Scientific Bulletin. Series F. Biotechnologies, Vol. XXV, No. 2, 2021
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

MICROBIAL LIPASES: OBTAINING, PURIFYING AND CHARACTERIZATION REVIEW

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Abstract

Microbial lipases present a real interest in the biotechnology field, due to their special properties, efficiency and easy obtaining. These enzymes are characterized by high rates of substrate conversion, versatility, resistance and wide applicability, being produced by many species of bacteria, fungi and yeasts. From a commercial point of view, due to their special abilities to efficiently hydrolyze fats or other esters, they are considered innovative, feasible technology because they can easily cover a complex and multi-branched spectrum of industrial applications.

Key words*: lipases, bacteria, yeasts, fungi.*

INTRODUCTION

Literature data report that a considerable range of over 3200 enzymes have been prepared isolated and purified, with varying degrees of purity. Such enzymes are present in all living organisms, in body fluids and secretions specific for different organs (stomach, pancreas, small intestine, kidneys), in plants and in different microorganisms, where the enzymatic content depends on the species and environmental conditions. The enzymes isolation methodologies are relatively easy due to the sources that have an abundance advantage, accessibility, with significantly reduced costs and time processing. Microorganisms as lipase sources, are grown rapidly, in large quantities, and the used culture media are economical (e.g. agricultural or food industry by-products or wastes).

According to the reported data, microbial enzymes account approximately 80% of the total enzymes produced today in the world. Some enzymes are of current interest, due to the ease of obtaining, their special properties, versatility (Singh and Mukhopadhyay, 2012), large-scale productivity (Chahinian et al., 2005), as microbial lipases case, presents a real industrial interest, with special implications in chemical, pharmaceutical, cosmetic or food applications (Vulfson, 1994).

By their origin, lipases can be classified into: microbial lipases; plant lipases; animal lipases (milk lipases; pancreatic lipases; lipoprotein lipase; hormone-sensitive lipases) (Pascoal et al., 2018). Rich sources of lipases are represented by bacteria, especially the genus *Chromobacterium, Pseudomonas* and *Staphylococcus*; by yeasts, *Candida lipolytica* (Ionita et al., 2001), fungi, especially *Aspergillus, Geotrichum, Rhizopus, Mucor, Penicillium* (Pascoal et al., 2018; Jurcoane et al., 2009).

An important criterion in the description of industrial applications is specificity, and from this point of view lipases can be classified as follows: (i) substrate-specific, (ii) regioselective and (iii) enantioselective (Sarmah et al., 2017).

Substrate-specific lipases can be used effectively in reactions, in which they act selectively on a specific substrate in a mixture of crude raw materials, facilitating the synthesis of the desired product, as demonstrated by the use of

lipases in biodiesel and diacylglycerol production of high purity. (Sarmah et al., 2017)

Regioselective lipases tend to lead the reaction in a favorable direction compared to other adverse reactions. Such a property of lipases is of extraordinary importance for the chemical and pharmaceutical industries, especially in the production of isomeric compounds which have an optimal function only in a specific configuration (Sarmah et al., 2017**)**.

Based on positional specificity (regiospecificity), lipases are divided into three groups: (i) nonspecific lipases, which have the ability to catalyze triglycerides to free fatty acids and glycerol (Kapoor and Gupta, 2012; Ribeiro et al., 2011); (ii) 1,3 specific lipases, capable of releasing fatty acids from positions 1 and 3 of triglycerides, but unable to hydrolyze the ester bonds in the secondary position; they can rapidly hydrolyze triglycerides into diglycerides (Kapoor and Gupta, 2012; Ribeiro et al., 2011); (iii) specific fat lipids with fatty acid selectivity, catalyzing the hydrolysis of long-chain fatty acid esters (Kapoor and Gupta, 2012; Ribeiro et al., 2011). Due to their diversity, wide range of actions and their independent production facilitated by genetic manipulation, microbial lipases are produced at industrial scale and represent the most common class of enzymes used in biotechnological applications (Dey et al., 2014; Lee et al., 2015; Priji et al., 2015; Ullah et al., 2015).

Some of the processes catalyzed by enatiospecific lipases include the transesterification of secondary alcohols in pharmaceuticals, the hydrolysis of menthol benzoate in cosmetics/food, and the hydrolysis of glycidic acid methyl ester in medical/health care products (Sarmah et al., 2017).

LIPASE-CATALYZED REACTIONS

Mainly used in bioprocess industries for the production of various significant products, such as biodiesel fertilizers, cosmetics, flavored foods. Although some applications are reported in ringopening polymerization reactions, generally lipase-catalyzed reactions are chiefly grouped in two categories, namely, hydrolysis and synthesis. Further synthesis reactions can be classified as esterification, aminolysis, interesterification, alcoholysis, and acidolysis. However, the interesterification, alcoholysis, and acidolysis reactions are together considered as transesterification reactions. (Sarmah et al., 2017). Triacylglycerol acyl hydrolases (E.C. 3.1.1.3) or lipases, belong to the family of serine hydrolases (Pascoal et al., 2018). These enzymes have multiple roles such as: fats and oils hydrolysis (Sarac et al., 2017); oils hydrolysis in the oil-water interface, with free fatty acids, diglycerides, monoglycerides and glycerol release (Jurcoane et al., 2009; Gopinath et al., 2013; Guldhe et al., 2015).

MICROBIAL LIPOLYTIC ENZYMES

Due to their diversity and versatility, lipases are of current industrial interest, ranking them on the 3rd place of the most used enzymes (Rios et al., 2018), after proteases and amylases (Javed et al., 2017). Of all lipase types, microbial lipases are preferred because of their high substrate specificity, higher stability and low production costs (Lee et al., 2015).

Microbial lipolytic enzymes differ in terms of pH range and optimum temperature activity, as well as in thermal stability duration (Table 1).

Table 1. Microbial lipases properties (Pascoal et al., 2018)

Producing microorganism	Optimum pH	pH stability	Optimal temp. (^{0}C)	Thermostability (^{0}C)
Candida sp.	6,0	5.0 - 7.5	40	45
Aspergillus niger	5.6	2.2 - 6.8	25	50
Rhizopus delemar	5.6	3.0 - 8.0	35	65
Geotrichum candidum	6.0	4.5 - 10.0	35	50
Penicillium roqueforte	8.0	-	37	50
Penicillium cyclopium	7.0	6.5 - 9.0	30	40
Achromobacter lipolyticum	7.0	-	37	99
Pseudomonas fragi	7.0 - 7.2	-	32	72

The microbial lipases are different and specific depending on the producing microorganism: type I lipases are obtained by *Pseudomonas aeruginosa, Candida lipolytica, Penicillium roqueforti*. Triglycerides have fast attack and the role of priority, namely the 1 or 3 position of fatty acids but have a low activity on mono- and diglycerides; type II lipases are produced by *Aspergillus oryzae, Rhizopus* sp. and hydrolyze a wide range of fats, natural oils and synthetic glycerides; type III lipases are produced by the fungus *Geotrichum candidum*, which possesses a unique but not absolute specificity to unsaturated fatty acids, regardless of the fatty acid position in glyceride structure (Fogarty and Kelly, 1990). The lipase action interface is environmentally dependent and manifests a "*closed*" or "*open*" conformation due to the presence of a "*lid*" type protection structure, with a hydrophilic and a hydrophobic area. The hydrophilic side realizes the contact with the aqueous environment, while the hydrophobic side is orientated within the core of the enzyme structure. In the absence of the specific substrate, represented by triglycerides, lipase has a "*closed*" conformation, and the hydrophobic side is exposed to the maximum. In the presence of the specific substrate, the lipases are receptive by making a signal to the active site, for hydrophobic side exposure, with "*open*" conformation enzyme form. This mechanism allows the enzyme to contact the preferred substrate, resulting in substrate catalysis (Lotti, 2007; Ugo et al., 2017). In the case of serine lipases, the active site is characterized by the triad serine-histidine-aspartic acid and it is essential for all reactions in which this enzyme is involved (Jaeger et al., 1999; Reetz, 2002). The mechanisms involved suppose a serine proton removal, a mechanism by which aspartate and histidine residues are required (Reetz, 2002; Brady et al., 1990). The hydroxyl group of serine reacts with the carbonyl carbon forming an intermediate substrate. The presence of an oxo-anionic spacer contributes to the load distribution stabilization and to reducing the minimum energy for intermediate substrate formation. The last step is deacylation, where the acyl group is transferred to enzyme and the catalytic center is regenerated (Brady et al., 1990; Pascoal et al., 2018).

OBTAINING MICROBIAL LIPASES OF INDUSTRIAL INTEREST

Microbial lipases are considered the most advantageous enzymes. They have wide applicability due to their special abilities, resistance and stability to the conditions imposed by the industrial environment. Bacterial strains with a high productivity degree which have been studied and evaluated so far, are part of Gram-positive bacteria (*Bacillus, Staphylococcus, Lactobacillus, Streptococcus, Micrococcus, Propionibacterium)*, Gram-negative bacteria (*Pseudomonas, Chromobacterium, Acinetobacter, Aeromonas*), fungi (*Rhizopus, Aspergillus, Penicillium, Mucor*), yeasts (*Candida, Rhodotorula, Pichia, Saccharomyces, Torulospora trichosporon)* or actinomycetes (*Streptomyces*) (Rohit et al., 2001).

SCREENING AND ISOLATION OF LIPASE-PRODUCING MICROORGANISMS

Lipase-producing microorganisms are present in various industrial habitats, in wastes, vegetable oil from processing companies, dairy factories, oil-contaminated soil, oilseeds, compost, coal quarters and thermal springs. A simple and safe method of detecting microbial lipase activity uses an agar culture medium with Tween 80 surfactant, following the opaque areas formation corresponding to lipases presence. Changes to this working technique include Tween surfactants, Cu^{2+} oils or salts (Cardenas et al., 2001), or Rhodamine B chromogenic substrates (Sharma et al., 2001) and are used in screening methodologies. Lipases are mainly produced by submerged culture (Ito et al., 2001), for which numerous studies have been carried out to optimize the culture media subjected to the influence of the reaction factors involved (substrate concentration, carbon and/or nitrogen sources, pH, temperature, dissolved oxygen concentration) (Elibol and Ozer, 2001). High yields of lipase production were also observed in vegetable oils, like olive, soybean, sunflower, sesame, cottonseed, corn and peanut oil, but the maximum production of lipase was recorded when using olive oil. For example, the

presence of 1% olive oil for *Bacillus* sp. culture, induces the production of lipolytic enzymes (Lee et al., 2015); for *Pseudomonas fluorescens S1K WI,* olive oil induced very good lipase biosynthesis yields, with 7395 U/mg specific activity at pH 8.5 (Lee et al., 1993); for *Penicillium expansum,* the presence of only 0.1% olive oil and a pH of 8.3 in the culture medium is favorable, and the yield of production and stability of the enzyme can be improved by the addition of Tween 20 and PX lubricant (Sharma et al., 2001). The thermophilic *Bacillus thermoleovorans ID-1,* isolated from Indonesia thermal springs, exhibited extracellular lipase activity with high growth rates on lipid substrates at high temperatures, using 1.5% olive oil as unique carbon source (Lee et al., 1999). Carbon sources such as fructose and palm oil are beneficial in lipase production, especially for thermostable lipases produced by *Rhodotorula glutinis. Bacillus Wai 28A 45*, in presence of tripalmitin, at 70°C, while *Bacillus A30-1* adds corn oil and olives (1%), or beef/palm oil for *Bacillus stearothermophilus L1* (Sharma et al., 2001). On the other hand, the addition of different oils (eg. rapeseed or maize) to the culture medium, leads to lipase yields stimulation of (e.g. for *Rhizopus oryzae*). An alkaline lipase with excellent ability to remove greasy stains in alkaline solution, is produced by *Pseudomonas alcaligenes M-1*, when culturing on citric acid and soybean oil substrate (Sharma et al., 2001). Fermentation with 2% castor oil is beneficial for *Pseudomonas aeruginosa KKA-5*, which produces lipases capable of hydrolyzing 90% of castor oil and is stable under alkaline conditions (pH 7-10) (Sharma et al., 2001).

The nitrogen source effect can be decisive in microbial lipases production. In the presence of 2-5% peptone at 7.2 pH *Penicillium citrinum, Pseudomonas* sp. *KW1-56* (Izumi et al., 1990, Sharma et al., 2001), lipase synthesis had high yields in culture medium. Similar effects were observed with corn extract (7% for *Rhizopus oryzae),* soybean meal (35% for *Acremonium structum*, 1% for *Pseudomonas alcaligenes F-111*). Thermostable lipases produced by thermophilic fungi as *Emericella rugulosa, Humicola* sp*., Thermomyces lanuginosus, Penicillium purpurogen* and *Chrysosporium sulfureum*, need yeast extract, and thermostable alkaline lipases produced by *Bacillus A 30-1* are obtained in good yields in the presence of 0.1% yeast extract and 1% ammonium chloride, at 60°C, optimum pH of 9.5. The presence of metal ions in the culture medium can beneficially influence the microbial lipases production: magnesium ions enhance biosynthesis in *Aspergillus niger, Pseudomonas pseudoalcaligenes F-111, Bacillus* sp*. A 30-1, Pseudomonas pseudoalcaligenes KKA-5,* those of iron and calcium in *Bacillus* sp*.,* those of magnesium, calcium, copper, cobalt for *Acinetobacter calcoaceticus, Bacillus* sp*. A 30-1.*

MICROBIAL LIPASES PURIFICATION

Most enzymes isolated from plant or animal tissues or microbial cultures, require purification steps, for removing the contaminants that can catalyze unwanted side reactions. Extracellular enzymes, like most microbial lipases, are secreted into the culture medium and the biomass is separated by centrifugation or filtration techniques. The resulting solution can be concentrated using ultrafiltration membranes, and the separation of different proteins is achieved by fractional precipitation. The solvation capacity of the different proteins by water can be modified by water-miscible organic solvents, in other to dissolve substances or by combining these methods, together with the change of temperature (Jurcoane et al., 2009). The main used techniques in lipase purification processes consist of: selective precipitation techniques using neutral salts; fractional precipitation techniques using water-miscible organic solvents such as ethanol or acetone; gel filtration; hydrophobic interaction and affinity chromatography (Jurcoane et al., 2009; Javed et al., 2018). Purification methodologies purpose is not only to isolate the enzymes from contaminants, but also to improve the activity, stability and shelf life of the enzymes (Javed et al, 2018). On the other hand, after the purification stages, structural and conformational studies can be carried out (Nadeem et al., 2015) following the kinetic and thermodynamic mechanism for substrate hydrolysis, the transesterification reaction and

the structure-function relationship (Javed et al., 2018). The lipase purification step is very important and necessary for product formulations with industrial or medicinal applications and uses. Purification is a key step, which is performed for enzyme specific function assessing. The cell-free culture medium is subjected to salts precipitation, up to column chromategraphy, depending on the proteins nature and the purification level desired. The variety of chromatographic techniques includes anion exchange, cations or exclusion chromategraphy. Data from the literature indicate that lipases have been purified with high yields (Javed et al., 2018).

Often ultrafiltration technique is applied for concentration and then ion exchange chromatography and gel filtration are performed.

Purification stages may vary depending on bacterial strains used in order to obtain lipases: generally, the first step is ammonium sulphate precipitation or solvent precipitation, followed by different chromatographic techniques using on different chromatographic materials (e.g. DEAE-Toyopearl 650 M, Phenyl Toyopearl 650 M, Toyopearl HW-60), Sephadex G-75, G-100 gel filtration, DEAE-Sephadex A-50 column, Sephacryl S-200 gel filtration chromatography and isoelectric focusing (Sharma et al., 2001). For example, anion exchange chromatography (for *Bacillus* sp*., Aneurinibailus thermoaerophilus HZ*), Sephadex G-100 gel filtration (for *Pseudomonas aeruginosa BUP2* and *NB-1*), DEAE-cellulose purification (for *Geobacillus stearothermophilus AH22, Idiomarina* sp., *Bacillus* sp.), ultrafiltration (for *Bacillus* sp*., NS5, Pseudomonas aeruginosa BN-1 and CS-2*), affinity chromatography (for *Acinetobacter* sp. *XMZ-26, Geobacillus* sp.), hydrophobic interaction fast performance liquid chromatography (FPLC) (for *Acinetobacter calcoaceticus)* (Sharma et al., 2001).

MICROBIAL LIPASES CHARACTERIZATION

The molecular mass has been determined by electrophoretic techniques. Also, the optimal pH range, as well as temperatures, at which the enzymatic activity is expressed at its maximum level were determined. The molecular mass was determined in 19-92 kDa range, the lowest being recorded for *Bacillus stratosphericus* lipase, and the largest one for *Pseudomonas gessardii* lipases. The optimum temperature range at which the lipase activity was maximal was determined to be between 15-80°C, this specific temperature leads to a decrease in lipase activity. Thus, the lipases produced by *Acinetobacter* have an optimum temperature of only 15°C, while the vast majority of microbial lipases have an optimum enzyme activity at temperatures above 35°C, reaching even 60 or 80°C (*Cohnella* sp*., Idiomarina* sp*., Janibacter* sp. lipases) (Javed et al., 2018).

MICROBIAL LIPASES APPLICATIONS

From a commercial point of view, microbial lipases are of great interest, because they have a good fat hydrolyzing ability being considered a feasible technology, as they can cover a wide range of applications (pharmaceutical, food, cosmetics, biofuels) (Israel- Roming, 2014).

The detergent industry needs high activity lipases capable of acting on different type of fats, in harsh washing conditions (alkaline pH, high temperature, surfactants) (Guerrand, 2017).

In food industry, there are many possibilities of using lipases in edible oil production, bread making, diary products obtaining and egg processing (Guerrand, 2017; Panyachanakul et al., 2019).

In the biofuel industry, lipases are able to synthesize biodiesel by catalysing the trans-esterification reaction in media based on oily plant material or waste oils or fats (Melani et al., 2019).

Bioremediation of wastes, but also grease contaminated soils and waters, represents an opportunity for enzymes to show their capacity of acting for environmental preservation in a sustainable way. Another application of great interest in the field of environmental protection is the use of microbial lipases in accelerating the degradation of polyurethane and polyesters derived from renewable resources (Rohit et al., 2001).

A new and successful approach is represented by the use of immobilized microbial lipases on different substrates, due to the many advantages, such as the continuous, repeated and easy use of biocatalyst,, as well as the

increased stability of the enzyme (Yunus, 1995; Ivanov and Schneider, 1997; Pascoal et al., 2018).

CONCLUSIONS

Due to the large field of applications that address lipases, a continues work is done for obtaining these enzymes from novel sources or by optimised processes that lead to higher activity, more stability against environmental factors and lower production costs.
In the meantime, because of the involvement in different reactions and due to the relative substrate specificity, new potential uses are investigated.

ACKNOWLEDGEMENTS

This work was possible with the support and conducted by National Institute for Chemical-Pharmaceutical Research and Development, ICCF Bucharest, through M. Moscovici PhD., and University of Agricultural Sciences and Veterinary Medicine Bucharest, Department of Industrial Biotechnologies, Faculty of Biotechnologies, with Prof. Florentina Israel-Roming . PhD.

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