

## LIPASES PRODUCED BY YEASTS: POWERFUL BIOCATALYSTS FOR INDUSTRIAL PURPOSES

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### ABSTRACT

The term “lipolytic enzymes” refers to the lipases and carboxylic ester hydrolases. Lipase production is widespread among yeasts, but few are capable of producing lipases with interesting characteristics and in sufficient amounts to be industrially useful. The literature concerning lipases produced by *Candida rugosa*, *Yarrowia (Candida) lipolytica*, *Candida antarctica* and other emerging lipase-producing yeasts is reviewed. The use of recombinant lipases is discussed, with emphasis on the utilization of heterologous expression systems and design of chimeras. Finally, the three approaches that aim the improvement of lipase production or the modification of the substrate selectivity of the enzyme (medium engineering, biocatalyst engineering, and protein engineering) are discussed.

**Keywords:** *Candida antarctica*. *Candida rugosa*. lipases. *Yarrowia lipolytica*. Yeast.

### 1 Introduction

The term “lipolytic enzymes” refers to the lipases and carboxylic ester hydrolases, and the former differs from the latter due to their ability to act in insoluble esters. Lipases act mainly in emulsified substrates with long fatty acid chains (triglycerides), while the carboxylic ester hydrolases act in soluble esters with relatively short fatty acid chains. This is the reason why lipases, in contrast to other esterases, need an oil– water interface for optimum activity [1-3]. Lipase production is widespread among yeasts, but few are capable of producing lipases with interesting characteristics and in sufficient amounts to be industrially useful. Industrial applications of lipases include roles in the food industry, resolution of pharmaceuticals, biocide making, biosensor modulation, biosurfactants, production of lipid-derived flavours and fragrances, and treatment of olive mill wastewater [4]. Literature concerning yeast lipases is overwhelming, thus this review intends to highlight the main issues of this topic, with special emphasis in the last 10 years, without the pretension to cover all the information available. We will start with *Candida rugosa* lipase, continue with lipases produced by other yeasts, recombinant lipases, and, finally, the approaches for improving yeast lipase production or selectivity.

### 2 Lipase producers

#### 2.1 *Candida rugosa* lipase

*Candida rugosa* lipase (CRL) is one of the enzymes most frequently used in biotransformations, and has the great advantage of being considered safe for food applications [4-6], therefore there is a plenitude of manuscripts dealing with it. The crude enzyme is composed by at least 5 isoenzymes, denominated LIP1 to LIP5 (CRL1 to CRL5), that share high sequence homology, but have different characteristics and substrate preferences [6-8]. Chang *et al.* [9-10] report the existence of 7 isoforms with distinct thermal stability and substrate specificity, codified by the *lip* gene family. Sequence divergence of the isoforms is located in the lid, a mobile loop that modulates access to the active site [11]. Commercial preparations are mixtures of the isoforms, and lack of reproducibility of biocatalytic reactions using this commercial CRL may be due to the presence of variable isoforms in the preparation, complicating the interpretation of results [5,7]. Purification of crude CRL improve its substrate specificity, enantioselectivity, stability, and specific activities<sup>5</sup>. Enantiopreferences of CRL are explained by its structure and conformational flexibility [12,13]. *C. rugosa* lipase has a tendency to form bimolecular aggregates in solution, altering the active center and modifying the enzyme functionality [14].

Regulation of CRL synthesis and secretion is complex [7]. De Maria *et al.*[15] studied the effect of several inducers in the production of new crude lipases from *C. rugosa*. These authors reported that LIP2 and LIP3 were always secreted, while

LIP1 was induced by n-docecanol in batch cultures and oleic acid in fed-batch experiments, concluding that the nature of the inducer controls the isoenzyme percentual in the crude preparation.

LIP1 (CRL1) gene codes for the major CRL form, and shares 40 and 30% sequence identity with *Geotrichum candidum* and *Yarrowia lipolytica* lipases, respectively [16]. It has many applications, such as non-specific, stereo-specific hydrolysis and sterification, and production of human milk fat substitute [9, 17]. LIP 2 and LIP3 have been obtained in pure and monomeric forms from *C. rugosa* 14830 pilot-plant fed-batch fermentation [18]. LIP2 (CRL2) has a high esterase activity towards long-chain (C12-C16) p-nitrophenyl esters, with optimum activity at pH7 and 30-50°C [19]. LIP3 (CRL3) isoform displays high activity towards cholesterol esters [11], and acts on triglycerides and plant-derived steryl esters, what is of considerable interest in papermaking waste treatment [20]. The optimum activity of LIP3 is at pH5-7 and below 55°C [20]. Lopez et al. (2004) [21] studied the reactivity of pure *C. rugosa* isoenzymes (LIP1, LIP2, and LIP3) in aqueous and organic media. LIP1 and LIP3 had similar stability, while LIP2 was the less stable. The highest differences were found in the hydrolysis of triacylglycerides. A short, medium and long acyl chain triacylglyceride was the preferred substrate for LIP3, LIP1 and LIP2, respectively [21]. The other isoenzymes have not been characterized yet.

CRL have been applied in several pharmaceuticals and chemical industrial process including enantioselective esterifications [22-23], hydrolysis and kinetic resolution of esters [24-28], transesterifications [29-30] in pure and immobilized forms [31-36].

## 2.2 Lipase produced by other yeasts

### 2.2.1 Pathogenic yeasts

The production of enzymes, such as aspartic proteinases (Saps) and phospholipases, by potentially pathogenic yeasts is a recognized mechanism of virulence [37]. Some pathogenic yeasts are capable of producing lipases [37-39], but the clinical relevance of this specific enzyme has yet to be established. Thus the issue of pathogenic yeasts lipase production will not be addressed in this review.

### 2.2.2 *Candida antarctica*

The second most studied yeast lipase is the one produced by *Candida antarctica* (CAL), particularly the lipase B (CALB) [40-44]. Lipase B has several applications, such as synthesis of flavors and fragrance esters [45-46], surfactants [47-50], biodiesel [51-54], waxes [55], acylated flavonoids [56] and modified glycerides [57-59] as well as kinetic racemic esters [60-

63] and amines [64] resolution. *C. antarctica* is considered an extremophile, since it is adapted to cold temperatures, and several researches aim the improvement of the thermal stability of its lipase [65-66]. Stability has also been improved using enzymatic synthesis in ionic liquids or nonaqueous media [67-68]. The largest group to fit lipase B active site is ethyl, restricting the number of secondary alcohols that are good substrates for CALB [69]. There is a commercial *C. antarctica* lipase denominated Novozym 435, that is the CAL B immobilized on acrylic resin [70].

In the last years, researchers have demonstrated more interest on the CAL A lipase, since it presents unique properties, being thermo-stable (over 90 °C), catalyzing reactions with tertiary or stereo hindered alcohols, showing selectivity to *trans* fatty acids, being stable at acidic pHs and presenting chemical-selectivity to amino groups (amino acids and esters aminics synthesis) [71].

### 2.2.3 *Yarrowia lipolytica*

*Yarrowia lipolytica* also produces several lipases, and LIP2, an extracellular lipase, is the most studied [72]. Fickers *et al.* [73] obtained 400 *Y. lipolytica* mutants from one original strain, and selected the mutant LgX64.81 as the one with the highest potential for lipase production. LgX64.81 exhibited lipase production uncoupled from glucose catabolite repression, with a high production on glucose medium, and showed a ten fold increase in productivity upon induction by oleic acid. Tryptone and oleic acid were the most suitable nitrogen and carbon sources for lipase production, and in the presence of oleic acid, lipase was cell-bound during the growth phase before being released in the medium [74]. LgX64.81 LIP2 expression is modulated by methylolate, thus an industrial culture medium for the production of lipase by this yeast was developed using methylolate as a substitute for olive oil [75-76]. The lower sensitivity of mutant LgX64.81 to glucose catabolite repression is due to a reduced level of hexokinase 1 (HXK1), and, consequently, to a low uptake of glucose [77]. Amplification of LIP2 gene in this mutant was used to select lipase overproducing *Y. lipolytica* strains [78]. Two other lipase genes (LIPY7 and LIPY8) were obtained from one strain of *Y. lipolytica*, and the purified enzymes were characterized [79].

Corzo *et al.* [80] describes an extracellular lipase produced by *Yarrowia lipolytica* strain 681 that presents 29.5°C and 4.7 as the optimum temperature and pH, respectively. This lipase is induced by Tween 80 in concentrations between 0,5 – 2g/mL, inhibited by glycerol and has high specificity to the substrates olive oil, corn oil and glucose.

### 2.2.4 Emerging lipase-producing yeasts

Although most yeast lipase studies deal with the above mentioned species (*C. rugosa*, *C. antarctica* and *Y. lipolytica*), there are some emerging lipase-producing yeasts. *Galactomyces geotrichum* (ou *Galactomyces candidum*) and its anamorph *Geotrichum candidum* are ubiquitous yeast-like fungi capable of producing lipases. Fernandez *et al.* [81] isolated the lip I gene from *G. geotrichum* strain BT107 and partially purified the enzyme. D'Annibale *et al.* [82] analysed the possibility of using olive-mill wastewaters as substrate for lipase production by fungi, including a strain of *G. candidum*. Holmquist [13] found a unique preference of *G. candidum* lipase for long-chain cis (delta-9) unsaturated fatty acid moieties in the substrate.

Other promising lipase-producing yeast is *Candida cylindracea*. Kim & Hou [83] reported the lipase production by strain NRRL Y-17506, which produces an extracellular lipase from oleic acid, and measured the lipase activity in flask culture, intermitent and stepwise feeding fed-batch culture. This strain showed the highest lipase activity among lipase-producing fungi tested in olive-mill wastewaters [82]. The same authors report that its lipase production was induced by the addition of olive oil, and was affected by the type of nitrogen source used. There is a report of an attempt to separate the racemic mixture of the drug proglumide, administered in the treatment of neuropathic pain, using the lipase from *C. cylindracea* [84].

Ciafardini *et al.* [85], studying lipase production by yeasts isolated from extra virgin oil, reported that *Williopsis californica* strain 1639 produced an extracellular lipase inducible by the free fat acids of olive oil. Lipase activity of yeast strains can lower the quality of olive oil, and it was demonstrated that acidity of oil inoculated with *W. californica* increased during storage<sup>86</sup>. This strain had an optimum lipase activity at pH6 [86].

*Candida utilis* is a yeast broadly used for single-cell protein production, and several alternative substrates have been tested for this purpose [87-88]. Fujino *et al.* [89] reported the purification and characterization of a phospholipase from this yeast. It had two pH optima: at pH3, it hydrolysed all phospholipids without addition of metal ions; but at pH7.5, substrate specificity required addition of metal ions.

*Arxula adenivorans* is an emerging yeast expression vector [90], thus the knowledge of natural protein secretion by this yeast is of ultimate importance. Boer *et al.* [91] cloned its ALIP1 gene, purified and characterized the lipase produced by this yeast. It has an aminoacid sequence similar to the lipases from *C. albicans* and *C. parapsilosis*, possesses a dimeric structure (100KDa), and optimum activity at pH7.5 and 30°C.

Some yeast species are scarcely studied for lipase production. *Kluyveromyces marxianus* was shown to produce a highly thermostable lipase in aqueous solutions that is also stable at acidic pH and has good tolerance to organic solvents [92].

Kakugawa *et al.* [93] reported the purification and characterization of a lipase from *Kurtzmanomyces* sp. strain I-11. This lipase has optimum activity at 75°C and pH 1.9-7.2, preference for C18 acyl groups, and is stable in the presence of various organic solvents.

*Issatchenkia orientalis* CECT 10688 has been investigated regarding to the ability to secrete lipolytic activity in submerged culture [94]. Tributirin was the best inducer of lipolytic activity and sugars as glucose and fructose have also been shown to possess inductive properties for this enzyme activity.

Finally, there are some reports of lipase production by the conventional yeast *Saccharomyces cerevisiae*. Its lipase activity is restricted to whole cells, that is, is intracellular, but is capable of lowering the quality of extra virgin olive oil [85-86]. The intracellular enzyme tgl1p (triglyceride lipase 1) isolated from this yeast has lipase and/or esterase activity, depending on the pH of the assay [95]. Interest concerning lipase production by *S. cerevisiae* is restricted due to its intracellular character.

Reports of lipase production by basidiomycetic yeasts are extremely scarce within the literature, but it is known that these yeasts survive in environments rich in lipidic substrates, such as plant leaf surfaces<sup>60</sup>. As an example, Masaki *et al.* [96] described a cutinase-like enzyme from a strain of *Cryptococcus* sp. capable of hydrolysing biodegradable plastics. , Kamini *et al.* [97] have been obtained a lipase from *Cryptococcus* sp S-2 whose production was dependent of inducers such as sardine oil, soybean oil and triolein. The enzyme showed high stability in presence of organic solvents at the concentration of 50% (v/v) for 60 min.

### 3. Recombinant lipases

The use of expression vectors for protein production usually aims the enhancement of protein secretion. In a general explanation, the gene of interest is isolated from the original yeast producer, and cloned into an expression vector. This vector is preferentially another yeast because these eukaryotic microorganisms require pos-translational enzyme modifications not available in bacterial expression vectors. There are some yeast expression vectors available, but the most used for recombinant lipase production is the methylotrophic yeast *Pichia pastoris*, due to the high protein secretion of this vector and to a methanol induced secretion mechanism [90]. The protein secreted by *P. pastoris* can be purified from the culture supernatant and characterized.

Besides the aim of lipase production enhancement, the use of the *P. pastoris* expression system has another purpose. As stated above, the most frequently used yeast for lipase production is *C. rugosa*, but the original yeast produces in fact a mixture of isoforms of the enzyme. This complicates the characterization

and analysis of the lipase activity. One way of producing each isoform in separate is by expression of the corresponding gene in a heterologous host [16]. By using this approach, several authors managed to obtain an efficient lipase production and/or purified and characterized the isoform [9-10, 19, 98]. But expression of *C. rugosa* lipase in *P. pastoris* has a technical problem. *C. rugosa* uses an alternative genetic code, reading the codon CTG as serine instead of the universal leucine, and a site-directed mutagenesis is necessary in order to allow *P. pastoris* to express the correct protein [9,19,98]. Mileto *et al.* [16] tried to overcome this problem by using the yeast *Candida maltosa* as the expression vector, since this yeast uses the same genetic codon as *C. rugosa*, but as *C. maltosa* is not a conventional expression yeast vector, this approach was not followed in more recent works. Other yeasts had their lipase genes expressed in *P. pastoris*, such as *G. geotrichum* [81], *Y. lipolytica* [79], and *C. parapsilosis* [38]. Neugnot *et al.* [39] expressed two lipase open reading frames (ORFs) from *C. parapsilosis* in *S. cerevisiae*, but only CpLIP2 was active.

Another approach in the recombinant lipases is the production of chimeras. Chimeras are proteins formed by the linkage of parts of different original proteins, usually after manipulation of the corresponding genes. Using this approach, Brocca *et al.* [11] was capable of transferring the *C. rugosa* CRL3 cholesterol esterase activity to CRL1, an isoform that was originally incapable of this activity. The same modification was performed by Secundo *et al.* [99], but the chimera was less active and enantioselective than the original isoforms. Suen *et al.* [66] created a chimeric *C. antarctica* lipase B with improved activity and thermostability.

#### 4 Approaches for improvement of production or selectivity

This section deals with the approaches that aim the improvement of lipase production or the modification of the substrate selectivity of the enzyme. There are basically three types of approaches [100]. The first one targets the design of culture conditions for enhancement of lipase production, enzyme immobilization and solvent manipulations for improved lipase activity (medium engineering), the second aims the genetic improvement of the producing strain (biocatalyst engineering), and the last one deals with the enzyme manipulation in order to alter its characteristics (protein engineering).

##### 4.1 Medium engineering

Lipases show considerable enzymatic specificity related to the substrate selectivity, functional group selectivity, regioselectivity (inside the molecule), stereoselectivity (resolution of enantiomers and diastereomers) and chemoselectivity (products generated) [101-103]. Lipases with

different specificities can be produced altering the medium composition [104], and the expression of isoforms of *C. rugosa* lipase is known to be governed by cultural or fermentation conditions [5,105]. By controlled fermentation conditions using glucose and oleic acid as carbon sources, it was shown that lipase-encoding genes of *Candida rugosa* may be grouped in two classes, one of which is constitutively expressed and the other is induced by fatty acids [106]. Similarly, Sánchez *et al.* [107] obtained a crude lipase preparation with higher protein content and lipase activity than commercial Sigma type VII CRL (*Candida rugosa* Lipase) by using oleic acid as inductor in a controlled constant feeding rate fed-batch system. Finally, lipase enantioselectivity can be increased by pH or temperature changes, solvent addition or elimination, addition of compounds like Triton X-100 and CaCl<sub>2</sub>, or still by enzyme immobilization in a support.

Some components of the culture medium can stimulate lipase production while others act as repressors. Lipase production suffers from glucose catabolite repression, unless the producing yeast strain is defective in using glucose in the metabolism, as is the case of *Y. lipolytica* LgX64.81 [77]. The most common stimulators are long chain fatty acids and organic nitrogen sources [74], while common repressors are glucose and glycerol [80,108].

The optimum temperature and pH for lipase production are extremely variable, being related to the genus, the species and even to the strain in study. *S. cerevisiae* lipase presents 30°C and 7.5 as the optimum temperature and pH, while *W. californica* optimum conditions are pH 6 and 20°C [86]. The optimum incubation temperature for *Candida (Yarrowia) lipolytica* and *Pichia (Hansenula) anomala* lipases is about 30 °C, while the optimum pH is between 5 – 6.5 and 6.5 – 7.5, respectively [109]. The optimum pH and temperature conditions for *Cryptococcus sp.* S2 lipase are 5.7 and 25 °C, respectively [85]. The genetically modified yeast *Y. lipolytica* LgX64.81 has optimum pH and temperature conditions of 7 and 37 °C, respectively [110]. pH has as important role in the hydrolysis of ibuprofen esters by the *C. rugosa* lipase (CRL) in an aqueous environment. At neutral pH (7.2), the flap opens and allows the bulkier substrate (butyl ester of ibuprofen) to diffuse into the active site. At acidic pH (5.6), there is a decreased opening of the flap, accommodating a more compact substrate (methyl ester). Thus, substrate selectivity of CRL is pH-dependent [111].

Tejo *et al.* [112] investigated the effect of organic solvents on the structure and dynamics of CRL. Although the organic solvent used (carbon tetrachloride) caused only minor structural effects, a highly specific effect on the flexibility of solvent-exposed side chains was noted. The polar side chains were more flexible in water but less flexible in the organic solvent, while the opposite was observed for the hydrophobic residues. Castillo *et al.* [102] demonstrated that lipases were more active in the solvents cyclohexane and octane. It was

possible to increase the specific activity and enantioselectivity of the lipase produced by *Candida rugosa* treating with 2-propanol [113]. Using organic solvents, enzymes can perform catalysis at temperatures far above those temperatures that denature enzymes in aqueous systems. Despite this, enzyme inactivation has been reported frequently for various organic solvents applied as reactional mean [102].

According to Kaieda *et al.* [114], it is possible to use *Candida antarctica* lipases for methyl ester biosynthesis in free solvent systems. The increasing of the water activity ( $a_w$ ) in the reactional mean decreases the reaction velocity, on the other hand some water activity is fundamental to the enzyme action. At very low  $a_w$ , the enzyme is not sufficiently hydrated and shows poor catalytic activity. At high  $a_w$ , thermodenaturation of the enzyme can occur. Intermediate  $a_w$  seems to provide an optimum in catalytic activity and stability.  $a_w$  control (principally for the water formed during esterification) can be done by the addition of salt hydrate pairs to the reaction mixture, or through a gas stream previously equilibrated with the salt hydrate pair [115].

Salts have a number of effects on protein properties including activity, conformational stability and solubility. These effects possibly arise from the binding of ions to specific sites on the protein, changing the degree of hydration. Yu *et al.* [103], studying the lipase of *Candida rugosa*, found that salt incorporation could keep the conformation of the enzyme, prevent the large change of optimum pH and improve the stability, thermostability and enantioselectivity of the lyophilized lipase compared to native one.

The importance of the lipase immobilization is well known in the industrial application of enzymes, as immobilized biocatalysts offer unique advantages in terms of better process control, enhanced stability, enzyme-free products, predictable decay rates, and improved economics. The immobilized enzymes can be used repeatedly or continuously in a reactional process and they can be easily separated from soluble reaction products and non-reacted substrates, thus simplifying work-up and preventing protein contamination of the final product. The methods frequently used are based on physical adsorption of the enzyme on a carrier material, on its entrapment or microencapsulation in a solid support or on its covalent binding to a solid matrix [116-117]. The most common immobilization matrices are polypropylene [118], polyethylene [119], Celite 545 [120], methyl acrylate divinyl benzene copolymer [121], and alkyl silane precursors [122], but chitosan can also be used [123].

#### 4.2 Biocatalyst engineering

The second most common approach for increasing lipase production is the genetic manipulation of the producing yeast. Most studies try to obtain mutants with higher lipase production

than the original strain, but the use of heterologous expression systems, such as *P. pastoris*, can also be viewed as biocatalyst engineering. An efficient way of enhancing lipase production can be achieved by gene amplification, as more copies of the *lip* gene will result in higher lipase production [78].

#### 4.3 Protein engineering

Lipase activity can be altered through structural modifications in the enzyme. Schmitt *et al.* [124] designed synthetic *C. rugosa* lip1 genes, expressed them in *P. pastoris*, and performed an assay for chain length specificity. He introduced mutations in different aminoacids in the protein, obtaining mutants with different chain length specificities. Magnusson *et al.* [69] also redesigned the size of the stereospecificity pocket of *C. antarctica* lipase B by changing the aminoacid Trp104, creating space for large secondary alcohols to interact with the lipase, thus increasing the number of good substrates for the enzyme. This approach is very promising and should be stimulated in order to obtain lipases with different specificities.

## 5 CONCLUSIONS

Most studies dealing with yeast lipase production were performed with ascomycetic yeasts, but basidiomycetic ones are promising and should be more explored. Although lipase production by *C. rugosa*, *Y. lipolytica* and *C. antarctica* are abundant in the literature, there are many emerging lipase-producing yeasts that represent a hope for biotechnological innovation in this area. Classical approaches aiming the improvement of lipase production are based on the manipulation of the cultivation conditions, including the culture medium, but new approaches are necessary in order to optimize lipase production and/or substrate selectivity. These new approaches are represented by the recombinant lipases and by protein engineering. It would be ideal that research groups with these different expertise worked together in order to obtain an efficient yeast biocatalyst capable of secreting a versatile lipase for industrial applications.

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### LIPASES PRODUZIDAS POR LEVEDURAS: CATALISADORES PODEROSOS PARA APLICAÇÕES INDUSTRIAIS

**RESUMO:** O termo “enzimas lipolíticas” refere-se a lípases e hidrolases éster carboxílico. A produção de lipase é ampla entre as leveduras, mas poucas são capazes de produzir lipases com características interessantes e em quantidades suficientes para serem industrialmente úteis. A literatura relativa a lipases produzidas por *Candida rugosa*, *Yarrowia (Candida) lipolytica*,

*Candida antarctica* e outras leveduras produtoras de lípases é revisada. O uso de lípases recombinantes é discutido, com ênfase na utilização de sistemas de expressão heteróloga e desenho de quimeras. Finalmente, as três abordagens que visam à melhora da produção de lipase ou a modificação da seletividade do substrato da enzima (engenharia do meio, do biocatalisador e da proteína) são discutidos.

**Palavras chaves:** *Candida antarctica*. *Candida rugosa*. *Lipase*. *Yarrowia lipolytica*. *levedura*

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