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Lipolytic Enzymes and Their Use in the Production of Human and Animal Biotechnology

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Abstract

Lipases are omnipresent in nature and they act as catalysts for hydrolysis reactions of triglycerides, or synthesis of esters from fatty acids and glycerol. Although they are differentiated by their origins and properties, these enzymes have been highlighted in several industrial sectors, from food products, textiles, cosmetics and the formation of diagnostic tools. Most lipases need a "key" that gives access to its active site, as well as something that stabilizes the molecule when it undergoes activation. Nevertheless, few studies are available to designate and classify the genetic sequence of lipases obtained from producing microorganisms. In this context, this literature review aims to search for the molecular determination, through gene expression and registering of eukaryotic lipases *in silico* to make the enzyme employment as an economic alternative for the production of specific and feasible alternatives for industrial needs. The production and thermostability's importance of some microbial enzymes are also approached.

Keywords: Lipolytic enzyme; Characterization; Applications

Introduction

Microorganisms, as bacteria and fungi, have remarkable ease of nutrition and cultivation, high rates of growth and production, as well as a variety of bioactive compounds and greater stability in enzymatic molecules, allowing bioengineering for the production of new bioproducts. When comparing microbial enzymes with animal and vegetable enzymes, the former exhibit properties that determine their preference in the most diverse applications [1-8].

Among the microorganisms, fungi are distinguished by the production of extracellular enzymes, which facilitates the separation of the substance produced from the medium [2,3,9-16]. In each environment, the determinant for the performance of its metabolic processes will be the sequence of nucleic acids expressed by each microorganism, which when translated into proteins determines its environmental need.

Each factor, such as carbon and nitrogen sources, pH and temperature, is variable and specific for each microorganism determining the activity of the proteins produced. In general, the preparation of optically active compounds has been a major challenge for biochemists and chemical-organic, due to the increasing need for thermostable substances [17,18] for use in the various industrial sectors.

Among these substances, the lipase enzyme stands out in the industries of its most varied sectors, from food products, textiles, cosmetics and the formation of diagnostic resources, acting as enzymatic markers [1]. Lipases are classified in the superfamily of α/β hydrolase and have as an example of sister enzymes esterases, proteases, peroxidases, lyases, among other [19].

Lipases are ubiquitous in industrial sectors and constitute the most important group of biocatalysts in biotechnological applications [1]. In researches of mutagenic lipolytic determination there is a search for ways to improve the protein sequence to determine functions different from those previously expressed [8,20-23]. Thus, the effect of pH, temperature, metal ions and substrate are specific in the bioproduction of any substance of high reactivity, not different with lipases.

It is possible to produce high amounts of optically active and improved enzymes for industrial use [24]. The choice of the microorganisms to produce the interest's enzyme will vary according to the estimated gene sequence, where the active site of the enzymes of some microorganisms is homologous.

Thus, most lipases will require a "key" that gives access to their active site, consisting of one of the two α-helices attached to the protein body by flexible structural elements [25]. It is necessary for the molecular determination of the active site of this enzyme, even on enzymatic variations, to measure its qualitative and quantitative action against industrial production. The studies available in the literature that address lipases do not explore them extensively, designating their characteristics and differentiating them by their origins and properties.

In this context, the present review proposes the determination of the enzymatic characterization, through the search of gene expression, molecular, structural and functional characteristics, and the cataloging of inelastic eukaryotes lipases with data from the last 20 (twenty) years, addressing the production, the importance of thermostability of some microbial enzymes as well as their biotechnological applications and in veterinary medicine.

Lipases (Triacylglycerol Acylhydrolase, Ec 3.1.1.3)

Lipases are enzymes capable of catalyzing the synthesis

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Table 1: Families of lipolytic enzymes. Modified from [43].

Similarity (%)						
Family	Subfamily	Enzyme-producing strain	No Access	Family	Subfamily	Properties
I	1	*Pseudomonas aeruginosa*	D50587	100	NA	True Lipases
		Pseudomonas fluorescens C9	AF031226	95		
		Vibrio cholerae	X16945	57		
		Acinetobacter calcoaceticus	X80800	43		
		Pseudomonas fragi	X14033	40		
		Pseudomonas wisconsinensis	U88907	39		
		Proteus vulgaris	U33845	38		
	2	*Burkholderia glumae*	X70354	35	100	True Lipases
		Chromobacterium viscosum	Q05489	35	100	
		Burkholderia cepacia	M58494	33	78	
		Pseudomonas luteola	AF050153	33	77	
	3	*Pseudomonas fluorescens SIKW1*	D11455	14	100	True Lipases
		Serratia marcescens	D13253	15	51	
	4	*Bacillus subtilis*	M74010	16	100	True Lipases
		Bacillus pumilus	A34992	13	80	
	5	*Bacillus stearothermophilus*	U78785	15	100	True Lipases
		Bacillus thermocatenulatus	X95309	14	94	True Lipases
		Staphylococcus hyicus	X02844	15	29	Phospholipase
		Staphylococcus aureus	M12715	14	28	Phospholipase
		Staphylococcus epidermidis	AF090142	13	26	Phospholipase
	6	*Propionibacterium acnes*	X99255	14	100	Phospholipase
		Streptomyces cinnamoneus	U80063	14	50	
II		*Aeromonas hydrophila*	P10480	100	NA	Acyltransferase secreted
		Streptomyces scabies	M57297	36		Esterase secreted
		Pseudomonas aeruginosa	AF005091	35		Binding membrane esterase
		Salmonella typhimurium	AF047014	28		Binding membrane esterase
		Photorhabdus luminescens	X66379	28		Esterase secreted
III		*Streptomyces exfoliatus*	M86351	100	NA	Extracellular Esterase
		Streptomyces albus	U03114	82		Extracellular Esterase
		Moraxella sp.	X53053	33		Extracellular Esterase 1
IV		*Alicyclobacillus acidocaldarius*	X62835	100	NA	Esterase
		Pseudomonas sp. B11-1	AF034088	54		Lipase
		Archaeoglobus fulgidus	AE000985	48		Carboxylesterase
		Alcaligenes eutrophus	L36817	40		Supposedly lipase
		Escherichia coli	AE000153	36		Carboxylesterase
		Moraxella sp.	X53868	25		Extracellular Esterase 2
V		*Pseudomonas oleovorans*	M58445	100	NA	Polyhydroxyalkanoate Depolymerase
		Haemophilus influenzae	U32704	41		Supposedly lipase
		Psychrobacter immobilis	X67712	34		Extracellular Esterase
		Moraxella sp.	X53869	34		Extracellular Esterase 3
		Sulfolobus acidocaldarius	AF071233	32		Esterase
		Acetobacter pasteurianus	AB013096	20		Esterase

VI		*Synechocystis sp.*	D90904	100	NA	Carboxylesterases
		Spirulina platensis	S70419	50		
		Pseudomonas fluorescens	S79600	24		
		Rickettsia prowazekii	Y11778	20		
		Chlamydia trachomatis	AE001287	16		
VII		*Arthrobacter oxydans*	Q01470	100	NA	Carbamate hydrolase
		Bacillus subtilis	P37967	48		P-Nitrobenzyl esterase
		Streptomyces coelicolor	CAA22794	45		Supposedly carboxylesterase
VIII		*Arthrobacter globiformis*	AAA99492	100	NA	Stereoselective Esterase
		Streptomyces chrysomallus	CAA78842	43		Cell Membrane Esterase
		Pseudomonas fluorescens SIKW1	AAC60471	40		Esterase III

NA: Not Analyzed.

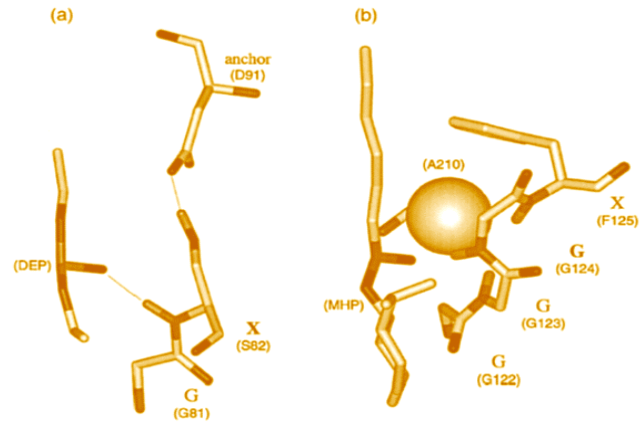

Figure 1: Illustration of two types of oxyanion holes. Modified from [25].

(development) and/or hydrolysis (breaking) of a broad spectrum of carboxylic esters, as well as the use or production of organic acids and glycerol [9,26], even in a disadvantaged environment of water molecules, according to the need of the microorganism [19,27].

During the catalysis the enzyme is produced in extracellular medium, facilitating its recovery from it [28,29]. This exoenzyme is susceptible to change in its structural conformations by changes in temperature, pH, nitrogen and carbon sources, as well as inorganic salts and oxygen concentration. Each characteristic, expressed by the enzyme, will be determined by the genetic sequence that transcribes it and is regulated by its affinity with the substrate.

Several studies demonstrate the production of this enzyme by fungi, either naturally or by molecular bioengineering, inducing them to produce specific enzymes [14-16,30,31]. Mobarak-Qamsari et al., [32] performed the genetic sequencing and also verified the increase in lipolytic activity through the improvement of production conditions through differentiated carbon and nitrogen concentrations for the selected bacterium.

In order to characterize the alkaline lipase enzyme for industrial applications, such as the use of lipolytic enzymes in detergents, animal leather processing industries and high quality chemicals, the authors used *Pseudomonas aeruginosa* strain KM110 (previously characterized for industrial use) from the wastewater of an oleic reprocessing plant located in the Vanak district of Tehran (Iran).

Nagao et al., [24] also verified the influence of carbon and nitrogen concentration on biological development and lipase production. For this, a transfection of the amino acid code present Saccharomyces cerevisiae was carried out in a strain of *Fusarium heterosporum*.

However, the authors observed that although the peptide expressions are very similar, the production of this enzyme is strongly influenced by the medium. This fact was also observed in the cultivation of strains with similar gene loads, even though of different genera, such as *Pseudomonas sp.* and *Burkholderia sp.* [33].

There are certain species of fungi that produce and degrade esters, using more stable lipases and better quality, being more active and stable in extreme environments, in the presence of detergents, alkaline pH and temperatures above 60ºC [34,35]. And it is these enzymes that the industry employs to dissolve solids coming from treatment plants, clearing and/or preventing oleic accumulation on wastewater surfaces [32,36-38].

Molecular characterization and protein sequencing

Lipases have different amino acid sequences, although they catalyze the same hydrolysis reaction. Although lipases: i) do not have any similarity between AA sequences; ii) do not operate with identical substrates and iii) do not have the same nucleophile (negative ion or neutral molecule acting as a Lewis base); Structural and spatial similarities are limited to folding designating its conserved catalytic region. Thus, although they do not have the same sequence of AA, after the packaging is observed in its conserved region, showing its common ancestry [20,39-42].

During the research of the mentioned authors, the Open Reading Frame (ORF) chains of 1,854 base pairs (bp), which coded about 617 AA, were identified. It should be noted that, in order not to confuse the terms used in the literature, the term nucleophile designates a compound (negative ion or neutral molecule) acting as a Lewis base, a potential electron-pair donor. The term nucleophilic denotes a reaction in which the core of the substance reacts with an ion acting through an available pair of electrons.

Lipases are classified into eight families (I to VIII), described in Table 1, according to their properties, structures and protein sequences, all of which are considered α/β hydrolases produced in

Table 2: Industrial applications of microbial lipases. Modified from [82].

Industry	Action	Product application
Detergent	Fat Hydrolysis	Oil stain removal from factories
Dairy Products	Milk and fat hydrolysis, cheese ripening, butter fat modification	Development of causative/flavor modifying agent, milk, cheese and butter
Cooked food	Flavor Development	Storage time extension
Drinks	Flavor development	Drinks
Food Adornment	Quality development	Mayonnaise, ornamentation and fiber breaking (meat)
Healthy food	Transesterification	Healthy food
Meat and fish	Flavor Development	Meat and fish products; fat removal
Fats and oils	Transesterification, hydrolysis	Cocoa Butter, Margarine, Fatty Acids, Glycerols, Mono-, and Diglycerides
Chemistry	Enantioselectivity, synthesis	Construction of chiral blocks, chemical compounds
Pharmaceutical	Transesterification, hydrolysis	Special lipids and digestion aids
Cosmetics	Synthesis	Emulsifiers, Moisture Controlling Agents
Leather	Hydrolysis	Leather products
Paper	Hydrolysis	Better fiber quality papers
Cleaning	Hydrolysis	Fat removal
Automotive	Biodiesel Synthesis	Transesterification of vegetable oils

extracellular form, and have the like enzymes esterases, proteases, peroxidases, lyases, among others [43]. After protein packaging these enzymes demonstrate the characteristic α/β structure, so they are considered as possessing a common ancestor only when active.

On the conserved sites, these enzymes become homologous only when activated after packaging, with α/β packaging structure, when finally the catalytic triad is shown [25]. Where the hydrophobic surface can be centralized, in the zone of lipid contact, there are proteic residues such as PHE, ILE, TRP, LEU, and TYR, where the first two probably have the function of coupling and penetrating the lipid surface [19].

Family I is the largest and most branched, covering seven other subfamilies (I.1 to I.7). Of these subfamilies, the first three are derived from gram-negative bacteria and thus considered true lipases. Families I.1 and I.2 have about 30% similarity in their genetic sequences, being secreted by the type 2 secretion system. Family I.3 have less than 20% similarity to the previous two, being expelled by the type 01 secretory pathway.

In family II, one does not have the conventional catalytic triad, but an association between GLY, ASP, SER, and LEU within the catalytic residue of the SER, so the same is closer to the amine terminus than any other lipase. The family III consists of monomers of acetylhydrolases that also function as a factor of activation of platelets and is constituted of the typical catalytic triad that plays the versatility of α/β hydrolases.

The variations of family IV lipases have a great similarity with mammalian hormone-sensitive lipase enzymes, demonstrating their origin in mesophilic bacteria. Those of the V family may also originate from mesophilic families, but are thermophilic in general, and can be adapted to cold or heat. The VI family has the smallest known esterases, thus not having activity in long chains of triglycerides, its active form is that of a dimer.

These latter enzymes are 40% like the eukaryotic lysophospholipases. The VII family has great similarity with acetylcholinesterases in the intestines of mammals and is particularly active even in the presence of the herbicide phenylcarbamate, in general, the protein family has a mean of 55kDa [44]. Finally, the family VIII has the most active-activesite β-lactamases, which suggests that its SER residue also has LYS, a hydropathic amino acid that assists in the formation of the oxyanion hole [43,45,46].

For Pleiss et al., [25] the classification is not based on numbers but on the name of each "superfamily". These superfamilies would support individuals of different species, but with the same characterization as previous authors, based on the properties, structures, and protein sequences that each superfamily exhibits. The classification from Arpigny e Jaeger [43], is the most used among the searches found in this review.

This process of stabilization occurs only by the formation of two hydrogen bonds between amide bonds in the forming residues of this electrophilic region (47).

Explaining this picture a little more (04), we have: a) GX type enzyme in Rhizomucor miehei lipase (PDB register 4TGL): stabilization of the substrate through the analogous diethylphosphate DEP inhibitor by hydrogen bonds in the first residue of the oxyanion hole (S82); Stabilization of S82 by hydrogen bonds anchored to residue D91. b) Enzyme type GGGX in Candida rugosa lipase (PDB 1LPM register): stabilization of the substrate through the analogous 1R-methyl hexyl phosphonate inhibitor where the first oxianion hole contains residue G (G124); stabilization of the interlaced oxyanion the A210 side-chain between G124 and the side chain of X (F125).

It is called "oxyanion" the internal hydropathic structure that, through hydrophilic forces, causes hydrophobic residues, generally: MET, CYS, PHE, LEU, VAL and/or ILE, to localize externally to the protein Figure 1.

How much more hydropathic (nucleophiles): i) plus the amino acids will be likely to be inside the protein; ii) better the equilibrium

Table 3: Some of the commercially available lipases of microbial origin produced by different companies. Modified from [57].

Fonts	Trade	Name	Vendor
Bacteria			
Alcaligene ssp.	LipasePL	MeitoSangyo, Co.	modifications in oils and fats/food additives
Pseudomonas cepacia	LipaseSL	Amano	synthesis of chiral compounds
Fungi			
Aspergillus niger	LipaseDS	Amano	food suplements
	Lipase	Sigma	organic and analytical synthesis
Rhizopus oryzae	Lipopan® F[a]	Novozyme	dough/paste (hardness)
	Lipomod™ 627P	Biocatalysts	flour / pasta dough (texture and shelf life)
	Lipomod™ 36P		Dietetics
Rhizomucor miehei	Palatase®[a]	Novozyme	development of dairy products flavors (Cheese)
Yeast			
Candida cylindracea	LipaseMY	MeitoSangyo, Co.	Dietetics
Candida antarctica	Novozym® 435[a]	Novozyme	olive oil specialties
	Noopazyme®[a]		pasta/noodles
Candida cylindracea/ porcine pâncreas	Lipomod™ 29P	Biocatalysts Ltd.	development of dairy products flavors (Cheddar Cheese)

[a]Trade names may change.

of the electric charges of the free carbonyl (identified in Table 2), which also increases its thermostability and iii) maintains the protein spatial arrangement in its active form [25,47-49].

Biotechnological applications in veterinary medicine

As regards the lipase enzyme in veterinary medicine, its importance starts with the interaction between the study of the maintenance of homeostasis and the pathogenesis. Historically, the determination of lipase serum activity, amylase, and trypsin immunoreactivity have been used for diagnosis [50].

The lipolytic concentration test is reported to be the most sensitive (65-94%) and specific (66-100%) non-invasive biomarker available for the diagnosis of pancreatitis in animals [51-53]. Thus, clinical enzymology is of fundamental importance to identify hepatic deficits and pancreatitis in animals through the analysis of the metabolic profile of the blood [54,55].

Lipolytic engineering began more than 150 years ago, but it was only after the mid-1980s that most of the enzymes produced came from microbial sources. Only when it became accepted that lipase enzymes remained active, even in organic solvents, that several investigations with these enzymes began as objects of study, rising to make them tools for the industry [49,56].

Over the years, it has been demonstrated that in order to obtain the production of enzymes with high quality and specialization it is necessary to prioritize and observe the production properties of the studied microorganisms, purification, and characterization of this production to achieve a stable and effective enzyme. Despite the expressive knowledge of the wide possibility of enzymatic production by microorganisms, only a small number of lipases are commercially exploited [13,33].

The development of lipolytic applications in the production and use in industries only increased the interest for their coding genes in the different microorganisms, because these enzymes are highly variable in composition, size, and structure.

The recent interest in lipolytic production is justified by the discovery of its most varied applications [57]. As food additives, lipases act in the synthesis of esters as flavoring agents and, in the hydrolysis of fatty acids (fats), in addition to acting as detergents and cleaning agents [58] and composition of medicines [59].

They can also be used in the treatment of wastewater by performing the decomposition and removal of oily substances [32,37,38], developed as an alternative to conventional treatment [60], on anaerobic biodigestion of swine manure [61,62], in the degreasing of skin and animal coatings and in cosmetics [63] in the removal of lipids.

Sometimes the use of the effluent for the production of lipases [32,60] is a viable and low-cost process. Effluents present high nutrient load still available for microbiological growth and subsequent enzyme production [64-66].

Lipase-producing fungi have already been isolated from greasy industrial waste [32], of soil contaminated with oil [64], factory processing of vegetable oils and dairy products [66-70].

Gomes et al., (2007) states that the same microorganisms that produce enzymes of lipase activity used in papermaking also perform the processing of starch and food [71]. Lipases can also be applied as an additive for animal nutrition, causing the previous breakdown of fats and oils from the mixture to be inserted into the feed [68,72] and/or preventing fats from being absorbed in the intestine [73].

With the enzymatic addition, absorption of fats by the intestine is reduced and the resulting composition of fats in foods is presumed to facilitate the development of lean and qualitatively high meat [68,72-76].

Over the past few years, lipases have been used in the synthesis of many biologically active compounds. For example, lipases to catalyze

the acylation of substances, forming novel optically active compounds [77,78]. As well as it is used in the regioselective esterification of diacetates, since this is impossible by chemical reactions like the alkaline hydrolysis [78,79].

As an example, pancreatic lipase has the ability to catalyze the ester emulsion hydrolysis in glycerol and long chains of derivative fatty acids [80]. These reactions of transesterification and enzymatic hydrolysis are complementary methods for the resolution of secondary alcohols in the synthesis of chiral drugs [81].

In animal production is recorded the use of products containing fats of dairy products. In this use is added a lipase that provides for the transformation of long-chain chains into short chains in order to facilitate absorption and accelerated the growth of goat, camel, cow, buffalo and pig pups [68,72].

Lipases have also been used in flavor enhancement [56,83], change in food coloring and creaminess agents [1], de according to the size and degree of unsaturation of the carbon chain [84,85], as shown in Table 3.

The most important element for lipolytic expression is its carbon source, i.e. from alternative carbon sources such as sugars and polysaccharides, to complex molecules [27,86] such as triglycerols, fatty acids, salts of bile and glycerol as well as other sources of carbon, although olive oil is usually the most used for lipolytic production in scientific studies [87-91].

Conclusion

There are few studies available in the literature that address lipases in a broad way, designating the gene sequence that produces this enzyme in the microorganisms studied, their molecular, structural and general classification characteristics, and the reasons for which they were not made are not identified.

Although they have a catalytic triad composed of conserved residues of SER-ASP/GLU-HIS/THR, lipolytic enzymes have a wide variety of characteristics and similarities with other protein groups, which makes the α/β family hydrolase so diverse, such as Candida antartica lipase, which has two regulatory protein layers and Fusarium solani cutinase.

Metal ions such as Ca^{2+} or Mg^{2+}, among others, are determinant components in lipolytic activation, as well as the oxyanion hole becomes indispensable through its stabilizing function of electrons in the molecular protein structure. The more hydrophilic, the more amino acids will be close to the protein core and, therefore, the better the equilibrium of the electric charges, maintains the protein arrangement in its active form and increases the molecular thermostability.

In the search for assisting veterinarians, microbiologists and biochemists in the complete understanding of the functioning of these enzymes, we try to elucidate and even confront the existing characteristics and knowledge. Therefore, further studies are needed to characterize and elucidate this enzymatic group. In industrial processes with the synthesis of new products, biocatalysis is a remarkable tool, and without any doubt, the lipases constitute one of the important current biocatalysts. The limitations found in the synthetic application of enzymes in their native form are currently being circumvented by altering stereospecificity, thermostability, and activity involving molecular biology techniques of site-directed or random mutations.

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