A Review on *Geotrichum* Lipases: Production, Purification, Immobilization and Applications

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Lipases are enzymes produced from innumerous microorganisms, plants and animal cells. They catalyze reactions of different lipid sources. The *Geotrichum fungi* are good producers of lipases with high hydrolytic activity and specificity for unsaturated fatty acids. A great number of studies have reported the importance of lipase from this genus and described important fermentation parameters for the enzyme production, such as nutrients, temperature, pH, inoculum, time of fermentation and others. Furthermore, different strategies have been used to purify and immobilize lipases from *Geotrichum* and innumerous applications are cited in different processes as polyunsaturated fatty acids enrichment, hydrolysis and esterification of fat and oils, synthesis of aromas, biodiesel, and many others. This review highlights fundamental aspects of the production, purification, characterization, immobilization, and the applications of lipases produced by the genus *Geotrichum*.

Key words:
characterization, fermentation, *Geotrichum*, immobilization, lipase, purification

Introduction

Enzymes are known due the extreme specificity that they present on their substrates. This characteristic results in high productivity, low energy costs, and low quantity of undesirable byproducts when enzymes are applied as biocatalysts in industrial processes. An ecofriendly appeal is obtained in the industrial process utilizing enzymes[1,2].

Lipases (triacylglycerol acylhydrolases EC 3.1.1.3) are enzymes that catalyze the hydrolysis of triacylglycerol into fatty acids and glycerol, mono- and diacylgylcerol at the water-lipid interface and the reversible reaction of lipids in non-aqueous solvents[3–5]. The versatility of lipases allows applications in reactions such as hydrolysis, esterification, interesterification and transesterification, alcoholysis, acidolysis and aminolysis. Several modern bioprocessesuse lipases, such as: biodiesel production[6,7], food industry[8,9], medicine[10,11], in pharmaceutical and cosmetic industries[12,13], pretreatment of industrial wastewaters[14,15], detergents formulation[16,17], and many others[18].

Lipases can replace alkaline catalyzers in biodiesel production facilitating the glycerol recovery and the purification of fatty methyl esters. However, high cost and low stability of the enzyme in the presence of alcohol are the largest disadvantages in this case. Whole cell, recombinant methods, protein and metabolic engineering are promising options to increase lipase applications in the biofuel processes[6,7]. In the food industry, lipases can be utilized for oil enrichment, flavor synthesis, quality improve-

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ment in bread, dough and dairy products, fragrance development, structured lipids and antioxidants production, etc.[8–9]. Lipases are capable of hydrolyzing triacylglicerol which facilitates the removal of greasy material from wastewaters, reducing the damage to the environment[14–15]. Furthermore, some lipases have high stability and activity in alkaline mediums, which allows the application of this biocatalyst in different types of detergent[16–17].

Lipases can be produced by different organisms, including animals, plants, and microorganisms, but only microbial lipases are commercially significant due to their higher production and greater variety. Filamentous fungi and yeasts are the most preferred sources for lipase production, since this enzyme is mostly described as extracellular, which facilitates its recovery from the fermented medium. Approximately 50 % of commercial lipases are produced from yeast and filamentous fungi, and some genus are frequently cited as good lipase producers, such as: *Aspergillus, Candida, Fusarium, Geotrichum, Mucor, Penicilium, Pseudomonas* and *Rhizopus, Rhodotorula*, etc.[4,7,19–23].

Geotrichum sp. is a group of dimorphic yeasts that can occur in cream-colored yeast-like and in white mould-like colonies. The genus is composed of 22 species (including 10 sp. nov.) and the ecology shows a rather unexpected degree of consistency given the large phylogenetic distances between the species, including a high degree of rDNA polymorphism[24–25].

Geotrichum is commonly related to the cheese ripening process, biodegrading of dyes, and lipase production[24–26]. It was undesirable in traditional cheese making until the 1970s because it can cause unstable slim or 'toad skin'. However, since the 1980s it has been used to reduce bitterness as well as to develop flavors (especially in Camembert cheese). In some cases, it shows an antagonist effect against *Mucor* spp. and *Listeria monocytogenes,* and it can contribute to the cheese ripening process via action of its proteases and lipases. These benefits depend on the morphotype and strain used[24].

Geotrichum consumes lactate, but it rarely consumes sugars and has a lower salt-tolerance during its growth. It is known for proteolytic, peptiodolytic and lipolytic activities, and for the production of long-chain free fatty acids and fatty acids esters, which have a minor flavor importance in different cheeses. It grows well in pH of 2.5 to 8.1 and with low level of oxygen. Its excess can inhibit other microorganisms during the cheese ripening process; however, the low quantity of its spores causes a lower flavor complexity[25].

Among them, *Geotrichum candidum* and *Galactomyces geotrichum* (the teleomorph state or sexual form) are the most common and studied species for lipase production. Lipases from *Geotrichum candidum* are known for their high specificity for unsaturated long chain fatty acids and esters, while lipases from *Galactomyces geotrichum* present specificity for other esters of long chain fatty acids and good stability regarding temperature, pH and organic solvents. These properties allow their utilization in different reactions utilizing various vegetable oils[22, 25,27–29]. These microorganisms have been isolated from soil and plants[29,30], dairy products[31,32], rotten vegetables and fruits[33], different infections[34], and many other sources[35].

This review is focused on *Geotrichum* lipases and their biotechnological applicability. The aim is to discuss the latest aspects related to their production, purification, characterization, immobilization and application. The databases utilized for this purpose were mainly: Science Direct, Wiley Online Library, Springer, Scielo and Taylor & Francis Online, between January, 2014 and October, 2016.

Production of *Geotrichum* spp. lipases

Geotrichum sp. can be obtained from different natural sources and its cultivation can occur under the most variable conditions of: nutritional requirements (carbon, nitrogen and lipid sources), growth parameters (pH, temperature, agitation and aeration), inoculum conditions, and bioreactor system. Table 1 presents a list of a few recently published papers about lipases produced by *Geotrichum* species. *G. candidum* is the most studied amongst the genus in relation to lipase production, but other strains also are related to different processes such as *G. fragrans, G. siamensis* sp. nov., *G. phurueaensis* and *G. klebahnii* [36–38].

Different substrates have been studied lately in search for better lipase productivity and/or lower process costs[39–64]. Organic and inorganic nitrogen sources are frequently investigated for lipase production from *G. candidum* (Table 1). Peptone, yeast extracts and urea as organic nitrogen sources, and ammonium chloride, ammonium nitrate and sodium nitrate as inorganic nitrogen sources, are the most cited. The concentrations used vary greatly, but the range from 0.1 to 5.0 % w/w is more commonly used, and organic sources are added in higher quantities than inorganic sources[44–64]. Concentrations (% w/w) of: 5.0 of peptone; 2.0 of yeast extract and 0.1 of $NaNO_3$ and 0.5 of NH_4NO_3 are commonly applied in the lipase production[43–46,49,57]; however, optimized studies about medium composition reduced or eliminated some nitrogen sources in the process. Burket *et al*. and Maldonado *et al.* showed that 3.58 % w/w of peptone is enough to obtain a high level of lipase activity from *G. candidum*. They demon-

Table 1 – *Different conditions for Geotrichum spp. lipase production*

Microbial source	Growth conditions	Results*	Reference
G. candidum (DBM 4012, 4013 and 4166) and *G. ludwigii* (DBM 48)	Liquid inoculum / Urea and olive oil / shaken tubes / 24 h / 30 °C/Liquid inoculum / Peptone, glucose and olive oil / shaken tubes / 24 h, 30 °C	– Extracellular DBM 4166: 10 μmol FFA / 10^6 cells min^{-1} – Cell-bound DBM 48, 4012, 4166: ~3 μmol FFA / 10^6 cells min^{-1}	44
G. candidum 4013	Liquid inoculum / Peptone and glucose / shaken flasks / 30 °C / 24 h	– Extracellular: 9.76 ± 0.93 μmol *p*-NP min^{-1} g^{-1} of protein – Cell-bound: 0.083 ± 0.006 μmol *p*-NP min^{-1} g^{-1} of extract	45
G. candidum	Liquid inoculum / Peptone and yeast extract / shaken flasks, 150 rpm / 30 °C / 54 h	3.66 g mycelium-bound lipase L^{-1}; 22.59 mmol FFA min^{-1} g^{-1} dry mycelia and 510 mmol FFA min^{-1} g^{-1} protein	46
G. candidum (41 strains)	Peptone, glucose and olive oil / shaken flasks, 120 rpm / 30 °C / 48 h	– Extracellular: 16.250–131.250 μmol FFA min^{-1} mL^{-1} – Cell-bound: 2.500–40.000 μmol FFA min^{-1} mL^{-1}	47
Geotrichum sp.	Liquid inoculum / Olive oil, citric acid and NH_4NO_3 / shaken flasks, 250 rpm / 28 °C / 72 h	23.15 μmol FFA min^{-1} mL^{-1}	48
G. candidum	Liquid inoculum / Olive mill wastewater / bubble column and settler bioreactor / aeration of 0.5 vvm / pH 6.0 / 30 °C / 72 h.	Better production in the bubble column / 28 μmol FFA min^{-1} mL^{-1} (70 h)	51
G. candidum	Liquid inoculum / Peptone and soybean oil / airlift, 2.5 vvm, and stirred tank reactor, 300 rpm / pH = 7.0 / 30 °C	20.0 μmol FFA min^{-1} mL^{-1} (both bioreactors)	52
G. candidum NRRLY 552	Solid inoculum / Corn steep liquor and soybean oil / shaken flasks and stirred tank and airlift reactors, 250 rpm/ airlift, 1 vvm / pH = 7.0 / 30 °C	Maximum activities of: 21 μmol FFA min^{-1} mL^{-1} (optimized in shaken flasks – 48 h); 25.67 μmol FFA min^{-1} mL^{-1} (airlift – 64 h) and 16.9 μmol FFA min^{-1} mL^{-1} (stirred tank reactor – 37 h)	53
Geotrichum sp. and *G. candidum* NRRLY 552	Solid inoculum / Corn steep liquor and soybean oil / shaken flasks, 150 rpm / pH = 6.0 / 30 °C	– *Geotrichum* sp: 11.0 μmol FFA min^{-1} mL^{-1} – *G. candidum*: 17.0 μmol FFA min^{-1} mL^{-1}	54
G. candidum	Liquid inoculum / Ammonium, leucine and glucose / batch reactor, 850 rpm / 25 °C / aeration of 13 L h^{-1} / 185 h	Maximum specific growth rate of 0.21 h^{-1} around 27 h	55
Geotrichum sp.	Ammonium nitrate, corn steep liquor and soybean oil / shaken flasks, 120 rpm / 30 °C / 8.25 h	20.0 μmol FFA min^{-1} mL^{-1}	56
Geotrichum like strain R59	Urea and sucrose and treolin / shaken flasks / pH = 6.0 / 30 °C / 48 h	146 μmol FFA min^{-1} mL^{-1}	57
G. candidum	Yeast extract, peptone and olive oil / shaken flasks / 30 °C / 6 days	87.7 μmol *p*-NP min^{-1} mL^{-1}	58
G. candidum	Liquid inoculum / Yeast extract, peptone, glucose and palm olein / shaken flasks / pH = 7.2 / 30 °C / 54 h	7.02 mmol FFA min^{-1} g^{-1} dry cell	59
G. candidum NRRL Y-552	Solid inoculum / Yeast hydrolysate and soybean oil / shaken flasks, 250 rpm / airlift, 1 vvm / pH = 7.0 / 30 °C	– 24.3 μmol FFA min^{-1} mL^{-1} (optimized in shaken flasks – 48 h) – 19.32 μmol FFA min^{-1} mL^{-1} (airlift – 32 h)	60
G. candidum NRRLY 552	Solid inoculum / Peptone and soybean oil / stirred tank reactor, 250 rpm / pH = 7.0 / 30 °C	16.0 μmol FFA min^{-1} mL^{-1}	61
Geotrichum sp.	Solid inoculum / Corn steep liquor and soybean oil / shaken flasks, 150 rpm / pH 5.0	35.2 μmol FFA min^{-1} mL^{-1}	62
G. candidum penicillatum M2	Liquid inoculum / Yeast extract (10 g L^{-1}), sunflower oil refining waste (10 mL L^{-1}) and minerals / shaken flasks, 170 rpm	Best cultivation after 48 h with a final pH of 6.0 which resulted in 0.6 μmol FFA min^{-1} mL^{-1} after medium centrifugation	63
G. candidum	Liquid inoculum/ Soybean molasses (32–368 g L^{-1})/ shaken flasks / pH (2.16 –3.84) / 21.6 – 38.4 °C / 130 rpm	12.0 U mL^{-1} (optimized condition of pH = 3.0, 30 °C and 200 g L^{-1} of substrate	64

*FFA = free fatty acid; *p*-NP = *p*-nitrophenyl

strated that sodium nitrate did not have a significant effect on the lipase activity[52,61]. Ginalska *et al.* showed that 0.4 % w/w of urea was the best condition of nitrogen source for lipase production compared to other organic sources. They also showed that the use of only inorganic nitrogen sources had an inhibitory effect on lipase activity[57].

The cost of these substrates is relatively high, and several studies have been conducted to find ways to replace these nitrogen sources with other cheaper sources. Corn steep liquor, yeast hydrolysate and soybean molasses are cited as good nitrogen sources due to their high concentration of nitrogen and amino acids. They have been used to replace the traditional nitrogen sources, maintaining or increasing lipase activity from *Geotrichum*. The used quantity of these sources varies greatly (3.0 – 15.0 % w/w) according to the substrate and fermentation conditions[53,54,56,60,62,64]. These agro-industrial residues present a certain quantity of salts and their use eliminated the necessity of supplementing the medium with inorganic salts.

The addition of lipids substrates is a known strategy to increase the lipase activity for different microorganisms. Long-chain fatty acids have been used as inductors for lipase production from *Geotrichum.* Shimada *et al.*, 1992, demonstrated that the use of unsaturated fatty acids (especially C 18:1, 18:2 and 18:3) results in high level of lipase activity in comparison to other fatty acids[43]. Several other studies had successful results utilizing vegetable lipids sources as inductors for lipase production from *G. candidum*. Some examples are babassu, cottonseed, fish, olive, soybean oils; almond, coconut, ginger, groundnut *Jatropha*, niger seed oil cakes, palm and sunflower effluents, and many others[39–64]. Olive oil contains a high concentration of C18:1 unsaturated fatty acid, and innumerous studies indicate the use of this oil as an inductor for lipase production[44,45,47–49,51,57,56]. Soybean oil also shows similar results due to its similar composition to olive oil, and its low cost is a strong appeal for its application. Concentrations of 1.0 % w/w are frequently utilized, but optimized studies showed that lower concentrations (0.5 to 0.7 % w/w) are enough to induce the lipase production, and concentrations above 1.0 % w/w do not have significant effects on the increase in lipase activity[52–54,56,60–62].

Glucose is the most used carbon source for the growth of *G. candidum* and the utilized concentration is usually 1.0 % w/w. Other carbon sources can be used, such as sucrose, galactose, lactose or lactic acid[44]. A lipid source can be a carbon source and inductor simultaneously. In fact, glucose contributes to cellular growth but it can also reduce the lipase activity, as verified by Burkert *et al.*[56].

The inoculum is another important variable in the lipase production from *Geotrichum,* and the liquid form[45,46], used at 10 % (v/v)[47] or 5 % (v/v)[48], is frequently applied. However, this type of inoculum can be an obstacle to the homogenization and standardization of inoculum, as shown by Resende-Maldonado *et al.*[49] The authors proposed the utilization of an initial solid inoculum (with a specific area) followed by an intermediate liquid inoculum that reduced the variability of the lipase activity by 33 % during the fermentation. In another study, the use of intermediate liquid inoculum doubled the level of lipase activity compared to the use of the spore solution. The reduction in size of the inoculum by 10 to 2 % v/v also increased the maximum lipase activity[59]. This technique was applied with success in other studies with *G. candidum* and *G.* sp[52–54, 60–62] and it was verified that the lower quantity of solid inoculum increases the lipase activity. The reduction in the quantity of inoculum probably reduced the crowding effect (a typical mycological phenomenon that causes self-inhibition in spore fungus germination under crowded conditions). A similar technique for sampling solid cultures of *G. candidum* and *P. camembertii* was applied by Aldarf *et al.*[50]

In relation to the bioreactor choice, several studies have been conducted in shaken flasks (Table 1), but other types of bioreactors have also been applied successfully for *Geotrichum* cultivation; in general, the scale up from shaken flasks to a bioreactor system requires optimized cultivation conditions that can be achieved with the factorial designs methods[49,51–54,65]. The air lift bioreactor was successfully applied for lipase production from *Geotrichum* because the absence of mechanical agitation reduces the damage in the mycelium and provides better conditions for obtaining high lipase activity[52,53,60]. The use of a low rate of aeration also contributes to increasing lipase activity since *G. candidum* grows well with low levels of oxygen[25]. Studies in stirred tank bioreactor indicated that aeration of 1.0 vvm or lower are the best conditions for lipase production from *Geotrichum candidum*[51–53,60]. The low level of oxygen is also efficient in shaken flasks; several studies have indicated agitation conditions below 200 rpm for lipase production from *Geotrichum*, which reduces the quantity of dissolved oxygen[46,47,54,56,62–64].

Geotrichum is a mesophilic yeast and most studies have shown that temperatures near 30 °C are more appropriate for its growth and lipase production. The optimum pH for lipase production for this genus is around 7.0, but some species are able to produce lipases in more acidic[62,64] or in more alkyne mediums[42]. In general, the pH increases during the fermentation time and at the end of fermentation it is possible to observe a great decrease

in lipase levels, mostly due to the pH increase (above 8.0) or proteases activities[53–56,60–62].

Lipases can be produced intra- (cell-bound) and/or extracellularly, as described by several authors[41,44,46,48,66]. The level of lipase activity and selectivity varies according to the location, strain and fermentation conditions. Hlavsová *et al.*[41] and Loo *et al.*[46] used a similar fermentation medium to produce cell-bound lipases, but in the first study the cell-bound lipase showed high selectivity for saturated fatty acids, while in the second study the highest selectivity was observed for polyunsaturated fatty acids, probably due to the difference in the strains used. In general, intracellular lipase production takes place in the initial hours of cultivation, followed by its release into the culture medium during the stationary phase when the highest levels of lipase are obtained[41,66], although the duration of the stationary phase can vary greatly and the maximum lipase activity can occur from 24 to 96 hours, depending on fermentation conditions[44,59].

Genetic engineering is very powerful and helpful in improving the enzyme expression. There are two ways to achieve this goal: *Geotrichum* species can be mutated to produce higher levels of lipases or *Geotrichum* genes for lipases can be expressed in bacteria. According to Cao *et al.*[67] an increase of 53 % in biomass and an increase of 158 % in lipid yield were obtained with the cultivation of a mutated strain of *G. robustum*. Mughal *et al.*[65] obtained a lipase activity almost 24 times higher after inducing the mutagenesis of *G. candidum*. Yan *et al.*[68] expressed *G. candidum* Y162 lipase genes in *Pichia pastoris* and obtained 55 U mL^{-1} of activity, and Pan *et al.*[69], by expressing *Geotrichum* sp. lipase genes also in *P. pastoris*, obtained (273 ± 2.4) μmol FFA min^{-1} g^{-1} of dry cells. In addition, *Galactomyces geotrichum* lipases have also been successfully expressed, mostly in *Picchia pastoris*[28,70]. In all these examples, the lipase activity or lipid contents in the clones or mutants of *Geotrichum* were much higher compared to original strains, indicating that these techniques can used successfully in industrial applications that need a high level of lipases activity. Furthermore, the clones or mutants were able to produce lipases that are more stable in high pH (above 8.0), high temperature (above 45 °C), and in different low polarity organic solvents, which normally does not occur with original strains of *Geotrichum*[28].

Lipase activity can be measured differently. The most common definition of U (unit of lipase activity) is the amount of enzyme capable of releasing 1 μmol of free fatty acids (FFA) per minute[71] and its variations, such as, the amount of enzyme capable of releasing 1 mmol of FFA per gram of dry cells[46] or the amount of enzyme that releases 1 μeq of FFA per min per mg of dry cell[72,73]. Another definition applied is the amount of enzyme capable of releasing 1 μmol of *p*-nitrophenol (*p*-NP) per minute[45]. When comparing results from different authors, it is crucial to pay attention to these peculiarities. According to Table 1, the obtained range of lipase activities is wide, since the methods for cultivation and the methods for lipase activity determination also widely vary.

Purification and characterization of *Geotrichum* spp. lipases

Crude lipases preparations from different *Geotrichum* strains have been studied and characterized throughout the years. Different degrees of specificity, in relation to type and positions of fatty acids in triacylglycerols and fatty esters, different molecular weights, stability, and biochemical properties have been observed[74–77]. Innumerous methods for purification and characterization of these enzymes have also been applied, as presented in Table 2. The simple concentration (partial purification) applied mostly for extracellular lipases has been conducted by precipitation with acetone[72], ethanol[77] and ammonium sulphate[79], ultrafiltration[83], extraction by aqueous two-phase system[84] and many other methods.

Among the precipitating agents, ammonium sulphate has been the most used[45,58,78,79,86,88]. Partial purification with this salt is easy to apply and results in high purification factors (in average 87 % of recovery factor[88]); including situations with enzyme hyperactivation[78], which is very interesting for improving the recovery factors in the subsequent purification process. On the other hand, the total time of this method is long, since a long time is required for complete enzyme precipitation, and a dialysis step is necessary to remove the excess salt from the precipitate[78].

In order to reduce the pre-purification time, other substances such as acetone[45,63], ethanol[78] and trichloroacetic acid[80] were also applied. In these cases, pre-purifications were conducted with cold solvent to prevent denaturation of the lipases since most of these enzymes were not stable in the presence of organic solvents. Acetone has been cited as a good option to wash and activate whole cell lipases[41,45], improving the catalytic activity. Ethanol resulted in a high concentration factor (64 – 66) with short time processes (1–2 h) [77,78], but the recovery factor was lower compared to ammonium sulphate precipitation because the lipases (in general) have low stability in contact with ethanol[78].

Specific purification steps can be achieved by chromatographic techniques such as: ion exchange[58,63,70,72,75,77,79,83,86,88], hydrophobic interac-

Table 2 – *Purification and characterization of Geotrichum lipases*

Microbial source	Purification steps	Characterization*	Reference
G. candidum 4013	Acetone precipitation for the cell-bound lipase; filtration and lyophylization for the extracellular lipase	– Cell-bound: opt. = 35 °C and pH 9.0; low selectivity to the PUFA; – Extracellular: opt. = 35 °C and pH 8.5; higher selectivity	41
G. candidum 4013	Ammonium sulphate precipitation (70 %) for the extracellular lipase, acetone for the cell-bound lipase and acetone and diethylether for the released lipase (cell-bound)	– Extracellular lipase: preference for *p*-NP decanoate (C10) and saturated triacylglycerols with C12–C18 and higher specificity with C18:2 and short-chain esters; (V_{max}/K_M) = 0.015 with *p*-NP C16 – Cell-bound lipase: preference for *p*-NP palmitate (C16) and triacylglycerols with acylgroup chain lengths between C13-C16 and short-chain esters; (V_{max}/K_M) = 0.085 – Released cell-bound lipase: (V_{max}/K_M) = 0.117	45
G. candidum	Filtration to remove mycelium and ammonium sulphate precipitation (70 %) followed by anion exchange and two gel filtration chromatographyc steps / Purification:1.03-fold (precipitation), 4.36-fold (anion exchange), 5.74-fold and 7.76-fold (gel filtration)	32 kDa; opt. = 40 °C and pH 7.0; ions Ca and Ba enhanced activity; good stability at 30 °C / 24 h at pH of 6.5 to 8.5	58
G. candidum penicillatum M2	Acetone precipitation (4:1 = acetone:medium) / Ion exchange chromatography and hydrophobic-interactive chromatography / Purification factors of 1, 1.15 and 11.27 and yields (%) of 100, 96 and 48, respectively	Good stability at 30–80 °C; maximum activity at 50 °C and pH 9.0;	63
Geotrichum-like strain R59	Centrifugation to remove mycelium	Opt. = pH 7.0 (with decreasing activity past pH 8.0) and at 37 °C and 50 °C (suggesting multiple forms); good stability at 60 °C and at pH 6.5–8.0	71
G. candidum (4012, 4013, 4166) and *G. ludwigii* (48)	Extracellular lipase: filtration Cell-bound lipase: filtrated cells were resuspended in buffer	It was observed a great influence of medium composition growth on the enzymes. In the blackcurrant oil hydrolysis, the enzymes from 4012 and 4013 induced in medium A and 4166 and 4013 induced in medium B released unsaturated fatty acids.	76
G. candidum NRRLY-552	Ammonium sulphate (80 g 100 mL^{-1}) or ethanol (70 mL 100 mL^{-1}) precipitation; with or without lyophilization / The highest recovery factors (139 %) were obtained with ethanol precipitation and lyophilization (LE-l) / The highest concentration factors (64.6) were obtained with the salt precipitation and lyophilization (LS-l).	At 37 °C, the lipases LS-l and LE-l presented reductions of 20 % and 15 % of its initial activities, respectively, after 6 h incubated in ethanol. In hexane, the reductions were around 40 % in the first 12 h.	78
Geotrichum sp. SYBC WU-3	Ammonium sulphate precipitation (30–60 %) followed by ion exchange and gel filtration chromatography / Recovery of 11.2 % (LipA) and 9.2 % (LipB)	– Lip A: 41.1 kDa; opt. = 20 °C and pH 9.5; preference for C2 to C16; – Lip B: 35.8 kDa; opt.= 15 °C and pH 9.5; preference for C2 and C4;	79
Geotrichum sp. lipase expressed in *S. cerevisiae*	Surface displayed: cells were harvested, washed and resuspended; Secretory lipase: purification by trichloro-acetic acid (TCA) precipitation at 10 %	– Surface displayed lipase: opt. = 40 °C and pH 8.5; maintained 89 % of its original activity at 40 °C/pH 8.5 / 3 h; preference for *p*-NP caprylate (C8); – Secretory lipase: opt. = 35 °C and pH 8.5; maintained 48 % of its activity at 40 °C / pH 8.5 / 3 h; preference for *p*-NP caprylate (C8)	80
G. candidum 4013	Purification from the cell-free medium by matrixes with different hybrophobicity and desorption with Triton X-100 / Purification factors of 24.2 (Lip1), 6.1 (Lip2) and 1.2 (Lip3)	– Lip1: 70.5 kDa; preference for *p*-NP laurate (C12); – Lip2: 75.5 kDa; preference for *p*-NP caprylato (C8); – Lip3: 43 kDa; higher preference for C8 and ability for hydrolysis of racemic *trans*-2-(4-methoxybenzyl)-1-cyclohexyl acetate	81
G. marinum ATCC 20614	Affinity and gel-filtration chromatography / Purification factor of 56 and 76, and yield (%) of 66 % and 46 %, respectively	62 kDa; opt. = 40 °C and pH 8.0; K_M = 11.5 mmol L^{-1} and V_{max} = 1000 μmol min^{-1} mg^{-1} protein; preference for *cis* double bonds, like trilinolenin (18:3*cis*-9,12,15)	82

*K_M: Michaelis-Menten constant; V_{max}: Michaelis-Menten maximum velocity; *p*-NP = *p*-nitrophenyl; PUFA = polyunsaturated fatty acids

tion[63,72,85,97], gel filtration[58,79,82,86], metal-affinity[74,87], etc. Many other techniques are possible for lipase purification[88].

Ion exchange is the most applied technique in purifying enzymes, and it is also applied to lipase purification as mentioned before. This type of purification is fast, easy to perform, requires low volume of sample solution, and is used for many other compounds. For lipase purification, ion exchange presents varied results for recovery factors (5 – 80 %), and for purification factor (1.1 – 33 fold) [58,63,70,72,75,77,79,83,86].

Hydrophobic interaction chromatography is another interesting method for lipase purification since the lipases have good affinity to hydrophobic compounds. The recovery factors are normally high (48 – 96 %), and under optimized conditions high purification factors can be obtained, such as 86.7-fold, which was achieved under optimized conditions for purification lipase produced using yeast hydrolysate[97].

Several studies have shown the use of more than one purification technique in order to obtain high levels of purify. Ammonium sulphate precipitation combined with ion exchange and gel filtration resulted in a 7.76-fold purification[58] and 11.2 % of recovery factor[79]. A purification factor of 11.3 and 48 % recovery were achieved with combined purification using acetone precipitation, ion exchange and hydrophobic interaction chromatography[63]. In another study, it was possible to obtain relevant data regarding a large-scale purification of a 61.6 kDa lipase from *G. candidum* with two consecutive chromatographic steps: ion exchange and hydrophobic interactions, resulting in a purification factor of 13.2 and a specific activity of 1.052 μmol FFA min^{-1} mg^{-1} of protein[89]. Examples of combined methods have shown that a high level of purity can be obtained, but the recovery factors reduce with the increase in the number of purification steps due to the application of sequential methods improving enzymatic denaturation. The choice of the type and sequence of purification steps depends greatly on the properties of lipases and the potential applications for these enzymes.

Aspects related to optimum pH and temperature, stability, molecular weight, etc., vary according to the strain investigated and the medium composition applied. The characteristics are quite different from one lipase to another, but a few similarities can be observed among them. Literature cites that most of the values for optimum pH and temperature for lipase from *Geotrichum* vary between 6.0–7.0 and 20–40 °C, respectively[28]. Some studies indicate the optimum pH and/or optimum temperature is within these ranges[58,72,77,78,86,90,95,97], however different results were obtained with modifications in several relevant factors for lipase production and purification, as shown in Table 2.

Genetic modifications, lipase location (cell-bound or extracellular lipase), use of new strains and modifications in fermentation medium, etc., can cause drastic changes in lipase properties. A clone containing genes from *Galactomyces geotrichum* (a species less studied within the genus *Geotrichum*) produced high levels of lipase activity with high optimum pH (8.0), high stability pH (until pH = 10.0), and high optimum temperature (50 °C)[28]. The cell-bound lipase showed higher activity and higher stability than the extracellular lipase from the same strain in pH = 8.5[41] and the range of pH stability was wider (5.2–9.2) for mycelium lipase than extracellular lipase (7.2–9.2)[90]. A new strain of *Geotrichum* sp. produced two new cold-adapted lipases, which showed optimum temperatures between 15–20 °C despite the cultivation conditions at 30 °C[79]. A lipase with high optimum temperature (47 °C) was obtained from *Geotrichum candidum* NRRL-Y-552 when clarified corn steep liquor was used as nitrogen source in fermentation medium[97]. The same strain exhibited an optimum temperature 10 °C lower when the microorganism was cultivated with yeast hydrolysate[97] or peptone[52,61].

According to Sharma *et al.*[4], most lipases obey Michaelis–Menten kinetics, and that is also valid for *Geotrichum* lipases as demonstrated in other studies[45,82,97] for different strains and cultivation conditions. It has also been stated that *Geotrichum* lipases can present even four types of enzymes with different molecular weight and substrate specificities[91], but Baillargeon and McCarthy[92], working with *G. candidum* NRRL Y-553, were able to obtain five glycosylated lipases with pI from 4.88 to 4.78 and with molecular weight also varying from 64 to 57 kDa. The isoelectric points of lipase from *Geotrichum* does not change much as do other parameters, and the results are normally in the range from 4.0 to 5.0[77,83,86,89,92].

Geotrichum lipases are frequently cited by their substrate specificity for fatty acids having at least one *cis*-Δ9 double bond[70,74,89,90,92,94], but different catalytic specificities are found in different lipases from this genus[93]. A few examples are shown in Table 2, and it is important to highlight that some lipases from *Geotrichum* showed high specificity for saturated fatty acids[41,74,76,90]. This characteristic can be related to one of the most important habitats of this genus, which is dairy products containing a significant quantity of saturated fatty acids. Differences in the specificity were mentioned for the position of the chain in the tryacilglycerol. There are three types of lipases – 1,3-position[72,76], 2-position[91] and non-position specifics[72,75,90]. Sometimes the same microorganism produces different isolipases with different position specificities[72]. This is a great

advantage because it allows application on different substrates.

Other examples can be cited to illustrate different possibilities regarding lipase properties: two lipases from *G. candidum* ATCC 66592[94] presented different velocities of hydrolysis of palmitic acid methyl ester, the 61 kDa lipase revealed a higher initial velocity compared to the 57 kDa lipase. An extracellular *Galactomyces geotrichum* lipase, with 57 kDa in the unglycosylate state and 62 kDa as glycosylated, showed a similar specificity to *G. candidum* lipase but a different amino acid composition[95].

Expressed *Galactomyces geotrichum* lipases have been also characterized, although less frequently than *Geotrichum* lipases. For example, Fernández *et al.*[70] purified 59 kDa lipase from *G. geotrichum* BT107 expressed in *P. pastoris* LF163 by ultrafiltration and anion exchange chromatography, which yielded 97.5 % and 42 %, respectively. Also, Yan *et al.*[28], by expressing a 64 kDa lipase from *G. geotrichum* Y05 in *P. pastoris* GS115 and pPIC9K, obtained 1.22-fold (ammonium sulphate precipitation), 2.8-fold (anion exchange chromatography), and 3.2-fold (gel filtration chromatography) purification. Additionally, Bertolini *et al.*[96] expressed *G. candidum* lipases I and II in *S. cerevisiae,* and significant differences between them were observed as lipase I presented much more affinity to long fatty acyl chains substrates than lipase II.

Immobilization of *Geotrichum* spp. lipases

Lipase has been successfully immobilized with different techniques[98–101] and the same rules for general enzyme immobilization are also applied for *Geotrichum* lipase immobilization. It is common sense among researchers that there is no 100 % perfect technique for it. All techniques will always present some disadvantages as well as advantages. A few examples of how *Geotrichum* lipases have been immobilized over the last years are presented in Table 3, and a few others examples will also be cited.

The entrapment of cells, instead of enzymes, seems to be a simple technique with good results for *Geotrichum* lipases. Pan *et al.*[106] immobilized *Geotrichum* sp. G38 cells in silicon granules and obtained the same pH profile for both forms, free and immobilized, but an increase of 5 °C in optimum temperature after immobilization was observed. In addition, the glucose supplementation into the medium reaction – for the cells maintenance – contributed to increasing cycles of use from 23 to 28 with 90 % activity. Bleve *et al.*[107] immobilized *G. candidum* cells in Ca-alginate beads, and Carballeira *et al.*[108] tested 6 different polymeric matrixes. In both cases, they were focused on other enzymes not lipases; Nakamura *et al.*[109] also immobilized *G. candidum* IFO 4597 cells, but by adsorption onto the surface of a water-absorbing polymer (BL-100®), and applied it for stereo-selective oxidation and reduction in organic solvent.

Immobilization by adsorption is a simple technique with good results and is considered the most applied enzymatic immobilization technique[110] even in combination with other techniques[111]. Two purified *Geotrichum* CMICC 335426 lipases, for example, were successfully adsorbed on hydrophobic macroporous polypropylene particles (Accurel EPI00) precoated with ovalbumin by Charton and Macrae[112] and, according to their results, immobilization and the use of an organic media (tri-*n*-butylphosphate) did not have a major effect on the specificities of lipases A and B. However, for the immobilized lipase B, the hydrolysis of 20 % of triolein presented a v_{max} value 357 times lower than the free lipase. The authors attributed this fact, among other reasons, to inactivation by the solvent.

Entrapment and adsorption can be simple techniques but they can also result in an undesirable leakage. For that purpose, the use of a stronger force, such as an ionic or covalent bond, can be of great help. As a brief example, Matsuda *et al*.[113] immobilized cells of *G. candidum* NBRC 5767 onto an ion exchange resin in order to use its alcohol dehydrogenase to reduce ketones.

Silica gel is a very common support for enzyme immobilization. It was used by Sonnet *et al.*[114] to adsorb a commercial *G. candidum* lipase preparation. Bhattacharyya *et al.*[115] also adsorbed carbonyl reductase from *G. candidum* NCIM 980 in silica gel, followed by cross-linking with glutaraldehyde for the immobilization. Several other materials, such as agriculture wastes[116], membranes[117] and ceramic materials[118] have been proposed as a support for lipase immobilization in general, and the decision for the right support and technique should only be based on the process characteristics.

When an enzyme is produced attached to the cell membrane it is possible to consider that it is already immobilized, using the cell as its natural support[44,48,69,76,80,119]. In cases like these, the biomass can be easily removed from the reaction medium and used again in a new medium, avoiding several enzyme purification downstream steps.

Applications of *Geotrichum* lipases

In general, *Geotrichum* lipases have the same broad applicability like any other lipase, since this group of enzymes is the most investigated and in-

Table 3 – *Immobilization of Geotrichum lipases*

Microbial source	Technique / Matrix	Results*	Reference
G. candidum 4013	Entrapment of cell bound lipase in calcium alginate beads.	Better composition of free fatty acids from the hydrolysis of blackcurrant oil and higher activity at SC-CO_2 than obtained with the lyophilized extracellular and the precipitated cell-bound lipases.	41
G. candidum strain NRRL Y-552	The filtered enzymatic solution was immobilized onto mesoporous PHB particles under continuous agitation (200 rpm) under room temperature for about 12 h.	The best immobilization yield (81.2 %) was obtained offering 1031.1 μmol FFA min^{-1} mL^{-1} of initial enzymatic activity and the resultant biocatalyst presented a hydrolytic activity of 284.3 μmol FFA min^{-1} g^{-1} of support. The immobilized enzyme presented, at pH 8.0, a maximum activity (51.3 μmol FFA min^{-1} g^{-1} of support) 1.5 times higher than the free enzyme. In relation to the optimum temperatures, the immobilization increased in 3 °C in relation to the free enzyme (37 °C).	42
G. candidum NRRLY-552	Entrapment in alginate beads, adsorption onto niobium-graphite particles, celite and zeolites.	Immobilization yield (%) / immobilized activity (μmol FFA min^{-1} mL^{-1}): 65.7 / 14.8 (zeolite), 49.0 / 4.9 (niobium-graphite), 31.8 / 23.8 (alginate) and 20.0 / 93.4 (celite).	78
Geotrichum sp.	Immobilization of the purified lipase onto NKA resin was conducted in four different medium: phosphate buffer (pH = 8.0) (IMLA); the same aqueous buffer added of octane (IMLAO), fish oil (IMLAF) and oleic acid (IMLAOA). The IMLAOA was frozen and lyophilized, the others were just filtered.	It was observed that the IMLAF and IMLAOA exhibited the highest fish oil hydrolysis degrees (around 35 and 40 %, respectively) in relation to the free lipase (around 10 %) and also a better thermal stability, with 60 % of the relative hydrolysis degree at 45–55 °C. In addition, the two biocatalysts showed similar operational stability, remaining 80 % of its relative hydrolysis degree after five cycles of repeated use.	102
Geotrichum sp. lipase gene expressed in *Pichia pastoris* GS115	The purified lipase powder aggregates were immobilized by cross-linking with glutaraldehyde with organic solvents (CLEA) and with polyethylenimine (PEI- CLEA).	Both techniques, CLEA and PEI-CLEA, avoided the pellet agglomeration and resulted in 33 and 42 % of hydrolysis degree, respectively, contrasting to the 12 % degree with the free enzyme. The PEI-CLEA biocatalyst also presented more thermal stability, better tolerance towards strong polar and non-polar organic solvents, no leakage of enzyme and better reusability after retaining 72 % of its relative hydrolysis degree after 5 cycles of use.	103
Geotrichum sp.	– PCMC methodology: lipase was dehydrated (in organic medium) on the surface of micro-crystals of K_2SO_4, coating them; – CLPCMC methodology: the obtained crystals described above were cross-linked with glutaraldehyde.	PCMC and CLPCMC presented a biodiesel yield of 69 and 72 % respectively; free lipase resulted in 29 %. CLPCMC also presented higher maintenance of activity after 5 successive batch reactions (80 %), better stability in pH from 4.0 to 6.0 and in temperatures from 45 to 50 °C.	104
Geotrichum sp. lipase gene expressed in *Pichia pastoris* GS115	The purified lipase was bioimprinted, pH tunned, salt activated, lecithin coated and adsorbed onto NKA, a macroporous resin.	The biocatalyst obtained presented a 18.4-fold enhancement in its sterification activity towards metyloleate synthesis, a highly improved operational stability compared to the crude form and after 10 cycles of use it was capable of retaining 90 % of its initial activity.	105

*FFA = free fatty acid

dustrially applied. Apparently, the majority of the *Geotrichum* lipases have a special characteristic that allows their utilization in different vegetable oil. They are very selective towards unsaturated long-chain fatty acids with *cis*-9 double bonds, such as oleic and linoleic acids, as mentioned before. Other applications can also be cited for these enzymes, as follows.

Polyunsaturated fatty acids enrichment

Polyunsaturated fatty acids (PUFA) are very important for different physiological functions[120], thus, their enrichment in medium is of great interest and *Geotrichum* lipases can be perfectly applied for that purpose. Pan *et al.*[69], when studying the enrichment of eicosapentaenoic (EPA) and docosahexae-

noic (DHA) acids contents from fish oil, using a *Geotrichum* sp. lipase displayed on the *P. pastoris* wall, obtained an increase of about 1.2-fold for each acid, reaching 46.62 % of total yield, for both. The best hydrolysis degree in fish oil for producing EPA and DHA was 40 % in both cases, using immobilized lipase from *Geotrichum* sp.[102] These results were about 2 and 4 times higher than with the use of free enzyme. In addition, γ-linolenic acid (GLA) was successfully enriched up to 40 % in the acylglycerol fraction, when Fregolente *et al.*[29] applied an enzymatic extract of *G. candidum* in borage oil, a 1.9-fold enrichment. Fogliaand Sonnet[121], with silica immobilized and free lipases from *G. candidum*, resulted in up to 70 % recovery of the same acid, GLA.

Conjugated linoleic acid enrichment

Conjugated linoleic acid (CLA) are C18 fatty acids mainly composed of *cis*-9, *trans*-11 isomers and are considered anti-carcinogenic, among other benefits, and are naturally found in many natural food sources, especially animal (ruminant) products[122]. A commercial *G. candidum* lipase preparation was applied by McNeil *et al.*[123], with safflower oil, in order to enrich the composition of the *cis*-9, *trans*-11 and also the *trans*–10, *cis*-12 isomers. According to the authors, obtained was a maximum fraction of about 98 % (between 13–35 % of conversion) of the *cis*-9, *trans*-11 in a much higher velocity. The same commercial enzyme preparation was also applied by Haas *et al.*[124] besides the recombinant *P. pastoris* with the *cis*-9 selective B lipase gene of *G. candidum* CMICC 335426 expressed; both enzymes demonstrated a highly selective production of the *cis*-9 isomer with compositions of 77 and 94 %, respectively.

Lipid hydrolysis

Lipases are capable of catalyzing both hydrolysis and synthesis of esters, and their specificities vary from strain to strain as much as their hydrolytic yield in di- and monoacylglycerol, glycerol and free fatty acids. The hydrolysis of triacylglycerols of black currant oil was evaluated for three different strains of *G. candidum* and one of *G. ludwigii* by Stránský *et al.*[76] and it was observed that the medium in which they were induced presented a great influence on the profile of the obtained hydrolyzed fatty acids. Diks and Lee[125] evaluated the selective hydrolysis of sunflower oil by the wild *G. candidum* CMICC 335426 lipase B and the one expressed by *P. pastoris* aiming at the production of a very slightly saturated fatty acid oil in which obtained degrees of hydrolysis of around 60 % (w/w) with more than 99 % (w/w) of unsaturated compounds in free fatty acid fraction, using sunflower oil for the hydrolysis. Other examples of *Geotrichum* lipases application in hydrolysis are cited briefly in Table 2 as Brabcová *et al.*[45,81] and Cai *et al.*[79]

Synthesis of aroma compounds

Lipids are an important source of aroma compounds[126] and several *Geotrichum* strains are responsible, for example, for the characteristic aroma/flavor of different cheese, not exclusively because of their lipases but also other enzymes produced during the ripening of cheeses[127–129]. In addition, according to Neto *et al.*[130], hydrolyzed castor oil and its fatty acid derivatives proved to be effective precursors of γ-decalactone, responsible for a fruit/chocolate aroma by the lipases from *G. fragrans* and *Geotrichum* sp. with the first lipase resulting in a better production (600 mg L^{-1}). Macedo and Pastore[131] evaluated, among others, a lipase from *Geotrichum* sp. in order to produce aromatic esters, and this lipase was able to esterify all the substrates tested, resulting in, for example, 80 % of esterification with butanol and acetic acid. Recently, Vong and Liu[132] evaluated different strains of yeasts to reduce the undesirable flavor of okara (a soybean residue obtained during the production of soymilk). During the fermentation of okara using the *G. candidum,* these authors observed a significant reduction in hexanal and *trans*-2-hexane, which were converted mainly into 2-propanol and pentanoic acid.

Conclusions

According to this review, it is possible to see that there are different aspects regarding production, purification, immobilization, and application of lipase from *Geotrichum* genus. Production is carried out to obtain extracellular lipases (mainly) and cell bond lipases using different substrates, operating conditions and bioreactors. Most studies produce lipases using submerged fermentation, and in recent years many alternative nitrogen sources have been explored in order to reduce the cost of production. Organic nitrogen sources, lipids for the induction and as carbon source, and glucose for growth are the most typically applied substrates for lipase production from *Geotrichum*. The application of different substrates and conditions allow production of lipases with different biochemical characteristics. In addition, genetic modifications have been evaluated to increase the lipase production using different species of *Geotrichum* or by expressing their genes in other species like *P. pastoris* and *S. cerevisiae*, for example.

Purification has been studied through different techniques, such as precipitation using salt solutions or organic solvents, ultrafiltration, and different chromatographic techniques, especially employing hydrophobic systems. A great number of techniques

have also been used to immobilize lipases from *Geotrichum* genus, especially adsorption in hydrophobic supports, entrapment using different supports, and in its own cell (natural support). Production, purification and immobilization processes can change the characteristics of lipases from *Geotrichum* drastically, however, most of the studies have shown that the lipases from this genus are good biocatalysts in pH near 7.0 and temperature near 30 °C. These characteristics can be changed with genetic modifications, use of different strain, and different fermentation conditions.

Finally, different applications have been mentioned in the literature about the use of lipases from *Geotrichum*. The most important characteristic of lipases from this genus is the specificity from unsaturated long-chain fatty acids with *cis*-9 double bonds, extensively studied for a long time. Furthermore, other applications are frequently cited, such as enrichment of both polyunsatured fatty acids (PUFA) and conjugated linoleic acids, hydrolysis of lipids, and synthesis of aromas. All this information is of great value to increase the potential for *Geotrichum* lipases applicability.

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