genus is found worldwide, and most species degrade plant polysaccharides [19]. It must be emphasized that among the different species, *Aspergillus oryzae* and *Aspergillus niger* are on the generally recognized as safe (GRAS) list of the Food and Drug Administration (FDA) in the United States. There are several species with great potential as sources of lipases [20] with important properties for industrial applications.

This review focused on the great diversity of *Aspergillus* spp. lipase, highlighting the production, purification, characterization, application and immobilization of lipases from several species of this genus. Different aspects of *Aspergillus* spp. lipase are also discussed.

2. Production of *Aspergillus* spp. lipase

Approximately 90% of all industrial biocatalysts are produced by submerged fermentation (SmF), frequently using specifically optimized media and genetically manipulated microorganisms. For this purpose, SmF processing can offer several advantages over solid-state fermentation (SSF), but on the other hand, almost all these enzymes could also be produced in SSF [21,22]. Interestingly, fungi, yeasts and bacteria that were recently tested in SSF exhibited different metabolic strategies under the two fermentation conditions, and a direct comparison of various parameters such as growth rate, productivity and volume activity favored SSF in the majority of cases. In addition, in most cases the cost-factor for the production of "bulk-ware" enzymes favors SSF over SmF [23].

Despite the great industrial importance of SmF, SSF shows some advantages when compared to the former, including economy of space for fermentation, simplicity of the media, no requirement for complex machinery, equipment or control systems, compactness of the fermentation vessel due to the smaller volume of water, superior yields, less energy demand, and less capital investment and recurring expenditure [24,25]. In addition, such systems have applications in solid waste management, biomass energy conservation and in the production of secondary metabolites. However, SSF has some limitations such as a limited choice of microorganisms capable of growing under reduced moisture conditions, and the controlling and monitoring of parameters such as temperature, pH, humidity and air flow [24,26].

Many studies have been undertaken to define the optimal culture and nutritional requirements for lipase production. These requirements are influenced by the type and concentration of the carbon and nitrogen sources, culture pH and growth temperature [27]. Lipidic carbon sources generally seem to be essential to obtain a high lipase yield, although a few authors observed that the presence of fats and oils was not statistically significant for enzyme production [20]. The use of agro-industrial residues as the substrate could result in a reduction in the costs of lipase production, considering that the culture medium usually represents 25–50% of the total production costs [28]. Table 1 lists the different residues that could be used for microbial lipase production.

Adham and Ahmed [29] screened four strains of *A. niger* for lipase production. Each was cultivated on four different media, differing in their mineral component contents and sources of carbon and nitrogen. Cultivation in medium 1, which contained 3% peptone, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.2% K_2HPO_4 and 1% olive oil:glucose (0.5:0.5), showed maximal lipolytic activities for *A. niger* A20 (19 U/mL), *A. niger* NRRL 599 (320 U/mL) and *A. niger* NRRL3 (325 U/mL), as compared to the other three test media. However, cultivation in a medium containing only glucose as the carbon source, caused lipase inhibition in all the strains tested. The most suitable media for lipase production were not the best ones for growth of the microorganism.

A response surface approach was used by Kaushik et al. [30] to study the production of an extracellular lipase from *Aspergillus carneus*, since this lipase shows properties of immense industrial importance. The interactions between five different variables (sunflower oil, glucose, peptone, agitation rate and incubation period) were studied using a one-at-a-time method, and found to influence lipase production. Using a statistical approach they obtained a 1.8-fold increase in production, with a final yield of 12.7 U/mL. After using response surface analysis, the optimum values obtained for the following more influential parameters were as follows: sunflower oil (1%), glucose (0.8%), peptone (0.8%), agitation rate (200 rpm) and incubation period (96 h) at 37 °C.

Lipase production by *Aspergillus japonicus* MTCC 1975 in solid-state fermentation was optimized by Sarat Babu et al. [31], using sugarcane bagasse and wheat bran as the mixed substrate. When response surface methodology (RSM) was used, the following three factors were considered to influence the optimization process and the best yields were obtained at pH 7.0 with 10 g of substrate and 80% moisture content.

In their studies, Yadav et al. [32] optimized the production of lipase by *Aspergillus terreus*. A yield of 7.78 U/mL was obtained using a medium containing corn oil (2%, v/v) and casein (0.1%, w/v). Maximum production was observed after 96 h at pH 9.0 and 37 °C. The presence of Ca^{2+} and Mg^{2+} ions enhanced the secretion of lipase.

According to Ohnishi et al. [33], *A. oryzae* produced large amounts of lipase in SmF (0.46 U/mL) using a modified GYP medium containing 2% glucose, 1% yeast extract and 2% polypeptone as substrates. The lipase activity was further increased by adding 3% soybean oil, and fixing the temperature at 28 °C in a shake-flask culture, and in a jar fermentor, 30 U/mL lipase activity was obtained after 72 h at 28 °C.

Table 1
Different agro-industrial residues for microbial lipase production in solid-state fermentation.

Microorganisms	Agro-industrial residue used as the nutrient	Ref.
A. niger	Wheat bran, soapstock and stearin	Damaso et al. [109]
Aspergillus sp.	Wheat rawa, corn steep liquor	Adinarayana et al. [110]
Penicillium restrictum	Babassu oil industrial waste	Palma et al. [111]
Penicillium simplicissimum	Soybean meal	Vargas et al. [112]
Rhizomucor pusillus	Olive oil cake and sugar cane	Cordova et al. [113]
Rhizopus rhizopodiformis	bagasse	
Bacillus coagulans	Solid waste from melon	Alkan et al. [114]
Candida rugosa	Coconut oil cake	Benjamin and Pandey [115]

Aspergillus wentii showed maximum growth and lipase production under SmF conditions in 3 days at 30 °C and pH 6.0. Maximum enzyme production was achieved when the medium was supplemented with glucose, followed by mannitol, fructose, galactose, sucrose, lactose and maltose. Of the nitrogen sources tested, maximum lipase yield was obtained with 2% peptone. Calcium and sodium citrates (0.1%) increased the yield of lipase, but synthetic and natural lipids, when added to the growth medium, reduced both growth and lipase production [34].

The above results show that, in general, several parameters must be discussed with respect to lipase production. For SSF, the water content had a great impact on the physical properties of the solid substrate [37]. Higher than optimum water levels decreased the porosity, lowering oxygen transfer and altering the wheat bran particle structure [38]. Likewise, a lower than optimum water content decreased the solubility of the solid substrate, lowering the degree of swelling and producing a high water tension [39].

Glucose is important in improving lipase production, possibly due to the rapid assimilation of this carbohydrate by the cells as compared to that of lipids, leading to a higher growth rate and greater lipase activity [40], in agreement with Mahadik et al. [39]. The types of lipid materials used are particularly important for lipase synthesis, considering that, as already mentioned earlier in this review, several studies have reported good results for the production of lipase by *Aspergillus* spp. when different oils were tested.

3. The purification and characterization of *Aspergillus* spp. lipase

Lipases have been extensively purified and characterized in terms of their properties of use in biotechnological applications. Homogeneity allows for the successful determination of their primary amino acid sequences and three-dimensional structures. X-ray studies of the structure–function relationships of pure lipases have contributed to an understanding of the kinetic mechanisms of the enzyme actions [41]. A variety of methods have been used to purify lipases from different sources, the most used strategies including non-specific techniques such as ammonium sulfate precipitation, hydrophobic interaction chromatography, gel filtration and ion exchange chromatography, followed by gel filtration. Affinity chromatography has been used in some cases to reduce the number of individual purification steps needed, with greater yields of purified enzymes [3].

The effect of various factors on the activity and stability of purified and crude lipases, such as the pH, temperature, effect of metal ions, organic solvents, detergents/surfactants and other inhibitors, can enhance or suppress the activity of the lipases. The characterization of a lipase can determine its suitability for use in different environments and industries, etc. [42].

Most of the microbial lipases such as those from *A. niger*, *Rhizopus delemar*, *Rhizopus miehei*, *Mucor javanicus* and *Yerrowia lipolytica* show 1,3-positional specificity [43,44] releasing 2-monoacylglycerol and 1,2- and 2,3-diacylglycerol as products from the substrate. Lipases preferring the 2-position are very rare in nature. Others lipases possessing no strict preference for position and fatty acid features hydrolyze all the ester bonds in various substrates, producing glycerol and fatty acids as the end products [45]. Mhetras et al. [46] purified an extracellular lipase from *A. niger* NCIM using ammonium sulfate precipitation, followed by phenyl Sepharose and Sephacryl-100 gel chromatography. This protocol resulted in a 149-fold purification with a final recovery of 54%. The purified enzyme was a monomeric protein with a molecular weight of 32.2 kDa, and exhibited optimal activity at an acidic pH of 2.5 and 50 °C. The enzyme seems to be unique since it only cleaved triolein at the 3-position, releasing 1,2-diolein. The present studies showed that this novel lipase is unique because it is active at high acidic pH (pH 1.5) and specific for 3-position in the ester bond. Chemical modification studies revealed that His, Ser, Carboxylate and Trp were involved in the catalysis. The lipase activity decreased significantly (50%) when the pH was increased to 4.0, but was little affected by lowering to pH 1.5, where it showed 70% of its original activity. The enzyme showed stability in the alkaline pH range (pH 8.0–11.0), retaining 100% of its original activity after incubation for 24 h.

The lipase from *A. carneus* was purified by Saxena et al. [3] and shown to be a monomer glycosylated to the extent of 12.3%. It was purified by ammonium sulfate precipitation, and SDS-PAGE of this partially purified enzyme showed four bands in addition to the lipase. The enzyme was then further purified by hydrophobic interaction chromatography, obtaining a 24-fold purification and an activity yield of 38.4%. This biocatalyst could tolerate pH 6.0–12.0, being stable in this range for 24 h and showing a pH optimum of 9.0. Even at pH 11.0, 52.4% residual activity was obtained. A high pH optimum for the lipase activity of *A. terreus* was also reported [47]. The enzyme was stable between pH 6.0 and 12.0, the greatest stability being observed between pH 8.0 and 10.0. The remarkable stability of the *A. carneus* lipase in this range justified it as a potential alkaline lipase. The maximum activity of the enzyme was at 37 °C, but it was active in the range from 5 to 90 °C. Nevertheless, at very low (5 °C) and very high (80–100 °C) temperatures, the activity was reduced. The purified enzyme from *A. carneus* retained 100% of its activity at 70 °C for 5 min, and approximately 55% of its activity even after 10 min. The effect of different ions and the chelating agent EDTA (20 mM) on the lipase activity was also tested. Mg^{2+}, Na^{+} and NH_4^{+} ions stimulated the lipase activity, while Cu^{2+}, Cd^{2+}, Hg^{2+}, Zn^{2+} and Pb^{2+} caused inhibition. EDTA and ferric ammonium citrate had no effect on the lipase activity, suggesting the absence of any requirement for a co-factor for this lipase activity.

Strains of bacteria, fungi and yeast were examined by Coca et al. [48] for a quantitative screening of lipase-producing microorganisms, using 2% of olive oil as the carbon source. The ideal carbon source for lipase synthesis was selected. Around 0.26 U/mL of *A. niger* lipase activity was obtained in 2% olive and sunflower oil on the 5th and 7th days, respectively, and 0.21 U/mL of *Aspergillus fumigatus* lipase activity was achieved in olive oil on the 4th day. The optimum pH and temperature to extract the enzymatic activities were pH 6 and 40 °C for *A. niger* lipase and pH 7 and 80 °C for *A.*

fumigatus lipase. Both enzyme extracts were stable for 5 h in neutral and weakly acid mediums at temperatures ranging between 20 and 40 °C.

Lipases show different kinetic behaviors depending on the substrate concentration, which determines their physical–chemical state [49]. The substrate forms an isotropic solution below the critical micellar concentration (cmc), whereas above the cmc, it forms a turbid emulsion. Therefore Mayordomo et al. [50] determined the kinetics of *Aspergillus nidulans* WG312 lipase in a wide range of concentrations, using pNPP as the substrate. The enzyme showed typical lipase behavior, with two Michaelis–Menten hyperbolas. The activity of the *A. nidulans* WG312 lipase was highly pH dependent. The optimum pH was found to be 6.5, and the activity decreased approximately 30–35% at pH 6 or 7. The pH stability profile revealed that the activity was well preserved at alkaline pH values over a 1 h period. The apparent optimal temperature for the activity of the enzyme was observed at 40 °C when assayed at pH 6.5, although the enzyme showed the ability to maintain high reactivity at lower temperatures. Even at 0 °C, the *A. nidulans* lipase conserved almost 30% of its relative activity, but it showed pronounced heat lability. Thus the enzyme lost its activity at an exponential rate when the temperature exceeded 40 °C. These thermal properties are similar to those displayed by cold-adapted enzymes [51,52], although reduced thermal stability seems to be a common property amongst the lipases isolated from many fungal species [53,54].

Different *Aspergillus* spp. strains are able to produce multiple iso-lipases. Thus, different enzymatic properties are reported. Fernández-Lorente et al. [35] purified different iso-lipases produced by an *A. niger* strain. The authors used two different supports, octyl agarose and octadecyl-sepabeads. The desorption of the adsorbed proteins were carried out using a gradient of Triton X100. At the end of purification process it was obtained three different iso-lipases and their molecular mass was 31, 43 and 65 kDa.

Höfelmann et al. [36] reported the isolation and purification of lipolytic activities performed by combination of DEAE-Trisacryl M ion exchange chromatography, Sephadex G 50 gel filtration and hydrophobic chromatography using Phenylsepharose CL-4B. Two homogeneous lipase isoenzymes (I and II) were isolated and presented the following properties: isoelectric points (I: 4.0; II: 3.5); molecular weights (I: 31,000 Da; II: 19,000 Da); carbohydrate contents (I: 6%; II: 9%) and compositions; pH optima (I and II: 5–6); substrate specificities and various effectors.

Based on the above reports, it appears that the lipases from different *Aspergillus* spp. strains could be purified with yields of interest, many researchers purifying the enzyme until just one monomer was observed in the electrophoretic analysis. The most widely used technique was protein precipitation by the addition of ammonium sulfate, apart from more refined techniques such as ion exchange chromatography, gel filtration and hydrophobic interaction. The majority of the *Aspergillus* spp. lipase showed an acidic optimum pH value for activity (5.0–6.0), an optimum temperature between 40 and 60 °C, and interesting thermal stability in a wide range of pH values.

4. Applications of *Aspergillus* spp. lipase

The estimated global industrial enzyme market has increased substantially to a current value of $2.1 billion (Novozymes 2005), and is expected to grow annually by 10–15%. After proteases and carbohydrases, lipases are considered to be the third largest group based on total sales volumes. The major applications of lipases are summarized in Table 2.

4.1. Organic synthesis using kinetic resolutions

One of the most important applications of lipases in organic synthesis is related to the resolution of racemates, considering the enantioselectivity they present towards a variety of substrates [55,56]. Chirality is a key feature in the efficiency of many drugs and agrochemicals, and consequently the production of single enantiomers of chiral intermediates has become increasingly significant in many industrial applications [57,58].

The therapeutic properties of the anti-inflammatory (*R*,*S*)-ibuprofen are mainly due to the active (*S*)-enantiomer [59]. Carvalho et al. [15] reported an *A. niger* AC-54 lipase with several properties of great industrial importance, such as the ability to preferably esterify (*R*)-ibuprofen. The lipase showed very high activity (18.2 U/mL) and was highly thermostable. In isooctane, it showed the best results in terms of the enantiomeric excess of the (*S*)-active acid (ee = 6.1%) and conversion value (c = 20%) in the esterification of the racemate with 1-propanol. In order to improve the enantioselectivity of this enzyme for (*R*)-ibuprofen, the same research group reported the optimization of the reaction parameters (enzyme concentration and molar ratio of propanol:ibuprofen) [60]. Under the optimum conditions (7%, m/v of enzyme and molar ratio of 2.41:1) the enantiomeric excess of active (*S*)-ibuprofen and total conversion values were 79.1 and 48.0%, respectively, and the *E*-value was 32, after 168 h of reaction.

All these attempts [15,60] to resolve (*R*,*S*)-ibuprofen were carried out in an organic solvent (isooctane), since a non-aqueous medium was necessary to favor the esterification reaction. However, organic solvents can be volatile and toxic to the environment [61]. An alternative was the use of ionic liquids (IL), which possess several interesting properties such as ease of preparation, reuse, high thermal stability and low vapor pressure. Thus the resolution of racemic ibuprofen was carried out using the IL 1-butyl-3-methyl imidazolium hexafluorophosphate [BMIM][PF6] as the co-solvent with isooctane. On the one hand, the presence of IL increased the solubility of the drug, and on the other hand, the esterification caused the reaction to proceed with higher enantioselectivity as compared to reactions in conventional solvents. This is due to the hydrophobic nature of [BMIM][PF6], because the enzyme needs a small amount of water to maintain its structure. After a thorough optimization of several reaction conditions (type and ratio of isooctane/ionic liquid, amount of enzyme and reaction time), an *E*-value of 9.2 was obtained for the *A. niger* lipase (15%, m/v) in a solvent system composed of [BMIM][PF6] and isooctane (1:1) after 96 h of reaction [62].

Enantiomerically pure alcohols are very important intermediates for use in organic synthesis to obtain enantiopure pharmaceuticals. Carvalho et al. [14] studied the kinetic resolution of (*R*,*S*)-2-octanol with octanoic acid in n-hexane by four *Aspergillus* spp. lipase (*Aspergillus flavus* AC-8, *A. niger* AC-54, *A. oryzae* AC-122 and *A. terreus* AC-241). They observed that lipases from *A. niger* and *A. terreus* showed the best results in terms of enantioselectivity (E = 4.9 and E = 4.5, respectively). These properties make these lipases good candidates for biocatalysis in organic media.

Enantiopure amines are important in organic synthesis as chiral synthetic building blocks for pharmaceuticals and agrochemicals [63]. Pilissão et al. [64] studied the enzymatic acylation of (*R*,*S*)-phenylethylamine, catalyzed by different lipases. It was observed that when the *A. niger* lipase was tested in n-heptane at 35 °C, a high conversion degree (30%) and *E*-value (E > 200) were obtained in 96 h of reaction with ethyl acetate as the acyl donor. The influence of different ionic liquids was also studied in a subsequent study. It was observed that this lipase showed better *E*-values in a two-phase system using n-heptane and 1-butyl-3-methyl imidazolium hexafluorphosphate [BMIM][PF6] or 1-butyl-3-methyl imidazolium tetrafluoroborate [BMIm][BF4] 9:1 (v/v). Values of 9

Table 2
Biotechnological applications of microbial lipases.

Microorganisms	Industry	Application	Ref.
Fusarium solani N4-2	Detergent	Removal of oil stains from fabrics	Liu et al. [6]
Burkholderia cepacia			Rathi et al. [116]
Candida antarctica	Food	Attainment of functional phenols	Buisman et al. [117]
Rhizopus sp.		Aroma ester synthesis	Macedo et al. [118]
Candida rugosa	Pharmaceutical	Kinetic resolution of (*R*,*S*)-ketoprofen	Chang and Hsu [119]
Serratia marcescens		Kinetic resolution of diltiazem intermediate	Shibatani et al. [120]
Pseudornonas cepacia	Fuel	Biodiesel production	Noureddini et al. [121]
Mucor miehei			Nelson et al.0 [122]

and 7, respectively, were obtained when vinyl acetate was used as the acyl donor, as compared to the use of pure n-heptane ($E = 2$) [65].

Numerous enantiopure carboxylic acids present biological activity, such as the (*R*)-2-phenoxypropanoic acids. Miyazawa et al. [66] reported the resolution of (*R*,*S*)-2-phenoxypropanoic acid) using *A. niger* lipase by transesterification of the vinyl esters. The enantioselectivity was affected by the alcohol as the nucleophile, by the organic solvent used and by the reaction temperature. The best results were found using methanol as the nucleophile, isopropyl ether as the solvent and a temperature of 25 °C.

The (*R*)-enantiomer of α-lipoic acid is much more active than the (*S*)-enantiomer against diabetes mellitus [67], HIV [68] and tumors [69]. Thus the kinetic resolution of α-lipoic acid was carried out using the lipase from *A. oryzae*. The optimum reaction conditions were found to be esterification with n-octanol at 50 °C in heptane, with an alcohol:acid molar ratio of 5:1. The conversion rate of a-lipoic acid was 75.2%, with an enantiomeric excess of 92.5% in 48 h of reaction time [70].

4.2. *Aspergillus spp. lipase in the food industry*

4.2.1. *Aroma compounds*

Aroma compounds are very important in several industrial fields such as the food industry. They can be obtained by traditional methods such as chemical synthesis or extraction from natural sources, but the use of biocatalysis provides some advantages. Esters from short-chain fatty acids and alcohols are important aroma compounds and can be synthesized by lipase catalyzed esterification.

Langrand et al. [71] studied the preparative synthesis of several short chain flavor esters using different microbial lipases. In the case of the commercial *Aspergillus* sp. lipase, the yields decreased with increasing number of carbon atoms in the alcohols. The *A. niger* lipase was very useful in the synthesis of terpene alcohol esters of lower fatty acids (C3–C6) [72].

Song et al. [73] studied the substrate specificities of nine lipases, including the fatty acid and positional specificities. The lipase from *A. niger* presented the greatest specificities for the different fatty acid esters (methyl acetate, methyl propionate, methyl butyrate and 2,2-dimethyl methyl valerate). Furthermore, the *A. oryzae* lipase showed the greatest specificity for methyl butyrate, a short-chain fatty acid ester. These results provided helpful information for the rapid selection of specific lipases amongst the many commercial lipases used to catalyze certain reactions. In addition, it can avoid unnecessary selection procedures.

4.2.2. *Functional lipids*

The modification and structuring of lipids can be used to design ingredients that have beneficial properties with respect to calorie management. Several structured lipids (SL) present unique nutritional and functional properties, depending on the molecular structures of the acyl chains [74]. Using *Aspergillus* spp. lipase, it was possible to promote acidolysis activity between the triacylglycerol fatty acids to produce SL. Tsuzuki [75] reported that a lipase from *A. oryzae* was a powerful biocatalyst to produce reduced-calorie structured lipids by an acidolysis reaction. The results showed that the lipase promoted the incorporation of more than 80% of triolein by butyric acid in the dried n-hexane at 52 °C, in 72 h of reaction time. Compared to the chemical process, this method is very interesting due to a reduction in the number of byproducts [76].

The *n*-3 polyunsaturated fatty acids (PUFA), such as eicosapentaenoic acid (C20:5 n-3, EPA) and docosahexaenoic acid (C22:6 n-3, DHA), are known to play an important role in human health. Lipases are known to catalyze hydrolysis reactions and have been used as a good alternative to obtain PUFA concentrates such as acylglycerols [77]. The *A. niger* lipase was the most effective enzyme in concentrating *n*-3 PUFA. The degree of hydrolysis (60%) led to an increase in the docosahexaenoic acid (DHA) content of from 14.4% in the original oil to 34.0% (2.4-fold enrichment) in the residual acylglycerol. The optimum conditions were an enzyme concentration of 500 U/g oil, reaction temperature of 45 °C, water/oil mass rate of 2:1 (m/m) and a 24 h reaction period. The enrichment of DHA by the selective hydrolysis of salmon oil catalyzed by *A. niger* lipase appears to be a feasible method for the development of salmon oil health products [78]. Some other authors have reported the use of *Aspergillus* spp. lipase to prepare PUFA [79,80].

4.3. *Aspergillus spp. lipase in the detergent Industry*

The major industrial application of lipases is in the detergent industry, considering their great ability to remove fatty stains from fabrics. In this case, the lipase needs to be thermostable, tolerant to an alkaline environment and present the ability to hydrolyze fats with various compositions. Enzymes are more interesting than conventional synthetic detergents, due to their ability to carry out washing processes at lower temperatures and also because of the reduction in pollution [81].

The application of a lipase from *A. niger* as an additive in a laundry detergent was reported by Saisubramanian et al. [82]. The enzyme showed increased stability in the presence of SDS, Tween 80 and in all commercial detergents. The washing process was optimized by RSM and the optimized conditions were 1.0% of commercial detergent, 75 U of lipase, pH 9.5 and a washing temperature of 25 °C. Under these conditions, 33% of olive oil was removed from cotton fabric.

The purified lipase from *A. carneus* shows interesting properties for use in the detergent industry. This lipase has an optimum pH and temperature of 9.0 and 37 °C, respectively, and stability in the pH range from 8.0 to 10.0 for 24 h, and at 70 °C for 5 min. In addition, the presence of several detergents stimulated its activity [3]. Other reports can be found in the literature regarding the potential application of *Aspergillus* spp. lipase in detergent formulations [83,84].

4.4. *Other biotechnological applications*

The textile industry is another important field for the application of lipases. Polyethylene terephthalate (PET) is the most impor-

tant synthetic fiber in the field. However, the PET fiber also has some undesirable properties due to its hydrophobic nature and its non-active surface. The treatment of PET fibers with lipases shows several advantages as compared to chemical methods. They act under mild and environmentally friendly conditions and do not harm the mechanical properties of the PET. Thus according to Wan et al. [85], *A. oryzae* lipase was capable of modifying PET fabrics, improving their hydrophilicity and anti-static ability.

Nowadays, industrial effluent treatment and environmental protection are two of the main industrial concerns, and must be dealt with in the most appropriate and cost-effective way, to avoid potential risks and costs. Wastewaters from dairies and slaughterhouses present high levels of fats and proteins, which can cause gross pollution of land and water. There are different methods for treating this effluent but it is always necessary to reduce the fat, oil and protein concentrations [86]. Industries pay severe fines for discharging these high chemical oxygen demand (COD) effluents into the sewage system, resulting in a loss of profit. Roux-Van der Merwe et al. [87] studied the growth of different fungal species in industrial oil effluents and observed that the reduction in COD promoted by *A. niger* (TUTC 120) was 83.8%.

5. Immobilization of *Aspergillus* spp. lipase

Despite the interesting properties of lipases, they are not perfect catalysts. In determined circumstances, these enzymes may be unstable and may show neither optimum activity nor optimum selectivity. In addition, the high cost of enzymes for industrial applications makes enzymatically driven processes economically unattractive. Thus the use of immobilized lipase is a possible solution to this problem, due to the increase in stability of the biocatalyst and the reduction in operational costs of the industrial processes with the selection of an appropriate immobilization method.

The immobilization techniques use adsorption, linkage or bonding of the enzyme to an insoluble support, entrapment of the enzyme in polymeric gels or encapsulation. The main advantage of using immobilized enzymes as biocatalysts is that it is possible to reuse them, since they can be easily recovered, thus making the process economically feasible. Various techniques, and even more support materials, have been studied, and consequently many immobilized preparations with a wide range of efficiency, stability and activity are available. Several parameters of the support are important and must be considered: mechanical strength, chemical and physical stability, hydrophobic/hydrophilic character, enzyme loading capacity and cost, amongst others [88].

The adsorption of lipases onto different suitable supports has been the more popular strategy for lipases. This technique is economically feasible, attractive and in some cases presents several advantages, mainly when the lipases are used in organic media. This is because the lipases are not soluble in such solvents. However in aqueous media, lipase immobilized by way of covalent bonding, can be reused more often than other available immobilization methods, such as adsorption and entrapment.

Finally, the technique of confining the biocatalysts in insoluble polymers (forming films) is one of the most used techniques, presenting the advantage of not interacting chemically with the polymer, thus avoiding its denaturation.

Immobilization of the lipases from *Aspergillus* spp. using different techniques results in alterations to the enzyme properties, such as those cited in the subsequent items. Amongst these the enzyme can present greater thermo-stability than the soluble enzyme since the biocatalyst can be protected. The selectivity, such as enantioselectivity can be decreased, increased or even start presenting selectivity for another substrate, different from that of the free enzyme.

5.1. Adsorption

On account of the relatively high surface hydrophobicity of lipases, simple adsorption onto suitably hydrophobic supports has been the more popular strategy. The enzymes are adsorbed by a combination of hydrophobic and Van der Waals linkages, electrostatic forces and so on. In addition, immobilization by adsorption is economically feasible and attractive [89,90]. Moreover, this technique shows several advantages when lipases are used in organic media. In this case, covalent linkages may not be necessary between the support and the lipase, and thus simple adsorption can be employed, considering that lipases are not soluble in those solvents [91].

Silva et al. [92] studied the immobilization of *A. niger* lipase, and several properties of the soluble enzyme as compared to that immobilized on Celite. The authors observed that both preparations showed similar biochemical properties, with maximum activity at pH 6.0 at a temperature of 30–40 °C. The most important effects observed when the lipase was immobilized were its thermal stability and improved esterification activity during the reaction of (*R*,*S*)-ibuprofen with 1-propanol in isooctane. In further studies, the authors [88] screened different supports to immobilize the *A. niger* lipase, and the immobilized biocatalysts were used in the kinetic resolution of (*R*,*S*)-ibuprofen. Only the adsorption method was tested with the different supports (Accurel EP-100, Amberlite MB-1, Celite, Montmorillonite K10 and Silica gel), and it was observed that the best support for this application was Amberlite MB-1, which gave an immobilization yield of approximately 62% and a biocatalyst with better esterification activity and enantioselectivity for (*R*)-ibuprofen.

Amberlite MB-1 consists of hydrophobic beads of a strongly basic and acidic resin [93]. On the other hand, Montmorillonite K10 is the commercially available acid activated form of smectite (2:1 dioctahedral). Other common hydrophobic supports include polyethylene, polypropylene, styrene and acrylic polymers. Celite and Silica gel belong to the hydrophilic group of supports, which also include Duolite, activated carbon, clay and Sepharose.

Lipases show affinity for hydrophobic interfaces, since an amphiphilic amino acidic chain, called the “lid” or “flap”, buries the active site [93]. During the adsorption process, the conformation of the lipase changes in the presence of the hydrophobic support surface, and it is thus adsorbed in an open conformation. The losses in activity during the immobilization processes have been attributed to the following possibilities: a situation in which only small quantities of lipase are immobilized; a change in the conformation of the lipase on adsorption into a form with reduced activity; a decrease in the ability of the hydrophobic substrates to reach the active site of the enzyme; or the existence of steric hindrance imposed by the carrier matrix, which constrains the flexibility of the lipase molecule [94].

An *A. niger* lipase was immobilized by adsorption on a polypropylene flat-sheet membrane, and used in the hydrolysis of butter oil. Compared to other proteins in the crude extract, the lipase was selectively adsorbed onto the support. The authors observed that when the temperature increased, the loading capacity of the hollow fibers decreased and the adsorption constant increased, which is more significant in the case of the lipolytic activity as compared to the total amount of adsorbed protein [95].

Fernández-Lorente et al. [35] reported the immobilization of purified lipases obtained from a commercial *A. niger*, via ionic adsorption on DEAE-Sepharose. The crude extract preparation gave a low enantioselectivity value ($E = 9$) in the resolution of (±)-O-2-butyryl-2-phenylacetic acid, whereas the three immobilized

preparations of purified lipases exhibited an increase in *E*-value from 11 (43 kDa lipase) to >100 (31 kDa lipase).

5.2. Covalent bonding

The immobilization of lipases by covalent bonding involves the chemical modification of one of the amino acids of the enzymes. This is due to the covalent bonding between the proteins themselves (cross-linking) or between the proteins and the support. Most of the immobilization protocols involving the amino groups of lipases can be carried out at pH 7.0–8.0, using different agents such as glutaraldehyde and cyanogen bromide [96,97]. A more specific study is necessary to improve the lipase properties. The enzyme can only be attached to the support at a single point, that gives the protein a rigidity similar to that of the soluble enzyme. However, immobilization of the lipase via multipoint covalent attachment might result in an enhancement of the rigidity and stability of the enzyme against any distorting agents [98].

A purified *A. niger* lipase was immobilized by different techniques (covalent attachment, anionic exchange and interfacial activation on a hydrophobic support) and was used in the regioselective monohydrolysis of different peracetylated-β-galactopyranosides. The immobilization method showed considerable influence on the activity and selectivity of the biocatalyst for the substrate. It was shown that the presence of different moieties at the anomeric position of the substrate also greatly altered the catalytic properties of these immobilized lipases [99,100].

Chatterjee et al. [101] reported the immobilization of *Aspergillus* sp. lipase in silk fibers via glutaraldehyde cross-linking, and used the preparation for the hydrolysis of sunflower oil. The activity of the lipase was very poor in the biphasic oil in water system, but increased with emulsification of the sunflower oil in an aqueous medium using a rhamnolipid biosurfactant, Triton X100, and ultrasonication, respectively. The immobilized lipase could be reused for the biosurfactant-mediated hydrolysis of sunflower oil up to the third cycle of the reaction.

López-Serrano et al. [102] reported the immobilization of different lipases, including a commercial *A. niger* lipase, as cross-linked enzyme aggregates. The authors tested the activity of this immobilized enzyme in the hydrolysis of *p*-nitrophenyl propionate. An activity yield of 116 was observed when the lipase was precipitated using ammonium sulfate and cross-linked with glutaraldehyde in the immobilization step.

An *A. oryzae* lipase was immobilized by covalent bonding onto a polyaniline nanotube based film, electrophoretically deposited onto indium–tin-oxide via glutaraldehyde. The bioelectrode was used to detect triglycerides and showed increased biosensing characteristics, such as linearity, fast response time and high sensitivity [103].

5.3. Entrapment

Lipases can be entrapped in different polymers, both natural ones like alginate and low-methoxyl pectin, or synthetic polymers, such as polyvinyl alcohol and poly (ethylene oxide). Nevertheless, this technique has been more extensively used for the immobilization of cells than for enzymes.

Reetz et al. [104] reported the immobilization of a commercial *Aspergillus* sp. lipase by entrapment in hydrophobic sol–gel materials, and an improvement was observed in the activity of the immobilized lipases as compared to commercially available lipase powders. According to Dalla-Vecchia et al. [105], a commercial *A. niger* lipase was immobilized onto poly (vinyl alcohol), carboxymethycellulose and a poly (vinyl alcohol):carboxymethylcellulose blend and used as biocatalysts in esterification reactions of lauric acid with n-pentanol. However, when compared to other lipases, *A. niger* showed the lowest esterification activities.

The use of a bioreactor with immobilized *Aspergillus* spp. lipase has been reported. Habulin et al. [106], carried out the high-pressure enzyme-catalyzed hydrolyses of oleyl oleate and sunflower oil with commercial *A. niger* in high-pressure membrane reactors. An optimal concentration of the free fatty acids was obtained when the concentration of the non-immobilized lipase preparation from *A. niger* was 16.67 g of lipase per (L of reactor volume). A problem with the distribution of the enzyme in the reaction medium appeared when the enzyme concentration was increased, the reason being the low purity of the commercial enzyme preparation.

Of the immobilization technologies under discussion, the use of whole cell lipase from *Aspergillus* spp. has also been reported. Romero et al. [107] studied the catalytic properties of mycelium-bound lipases from *A. niger* MYA 135, and the authors observed that the mycelium-bound lipase activities were very stable in reaction mixtures containing methanol and ethanol. This lipase had two pH optima at pH 4 and 7, and was cold-active showing high catalytic activity in the temperature range from 4 to 8 °C.

Ellaiah et al. [108] studied different supports for the immobilization of *A. niger* for the production of lipase. The authors observed that the enzyme activity of whole cells immobilized in polyacrylamide gel and κ-carrageenan was low in comparison to those immobilized in alginate, and the maximum production was observed with an alginate concentration of 3%.

6. Conclusions

Lipases are important enzymes that are widely studied for industrial applications, and different microbial lipase sources have been amply reported. The *Aspergillus* genus produces lipases with tremendous potential, and within the diversity of their properties, their stability and selectivity are highlighted. Thus high-value products (pharmaceuticals, agrochemicals, aroma compounds, etc.) can be obtained with the use of several *Aspergillus* spp. lipase, and in addition, the number of byproducts and effluents are reduced. However, many factors limit the industrial application of *Aspergillus* spp. lipase, such as the high cost of lipase production and a lack of enzymes with the optimal range of catalytic specificities and properties required in the various applications. Thus, several points must be improved: fermentation technology, allowing for a feasible production process of the enzyme on a large scale; immobilization methods, which are important to enhance lipase stability, activity and selectivity, added to the possibility of the continuous use or reuse of the biocatalysts; and protein engineering to obtain *Aspergillus* spp. lipase with desired properties.

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