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Review

A Temporal Evolution Perspective of Lipase Production by *Yarrowia lipolytica* in Solid-State Fermentation

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Abstract: Lipases are enzymes that, in aqueous or non-aqueous media, act on water-insoluble substrates, mainly catalyzing reactions on carboxyl ester bonds, such as hydrolysis, aminolysis, and (trans)esterification. *Yarrowia lipolytica* is a non-conventional yeast known for secreting lipases and other bioproducts; therefore, it is of great interest in various industrial fields. The production of lipases can be carried on solid-state fermentation (SSF) that utilizes solid substrates in the absence, or near absence, of free water and presents minimal problems with microbial contamination due to the low water contents in the medium. Moreover, SSF offers high volumetric productivity, targets concentrated compounds, high substrate concentration tolerance, and has less wastewater generation. In this sense, the present work provides a temporal evolution perspective regarding the main aspects of lipase production in SSF by *Y. lipolytica*, focusing on the most relevant aspects and presenting the potential of such an approach.

Keywords: *Yarrowia lipolytica*; solid-state fermentation; enzyme production; agro-industrial residues; bioreactors

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1. Introduction

The global market of enzymes is majorly represented by carbohydrolases, proteases, and lipases, and it was estimated at USD 9.9 billion in 2019 and is expected to grow at a compound annual growth rate of 7.1% from 2020 to 2027 [1,2]. Enzymes have gained interest in the industry mainly due to their widespread uses under mild conditions [3], highlighting the use of lipases among the enzymes.

Lipases are enzymes that, in aqueous or non-aqueous media, act on water-insoluble substrates, mainly catalyzing reactions on carboxyl ester bonds [4], such as hydrolysis, aminolysis, and (trans)esterification [5]. Therefore, they have a broad substrate specificity and are "promiscuous", since they catalyze reactions other than their natural physiological ones [6]. Fungi and bacteria are known to produce lipases. In some cases, the fungal lipases are secreted, which is advantageous for production purposes [7]. *Yarrowia lipolytica* is a non-conventional yeast known for secreting large amounts of enzymes, including lipases (either wild-type or genetically modified strains) [8].

Among the possibilities of lipase production, solid-state fermentation (SSF) presents several advantages. Initially, industrial use of solid-state fermentation could be of great interest within countries that benefit from the availability of large amounts of agro-industrial residues, estimated worldwide to generate 1.3 billion tons a year of material that is no longer

consumed or transformed from appropriate processes [9]. These are low-cost materials, which aid in economic viability [10].

SSF utilizes solid substrates in the absence or near absence of free water [11] and presents minimal microbial contamination problems due to the medium's low water content. Moreover, SSF offers high volumetric productivity, targets concentrated compounds, high substrate concentration tolerance, and less wastewater generation [12,13].

In solid-state fermentation processes, residues from oleaginous cultures are preferable once they already have some lipid content that acts as an inducer for lipase production. Residues from soybean (*Glycine max*), olive (*Olea europaea*), and other systems could be employed [14]. When the residues lack any component for microbial development, medium supplementation can also be used to fulfill these requirements [15].

Among the agro-industrial residues generated, the components produced during soybean processing stand out. Soybean hulls are the first by-products obtained once it is removed from the seeds before oil extraction and meal processing [16]. Their composition varies in a large range because it depends on local and seasonal growing conditions and the applied treatments [17].

The low lignin content is a desirable characteristic for the use of this major source of carbohydrates as fermentable sugars [17]. Thus, they have been used as a single component or in association with other waste for the cultivation of several fungi aiming at the production of cellulolytic enzymes by *Trichoderma reesei*, *Aspergillus oryzae*, and *Phanerochaete chrysosporium* with corn residue [18]; by *A. niger* with waste paper [19]; *T. reesei* and *A. oryzae* with wheat bran [20] or without it [21]; lipids by *Mortierella isabelline* [22]; monacolin K and isoflavones by *Monascus pilosus* [23]; oxygenase by *Funalia trogii* with wheat bran [24]; and peptidase by *A. niger* with orange peels [25]. However, there is a gap regarding soy hulls for lipase production using *Yarrowia lipolitica* since the main information presented concerns the use of soybean meal and oil.

Thus, the present work provides a temporal evolution perspective, focusing on the most relevant aspects concerning the use of *Yarrowia lipolitica* for lipases production in solid-state fermentation (SSF).

2. Lipases

Lipases belong to the group of enzymes characterized as serine hydrolases (triacylglycerol acyl hydrolases, E.C. 3.1.1.3) [7] and are relevant for industrial use, mainly due to their selectivity. They are resistant to organic solvents, catalyze synthesis reactions [26], and act on diverse substrates with varying reaction rates [27]. They also allow the design of molecules with specific functional and nutritional properties [28].

In addition, from the X-ray crystallographic data, it was also verified that lipases have the following structural characteristics: they belong to the αβ-hydrolases fold family; have a conserved catalytic triad made of the amino acids serine, histidine, and aspartic/glutamic acid; present a "nucleophilic elbow", where the nucleophilic serine is found between a helix and a β strand (except for Lipase B from *Candida antarctica*); show a structural motif (lid or flap) covering the catalytic site, and display an oxyanion hole and three pockets for fatty acids (for triglycerides) [29].

The lid is a mobile motif that controls the substrate molecule access to the catalytic pocket [30], and some of their characteristics can be predicted by their structure, making them a target for protein engineering [31]. However, its presence is controversial. According to their lid domain, Khan et al. [31] classified a selected group of lipases from the Protein Data Bank.

In their classification, lipases may or may not present lids with one, two, or more loop/helices. The authors also concluded that a higher optimal temperature was related to larger lids, while small loop/helix lids were found in all mono- and diacylglycerol lipases.

The lipase catalytic activity depends on changes in the enzyme and substrate conditions. The interfacial activation, commonly observed in lipases, is the result of combined effects of the amino acid composition of the lid (and therefore the structure resulting from

interactions with the environment) and the properties of the interface (such as the dielectric constant and ionic strength) [4]. The enzyme undergoes rearrangements at the water–oil interface upon exposure to a less polar solvent, making the catalytic site available for substrate access [32]. The exception is the lipase B from *Candida antartica*. Although it presents lids with open and closed structures confirmed, the activation depends on pH and is associated with an unusual mechanism [4]. Despite this, conformational changes in response to the environment are still responsible for its activation.

The hydrolysis reaction mechanism requires the action of the three amino acid residues found in the conserved catalytic triad: histidine and aspartic/glutamic acid activate the hydroxyl group of serine, which attacks the substrate carbonyl, forming a stable tetrahedral intermediate stabilized by the oxyanion hole residues. Next, an available nucleophile (found at the interface) proceeds with the deacylation step, leading to product formation and enzyme regeneration [27].

The key feature that differentiate esterases from lipases is that esterases act on the water-soluble substrate [27], which is relevant for enzyme identification, given that simultaneous production of these enzymes is possible.

Esterases show activity before the critical micellar concentration (CMC) and may be produced concurrently with lipases. After interaction with an interface, the latter acts majorly, needing a structural rearrangement to reach an active conformation [32]. Therefore the reaction media may be formulated differently to favor either type of enzymatic activity. Detergents used to form mixed-micelles structures may not interfere with enzyme activity if they do not interact with the enzyme [33]. In contrast, if enzyme-detergent interaction occurs, this can result in enzyme inhibition or denaturation. In addition, anything that alters the properties, or the size of the surface, may change the reaction rates. For instance, smaller droplets are beneficial for higher reaction rates [34], and calcium ions tend to neutralize negative charges maintaining a low dielectric constant at the interface [32].

As esterases show no interfacial activation, their reaction rates may be described by Michaelis-Menten equations, as shown in Equation (1) [27], considering that water is an excess reagent in the reaction. In this case, S is the substrate bulk concentration (mol L^{-1}), E is the enzyme concentration (g L^{-1}), K_{cat} is the catalytic turnover (min^{-1}), and K_M is the Michaelis-Menten constant (mol L^{-1}).

$$\nu = \frac{E\kappa_{cat}S}{K_M + S} \tag{1}$$

In contrast, lipase reaction rates increase considerably after an interface is formed [27], and reaction occurs at the two-dimensional space of the interface. Theoretically, substrate concentration should not be calculated in relation to the bulk concentration but in relation to the surface concentration ($S_{surface}$) (mol $area^{-1}$) or as a mole fraction. The bulk concentration of the surface should also be described, as shown in Equation (2) [33]. This equation represents the two-step process of enzyme sequestration at the interface followed by catalysis.

$$\nu = \frac{Ek_{cat}S_{total}S_{surface}}{K_S K_M + K_M S_{total} + S_{total}S_{surface}} \tag{2}$$

Here, S_{total} is no longer the substrate bulk concentration but the total surface available; that is, the sum of the bulk molar concentration of the components forming vesicles, micelles, or any other type of structure with the substrate that interact with the enzyme. K_S is the dissociation constant for the surface and has the same unit as S_{total}. K_M now is shown in the same unit as $S_{surface}$ [33]. Since the substrate is now diluted on the surface, a solubility value is also expected in this case, limiting the highest values of substrate that can be used in the reaction. For instance, Berg et al. [35] found the maximum mole fractions of 0.37 and 0.15 for 4-nitrophenyl butyrate and tributyrin in 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG) vesicles. This equation may also reflect Michaelis-Menten behavior if one fixes the concentration of surfaces and varies the substrate mole fraction

and vice-versa. Moreover, inhibition effects may still occur, leading to alterations in the mathematical description.

3. Yarrowia lipolytica

3.1. General Features of Yarrowia lipolytica

Yarrowia lipolytica, an ascomycete previously known as *Candida lipolytica*, was named Yarrowia after David Yarrow identified it in 1972 and from its relation with hydrophobic substrates consumption. It has emerged as a model for many types of studies, including lipase production, dimorphism, lipid body biogenesis, among others [8]. The main characteristics for growth have already been described, including protocols for media preparation, genetic manipulations, and environmental conditions such as pH, temperature, oxygen demand, biotechnological applications, genetics, and physiology [36–38]. Therefore, only specific topics will be mentioned briefly in the text since a deeper description can be found in the literature aforementioned.

As a strict aerobe organism, aeration is essential to maintain high growth rates and appropriate temperature (until 34 °C) and pH (from 3 to 8). Furthermore, these conditions influence the morphology of the cells that can be ovoid, pseudo-hyphae, or true (septate) hyphae. Septate hyphae can be millimeters long and 3 to 5 µm in width [39].

The environmental conditions, namely physicochemical (pH, temperature, dissolved oxygen concentration and, osmotic stress), mechanical (agitation rate and pressure), and nutritional effectors (carbon and nitrogen sources and metal ions), has an impact on morphology and product formation (enzymes, citric acid, and lipid) [40]. Information about morphology is found in Table 1. Specific strain behaviors may be determinant, and a further study comparing strains in the same conditions need to be performed, especially in controlled bioreactors, to observe mono-effector responses. One example that reassures this statement is the observed effect in different agitation rates, which correlates with dissolved oxygen. Braga et al. [41] proposed that the mechanical stress could have induced pseudohypha formation, but the oxygen supply was not the same, and the medium in the experiment did not have glucose. Thus, multiple effectors could have provoked the final results.

Table 1. Morphology responses of *Y. lipolytica* to different environmental conditions.

Environmental Condition	Effector	Observed Effect
Physico-chemical	pH	Controversial observations of ovoid and mycelium forms in acid and neutral values
	Temperature	A decrease in intracellular cAMP *, Heat shock (4 or 37 °C) increases filament formation
	Dissolved oxygen	Limited oxygen favors filament formation, except for glucose-limited conditions
	Osmotic stress	Osmotic stress block hyphae formation in media with N-acetylglucosamine and cells become round and smaller, but not in serum
Mechanical	Agitation rate	Controversial observation regarding mechanical and pneumatical agitation
	Pressure	Until 8 bar no effect is observed (no oxidative stress)
Nutritional	Carbon source	Glucose, N-acetylglucosamine, serum, and hydrophobic substrates (oils in general, except for castor oil) induce hyphae formation
	Nitrogen source	Controversial observations regarding organic and inorganic sources
	Metal ions	Deficiency of Mg^{+2} ($<2 \times 10^{-5}$ M), Fe^{3+} ($<10^{-7}$ M) suppress mycelia development Controversial reports over Ca^{+2} ions

* cAMP: cyclic adenosine monophosphate. Adapted from Timoumi et al. [40].

A novel process with immobilization, efficient and stable microorganism colonization (12 repeated batches with a total process time of 460 h) on the cotton towel was observed.

Using the same cell concentrations in free and immobilized cultures, as proven by scanning electronic microscopy, increased production rates were achieved. However, the authors did not define the morphology distribution of the immobilized cells. Therefore, it is not clear whether there is a correlation between morphology and production [42].

More recently, Vandermies et al. [43] obtained a morphological mutant strain (deletion in the YlHSL1 gene involved in mitotic cell cycle regulation) growing in a pseudohyphal form that could efficiently colonize stainless steel structured packing (98.3% of attached cells). They emphasized that the yeast could attach to the material without an "immobilization step" and a higher total cell concentration. Thus, the total time would be reduced for an immobilized-cell process, being more efficient, and the cells could be removed.

Studies of morphological transition normally use carbon sources like glucose, N-acetylglucosamine, and serum to induce a morphological transition in this yeast [44,45]. Besides these, *Y. lipolytica* can consume most of the commonly available sugars (glucose, fructose), including hydrophobic substrates, such as alkanes [46], triacylglycerols, fatty acids, glycerol, and acetate [47]. However, it cannot hydrolyze industrial polymeric raw materials, such as starch, inulin, xylan, cellulose, hemicelluloses, and monomeric sugars like xylose and arabinose rhamnose, galactose, and molasses. Non-consumption of dimeric polymeric substrates is due to the lack of genes encoding for the enzymes, or they are very poorly expressed, and synthetic biology tools are of interest to build robust industrial strains [48]. Nevertheless, despite the efforts to obtain promoters for reliable and reproducible mutations that add desired features to this microorganism, Larroude et al. [49] have reviewed the available tools (including five genomic-scale metabolic models) and stated that synthetic biology alternatives for strain development are emerging as tools to make *Yarrowia lioplytica* a model strain, but still need further advances.

3.2. Regulation and Characteristics of Lipases from Yarrowia lipolytica

Genomic data from this yeast has revealed the presence of protein families of 16 lipases, 4 esterases, 38 aspartyl proteases, and 10 alkaline proteases [36], indicating the ability to produce a diverse amount of proteins. Among those, lipases have been extensively studied regarding biochemical characterization, culture conditions for production, industrial and in vivo applications [50–52].

Lipases expression is proven to be repressed by glucose. Overexpression of the hexokinase Hxk1 resulted in reduced lipase production in a superproducer mutant strain incapable of performing hexose phosphorylation as the wild-type strain [53], suggesting that the activity of this enzyme was correlated to repression of Lip2 expression and lower activities.

Lipase is also regulated by triacylglycerol molecules which activate the Soa (specific oleic acid) genes. This regulation is somehow performed by two intracellular proteins unrelated to β-oxidation POX2 gene or oleic acid induction. The deletion of these genes still allows the induction of Lip2 expression by oleic acid and thus indicates the existence of another mechanism for free fatty acid consumption [54].

Recently, Sassi et al. [55] observed that co-fermentation of oleic acid and glucose in complex media composed of tryptone was able to induce Lip2 expression ten-fold higher than without glucose. Thus, the authors concluded that as glucose is also present, cells metabolize both carbon sources simultaneously, glucose being used for energy production and oleic acid for induction instead of lipid storage. Lastly, a complex nitrogen source also seems to regulate lipase production, and once Turki et al. [56] observed that pepsin and tryptic casein digests promoted high lipase synthesis. However, the authors verified that commercial peptone and tryptone worked better than homemade digests or yeast extract and attributed this to the degree of casein hydrolysis. Complete hydrolysis of proteins is not of interest; instead, small peptides are the ones that induce the production of lipases.

Some lipases expressed by *Yarrowia lipolytica* in submerged fermentation (SmF) have been characterized and the main features reviewed by Fickers et al. [26] They include Lip2p, Lip7p, and Lip8p. These authors concluded that these lipases have complementary

functions based on their substrate specificity for medium and long-chain acids, but Lip7p should be classified as an esterase due to its specificity for smaller ester molecules. Besides, specific information of lipases produced from the strain IMUFRJ 50682 has also been reported [57–59]. More recently, naturally immobilized lipases (possibly a mixture of Lip2p, Lip7p, and Lip8p) from this strain were also studied. These were obtained either from cell debris (after SmF and followed by cell disruption, named cell-bound lipase fraction-CBLF) [60–62] or from the lyophilized solid biocatalyst (SB) [63]. All these data are summarized in Table 2. A critical aspect of produced lipases Lip2 and Lip8 is that they seem to be irreversibly inactivated by extreme pH changes and also by the lack of an emulsifying agent in the reaction media, as was shown by Aloulou et al. [50] and Kamoun et al. [64] using the lipolytic method.

The crystallographic structure of the main lipase produced by *Y. lipolytica* has already been published by Bordes et al. [30], revealing that it is an α/β-hydrolase with its catalytic triad composed of the serine (S162), histidine (H289), and aspartic acid (D230) residues. However, one of the amino acid residues of the oxyanion hole (T88) differs from other lipases. Homology studies and molecular dynamic simulations by these authors indicated that this lipase requires drastic conformational rearrangements to have the lid opened. This is done in a two-step process. In the first step, the lid was found in a semi-open conformation at the octane–water interface that did not prevent substrate binding. The lid is then fully opened after substrate binding. Meanwhile, lid opening and closing follow different pathways. In organic solvents (which are relevant in esterification reactions), hydrophilic residues must be exposed for their closure, which explains the loss of activity when methanol is used. In contrast, the weaker interaction of hexane, a nonpolar organic solvent, can only leave its lid partially opened [65].

The structural information is essential for selectivity and process application [30]. For *Y. lipolytica* Lip2p, Cao et al. [66] used β-cyclodextrin to keep its activity in methanol, demonstrating by molecular simulation that this substance stabilized the "pathway" for lid closure, holding it partially opened. Sheng et al. [67] rationally chose a region close to the lid one for mutagenesis of Lip8p, altering its optimal operating temperature and substrate specificity towards longer acyl-chain esters. Augustyniak et al. [68] used the available crystal structure of *Bacillus subtilis* lipase to improve its thermostability, which resulted in a modified protein with a reduced tendency to precipitate in high temperatures due to changes in the superficial amino acid residues.

Other characteristics are also found in the literature for these lipases, such as enzyme aggregation. Brígida [59] attributed the observed high molecular weight bands while studying the purification of lipases from *Yarrowia lipolytica* to the aggregation of enzymes in the concentrated extracts. Aloulou et al. [50] verified lip2p aggregation to lipids after performing gel-filtration on the supernatant from production with oleic acid as lipase inducer. Kamoun et al. [64] also reported aggregate formation due to the presence of lipids in samples of Lip8p. After purification with gel filtration and anion exchange chromatography, the authors have immunodetected Lip8 in supernatants as 2 species, one of 39–40 kDa and another of 37 kDa (majority), and speculated that this difference could be related to the production of a pro-enzyme that undergoes further processing outside the cell or a membrane-anchored enzyme (most likely) that suffers some cleavage to be released into the extracellular media. Similar results were observed by Fickers et al. [69].

Deglycosylation of the enzyme showed that sugar moiety corresponded to 4% of the enzyme mass in its mature form. Lip7 seems to have a similar molecular size compared to Lip8. Aloulou et al. [50] found that multiple isoforms of Lip2 are generated through the post-translational processing of proteins related to glycosylation inside the Golgi apparatus. Until leaving the endoplasmic reticulum where, as a net result, a 10-residue Man8GlcNAc2 is transferred to the protein [70]. Yeast glycosylation is conserved among other types of eukaryotes (either higher or lower eukaryotes), differing in the Golgi apparatus after the action of glycosyltransferases [52].

Table 2. Compilation of general features of lipases from *Yarrowia lipolytica*.

Lipase and Production System	Activators	Inhibitors	pH and Temperature (Optimal)	Localization and Glycosylation Sites	Solvent Stability	Substrate Specificity	References
Lip2p, SmF	Ca^{+2}, Mg^{+2}, bile salt	Zn^{+2}, Ni^{+2}, Cu^{+2}, oleic acid	6–10, 37 °C (most reported), 55 °C	Cell wall (growth phase) and extracellular (end of growth phase), 2 sites (N113 and N134)	90% in acetone, methanol, ethanol, isopropanol, DMSO (10%), 60% in methanol (20%) and DMSO 95%)	$C_{8:0}$ and $C_{18:1}$ triglyceride, $C_{12:16}$ methyl fatty acid	[26,57–59]
Lip7p, SmF	-	-	-	Cell wall (phosphate buffer extraction), 1 site at aa 140	-	*p*-NPC6	[26]
Lip8p, SmF	Bile salts	-	7.5–9.5, <30 °C	Cell wall (phosphate buffer extraction), 1 site at aa 140	-	*p*-NPC8, *p*-NPC10, and *p*-NPC12 (after enzyme engineering)	[26,64,67]
CBLF, SmF	-	-	7 (37 °C)	bound to cell membrane/wall	80% in 10% *v/v*) in ethanol and acetone	*p*-NPC16	[60,62]
SB, SSF	Ca^{+2} (2 mM)	Ca^{+2} and Mn^{+2} (>20 mM), Hg^{+2}, Fe^{+3}, EDTA	7 (50 °C), 8 (37 °C)	-	Stable (at 9% *v/v*) in hydrophobic solvents, glycerol, methanol, ethanol, acetone, acetonitrile, ether, DMSO; Stable (at 30% *v/v*) in hexane and heptane	$C_{8:0}$ triglyceride, coconut oil, and palm kernel oil	[63]

SB: Solid biocatalyst obtained by lyophilization. SmF: submerged fermentation; SSF: solid-state fermentation; aa: amino acid; *p*-NPCX: p-nitrophenyl alkanoate with X carbons in the acyl chain; CBLF: cell-bound lipase fraction.

However, the N-glycosylation patterns at N113 and N134 of lipase produced by *Yarrowia lipolytica*, that is, the ones obtained from Lip2 expression, do not seem to be critical for its catalytic activities (retain more activity for longer chain acyl substrates than for p-nitrophenyl butyrate) and secretion [71]. Even though only two sites of N-glycosylation are observed, it seems that the occurrence increases the adsorption of the enzymes at the lipid–water interfaces [72]. Thus, the level of glycosylation depends on the microorganism, ranging from 8.3% in *Pichia pastoris* [72] to 13.5% in *Yarrowia lipolytica* [50,71] in mass, representing highly glycosylated proteins. Moreover, increased resistance to proteolytic activities was observed through assays with pepsin (aspartic protease or "acidic" protease), chymotrypsin, and trypsin (serine proteases), which are observed in samples of the wild-type strains with regular glycosylation patterns, with a higher sensibility towards the serine proteases [72].

Taking into consideration that *Yarrowia lipolytica* is also capable of producing high amounts of protease, as previously mentioned by several protease families, it is important to have in mind that for the accumulation of lipases in the fermentation media, any sort of strategy may be crucial for maintaining its activity, including the absence of production or inhibition of protease activities.

4. Solid-State Fermentation (SSF)

4.1. Lipase Production by Yarrowia lipolytica in Solid-State Fermentation

Among the publications available that use solid-state fermentation for *Yarrowia lipolitica* growth, the majority (60%) are focused on the production of lipases, presenting experiments performed in Erlenmeyer flasks, Petri-dish plates or slants, being considered as tray bioreactors-except for the work of Try et al. [73] that employed a forcefully aerated bioreactor. The main characteristics of these processes regarding the vessels and environmental conditions are summarized in Table 3. Regardless of the product, the temperature was mostly 28 or 30 °C, the usual temperature range for increased growth, while particle diameter was usually smaller than 2 mm. The moisture content, however, varied between 50 to 90%. When considering soybean products, Okara has also been used as a source of nutrients for *Yarrowia lipolytica*.

Specific reports from lipase production by *Y. lipolytica* in solid-state fermentation date from 2003. The strain *Yarrowia lipolytica* CECT 1240 (ATCC 18942) was used for individual investigation of nylon sponge (inert support employed as control), barley bran (supplemented or not with corn, sunflower, or olive oil), and triturated nut, all of them impregnated with culture media composed of glucose, urea, minerals, and vitamins, with the production of 23 kU L^{-1} of lipolytic activity on tributyrin for the triturated nut (the highest one) [74].

In Asia, Indian researchers started publishing a series of papers on this subject in 2007. The first, by Imandi et al. [75], aimed at evaluating the combination of abundant solid substrates in India, namely sugarcane bagasse, wheat bran, and rice bran, with carbon, nitrogen, and humidity sources by univariate exploration in slanting position, resulting in 9.3 U g $D.W^{-1}$ (hydrolytic activity) for 10 g of sugarcane bagasse with wheat bran, 80% moisture content and supplementation with 1 g of urea and glucose. Later on, in 2010, Imandi and contributors published two reports on media formulation for the same strain (*Y. lipolytica* NCIM 3589), but at this time, the solid material was niger seed (Guizotia Abyssinica) oil cake [76] and palm kernel (Elaeis guineensis) cake [77], using Plackett-Burman designs for evaluation of carbon and nitrogen sources. They obtained 26.42 and 18.58 U g $D.W^{-1}$ of hydrolytic activity, with 60 and 50% moisture content, 5 and 6% m/m glucose, respectively, with 1.5% m/m urea on both cases. Finally, Imandi et al. [78] reported lipase production on mustard oil cake, with the same type of methodology for the verification of influencing variables, with the final production of 57.89 U g $D.W^{-1}$ (hydrolytic activity), for 50% moisture content, 1.5% m/m urea, and 7% m/m glucose. Common issues investigated by these authors within their publications are the diverse

range of nitrogen (1 to 5% m/m) and carbon sources (1 to 9% m/m). In all cases, glucose and urea were the best options.

In the same year of the last report by the Indian group, Moftah et al. [79] used crude olive oil cake (5 g in 150 mL Erlenmeyer flasks) with 0.5 mL inoculum of the strain NRRL Y-1095. The authors found 40 U g D.W^{-1} (hydrolytic activity) for the alkaline pretreated (NaOH 3% *w/v*) with yeast extract (3% m/m) as the better nitrogen source to be supplemented.

In Brazil, production started with the strain IMUFRJ 50682 in 2014. Farias et al. [80] examined for 48 h the production of lipases and proteolytic enzymes having cottonseed cake and soybean cake supplemented with its sludge. Proteolytic activity was always observed after the peak of lipase production for the two agro-industrial wastes, corresponding to hydrolytic activities (in p-nitrophenyl dodecanoate) of 102 and 139 U g D.W^{-1}, respectively.

Lopes et al. [81] investigated the combination of two-phase olive mill waste and wheat bran with nitrogen sources, such as urea and ammonium sulfate, finding that the latter was the best option. pH remained acidic throughout the cultivation time with this nitrogen source, and this was stated as one of the possible reasons that could have avoided the decay of hydrolytic activity once alkaline proteases would not be produced. The highest activity of 486 U g D.W^{-1} was obtained at 96 h.

In a more detailed study, Souza et al. [82] applied the fractional factorial, and central composite rotational (CCR) designs to investigate how to increase lipase production with soybean cakes and compared it to canola cakes without supplementations. The evaluated variables were inoculum size, moisture content, soybean oil, and urea concentration. Oil supplementation was in the highest case equal to 1.8% m/m, once the purpose was to induce lipase production and avoid that cell consume the added oil as the main carbon source. For canola cake, the highest lipolytic activity (for olive oil as substrate) of 72.6 U g D.W^{-1} was obtained with 62% moisture content and 1.44 mg g D.W^{-1} inoculum. In comparison, for soybean cake, it was 93.9 U g D.W^{-1}, with 55% moisture content, 0.73 mg g D.W^{-1} inoculum, and 1.5% m/m soybean oil. The cell growth was assayed in both cases, and different profiles were obtained. Linear growth was observed for canola cake, and it was observed along with protein production. However, cell growth was verified only after the lipase activity decay with exponential behavior for soybean cake, which could be confirmed for a longer fermentation process. For these cakes, the optimal pH and temperature for lipolytic activity were also evaluated by CCR. The results were 7.6 and 41.9 °C for lipases from canola and 7.2 and 40.3 °C for soybean meal.

The latter served as a basis for other studies on lipase production and application. Silva et al. [85] used solid enzymatic preparation (SEP) in esterification reactions to decrease the free fatty acid content in acid oils used for biodiesel production. The authors also evaluated the catalyst properties regarding its reuse, reaching 6 batches of reused SEP. Souza et al. [63] improved lipase production by using higher inoculum and soybean oil supplementation. The authors also investigated its application, in short, medium, and long-chain esters synthesis. Additionally, aiming at polyethylene terephthalate (PET) degradation, Sales et al. [86] tested the combined use of watermelon peels and soybean meal to induce esterase and lipase production. This enzyme pool led to a higher concentration of terephthalic acid during PET hydrolysis than the enzyme pool obtained with soybean meal alone, showing potential for other hydrolysis applications.

Table 3. Main process conditions used for solid-state cultivation of *Y. lipolytica*.

Y. lipolytica Strain	Support/Substrate	Maximum Activity	Product	Temperature (°C)	Initial Moisture Content (%)	Particle Diameter (mm)	Ref.
CECT 1240 (ATCC 18942)	Barley bran and Triturated nut	23 kU l	Lipase	30	90	-	[74]
NCIM 3589	Sugarcane bagasse, wheat bran, and rice bran	9.3 U/gds	Lipase	30	80	-	[75]
NCIM 3589	Niger seed oil cake (*Guizotia abyssinica*)	26.42 U/gds	Lipase	30	60	2	[76]
NCIM 3589	Palm Kernal cake (*Elaeis guineensis*)	18.58 U/gds	Lipase	30	70	2	[77]
NCIM 3589	mustard oil cake (*Brassica napus*)	57.89 U/gds	Lipase	30	50	2	[78]
NRRL Y-1095	Olive mill wastewater and crude olive oil cake	850 IU dm^{-3}	Lipase	30	55	0.2–0.5	[79]
NRRL Y-1095	Canola meal, almond meal, coconut oil cake, soybean meal (better producer), barley bran, and wheat bran	~0.6 U	Lipase	30	-	1.5	[83]
IMUFRJ 50682	Cottonseed cake; and synergism of soybean bran + its sludge (better producer)	139.0 U/g	Lipase	28	58	<1.18	[80]
IMUFRJ 50682	Two-phase olive mill waste supplemented with a mineral nitrogen source	486.0 U/g	Lipase	28	53	<2	[81]
IMUFRJ 50682	Soybean meal	93.9 U/g	Lipase	28	55	<1.18	[82]
M53	Buckwheat husk and okara	22.1 U/gds	Lipase	30	70	-	[84]
IMUFRJ 50682	Soybean bran	16 U/g	Lipase	28	55	<1.18	[85]
IMUFRJ 50682	Soybean meal	106.7 U/g	Lipase	28	55	<1.18	[63]
IMUFRJ 50682	Watermelon peels	75.22 U/g	Lipase and esterase	28	55	<1.18	[86]

4.2. Solid-State Fermentation, Substrates, and Bioreactors

SSF utilizes solid substrates in the absence or near absence of free water, which is a more appropriate condition for the growth of filamentous fungi [11] and offers a series of advantages over submerged (or liquid) fermentation, which resemble the natural habitat for several microrganisms [87]. The low water contents in the medium present minimal problems with microbial contamination, especially with a lesser risk of bacterial contamination. The microbial growth and product formation occur on the surface of a solid substrate that works as support or on an inert material impregnated with nutrient solution [88]. After the SSF process, the target compounds are recovery through extraction and separation steps. SSF offers high volumetric productivity, target concentrated compounds, tolerance of high substrate concentration, and less wastewater generation [12,13], unlike submerged fermentation, where the target products tend to be diluted at the end of fermentation [89,90].

The SSF presents extended stability of products, lower production cost, lower protein breakdown (which is especially important if an enzyme is the target product), lower energy requirement, lower energy costs for sterilization, lower fermentor volume, and lower catabolic repression [91,92]. Furthermore, the SSF presents a scarce operational problem, better oxygen circulation, and less effort in downstream processing [87].

Technical and economic viability is directly related to the choice of essential parameters, including the nature of the solid substrate, chemical compositions, and physical state of the substrate (particle size), which affect the microbial physiology and, consequently, the productivity of the process. Other important factors include the selection of microorganisms, substrates, physical-chemical and biological parameters of the process [91,92].

Another advantage is related to the fact that in SSF, agro-industrial wastes and by-products can be utilized as a nutritional source for microorganism species to obtain the lipase enzyme. The choice of substrate will depend on the nutritional needs of the microbial species to produce the target compound [93]. Among the substrates used for the production of lipase, stand out cottonseed cake [80], soybean bran, sugarcane bagasse [94], wheat bran, spent barley [95], olive mill waste [81], by-products from corn oil refining [96] and others reported in the literature efficiently used individually or supplemented with other nutritional components. In addition to solving an environmental problem, through the alternative use instead of improper disposal, the use of agroindustrial wastes and by-products allows the reduction of the costs of the biotechnological process since they are abundant and have a low cost [93,97,98].

As general characteristics, substrates must have a considerable lipid content, and composition (oil or triacylglycerol or any other inductor) since the induction of lipase production depends on the type of lipid used [99] as a carbon source [100]—lipidic carbon sources seem to be essential for obtaining a high lipase yield [101]—being the major factor for the expression of lipase activity. Furthermore, other materials, such as surfactants, fatty acids, some esters, glycerol, and biliary salts, can be used to induce lipase production [102].

Lipase production requires carbon and nitrogen sources as required by any fermentation process. Most lipase production studies do not use simple sugars as carbon sources. They instead use lipid substrates as sole carbon sources. On the other hand, the protein content must be established appropriately, since a large amount of protein present in agro-industrial wastes can induce the microrganisms to synthesize higher amounts of proteases during fermentation, somehow decreasing lipase production [103].

Generally, the production of lipases increases when the relative percentage of C18:n fatty acid esters in the respective substrate is increased or is present in higher concentrations. The acid is transported, consumed, transformed, and stored in the cell. The produced lipase is then excreted and can be separated. The synthesis and secretion of the lipase (intra and extracellular) increase with increasing lipid concentrations, although excessive levels in the growth medium may be cytotoxic [104].

It is important to note that the mechanisms regulating lipase biosynthesis vary widely in different microorganisms. Although the induction mechanism is well known, it is essential to properly establish the conditions of process and composition of the medium to guarantee the nutritional needs of the microorganisms used [80]. In this sense, consideration should also be given to the possibility of combining agro-industrial by-products and residues to supply the species' metabolic needs, especially in the absence of specific lipid components, which can suppress enzymatic activity [75].

Vessels for fermentation processes vary significantly in geometry and mode of operation, and their main objective is to guarantee the appropriate environment for the development of the cells. The main operational variables that may impact growth are monitored whenever possible to accomplish that. Hence, monitoring and modeling the physicochemical and biological phenomena happening inside the reactor allows design and scale-up of better processes, prediction of process behavior, development of control strategies, and optimization of the operating conditions. According to Mitchell et al. [105], there are many types of bioreactors for solid-state fermentation, and they are classified into

four groups concerning their operation: static bioreactor with or without forced aeration through the bed and agitated beds with or without forced aeration through the bed. The air supplied to the systems can have its temperature, humidity, and airflow controlled in all cases. The air, which contains water, is necessary to provide oxygen to the cells and remove CO_2 and heat from the bed. Drying happens as water is consumed by the microorganism and evaporates from the solid's surface as heat is generated. Therefore, the authors state that axial and radial temperature gradients are inevitable and summarize the main phenomena inside the bioreactor as metabolic reactions that produce heat, heat conduction (which is less important in forcefully aerated reactors such as the packed bed) and convection, evaporation, and convective mass transfer. Furthermore, pressure drop tends to intensify as the microorganism grows and penetrates the solids, and the flow regime approximates to the plug flow as a result of passing through the void part of the bed.

4.3. Cell Analysis in Solid-State Fermentations

Biomass estimation is still a relevant topic of research in solid-state fermentation. The natural limitations imposed in this type of process, such as system heterogeneity and microorganism growth into the solid matrix, make direct biomass determination challenging [106–108].

When microorganisms grow attached and even linked to solid matrices, like in most solid-state fermentation, offline cell analysis methods are majorly based on the estimation of cell components, such as the cell wall components like chitin [109–111]. However, extraction of cellular material is time-consuming, including hydrolysis and chemical reactions based on the compound to be assayed. Beyond that, hydrolysis of the fermented material may generate a background signal from the solid matrix [107]. Aidoo et al. [110] compared four different methods based on chitin quantification and found that alkaline depolymerization and deacetylation of Koji fermentation samples produced the lowest background signals.

Souza et al. [82] evaluated the growth of *Y. lipolytica* IMUFRJ 50682 in canola cake and soybean meal after acid hydrolysis with 6 M HCl, and no variation of N-acetyl glucosamine was observed in both growth phases after 4 and 20 h. The values seem to have been blankly discounted since Aidoo et al. [110] found high initial values of this component at the beginning of Koji fermentation. Thus, it is difficult to determine whether cells did not grow and remained in a lag phase or if they initially decreased in number, and this was masked by the supposed blank discounting.

Besides the existence of the target component in the solid matrix, cell composition and products may vary in response to the environmental conditions [107]. For example, Vega et al. [112] analyzed the wall components of *Y. lipolytica*. They found that chitin represented approximately 7 and 15% of the wall in yeast cells and septa in hypha, respectively, after chemical hydrolysis. Hence, two main aspects should be taken into consideration: cells may or may not undergo morphological transition, meaning that the increase in the chitin content may not be appropriate to evaluate growth in SSF; if cells do not grow well on the solid matrix or they are from the beginning of fermentation, data interpretation may be disturbed by the influence of the solid support since the chitin percentage on the cell wall is quite small.

A systematic approach to quantification methods has been performed by Steudler et al. [107] After evaluation of several methods of analysis (quantification of ergosterol, glucosamine, nuclei number, nucleic acids, protein content and genomic DNA, ligninolytic and cellulolytic enzymes, glucose content and respiratory analysis), the authors verified that respiratory analysis, nuclei count (aided by flow cytometry for a rapid procedure) and ergosterol quantification were the most suitable for monitoring of the fungus *Trametes hirsuta*. Respiratory analysis has been used as an indirect online method [108,113]. This analysis is based on the off-stream gas leaving the bioreactor in solid-state fermentation. CO_2 and O_2 are measured with online sensors in the gas stream, which sometimes passes by a silica column to remove water and other components. A simple mass balance in the gas phase is used to quantify the production and consumption of these gases. Botella et al. [108]

also used capacitance measurement as a potential new method, although developments are still required to have absolute biomass values. Other than that, these authors also used a total mass balance to estimate fungal growth, assuming that the only product removed from the bed is CO_2 and that product formation followed a Luedeking-Piret model.

To circumvent the time-consuming and labor-intensive procedures used in ergosterol quantification, Mansoldo et al. [114] studied strategies for rapid ergosterol extraction and simple quantification using ergosterol autofluorescence. Their results proved that ultrasound-assisted extraction for 20 min was sufficient for later detection by excitation-emission matrix measurements, even in the presence of an interferent component. However, it is unclear whether or not the method could present a limitation in extraction with real fermented material since the samples consisted of solid residue commonly used in SSF processes mixed with the target fungus (*Schizophyllum comune*).

Even though these indirect quantification procedures give an idea of growth, no physiological state information is obtained. Cell viability is also important because it reflects its active state, and as reactions rates depend on the cells, monitoring becomes necessary [115]. Various methods were already used to distinguish morphologies and size distribution of *Y. lipolytica*, including morphonogranulometry, coulter counter, density gradient centrifugation, diffraction light scattering, flow cytometry, and electronic or optical microscopy, as it was reviewed by Timoumi et al. [40], being the techniques cited in ascending amount of reports. The authors state that the more recent techniques provide information on a single-cell level, allowing studies of the subpopulations.

Digital image analysis has also received some attention as a potential tool for biomass monitoring. Once images have been acquired, the processing is required to extract information. The main steps of image processing include preprocessing, where contrast enhancement and filters are used; segmentation, where cells are separated from the image background; and classification and information extraction, when cells are separated into different populations, usually using measured properties [116]. Besides, proper cell preparation and visualization before image acquisition are necessary. Coelho et al. [117] had to focus the cell borders for images with more significant differentiation from the image's background. In contrast, Peclat [118] and Braga et al. [41] used safranin to stain the cell surface.

In this sense, dark-field microscopy has already been applied to extract information for various types of cells. The dark background and the objects provide enough contrast, which is advantageous when compared with bright field microscopy [119]. Moreover, the light scattering produced by the microscope allows expansion of the resolution of the images, with an increased signal-to-noise ratio. Nevertheless, errors from light scattering may occur if cells are in contact, since it happens at the cell edges, and therefore the signal will not be proportional to the number of cells [120]. Examples of application of this type of microscopy include examination of bacterial biofilms and their motility [120], cell tracking of amoebae [119], in situ viability classification of *S. cerevisiae* [115], and viability classification of animal cell cultures [121].

For the yeast *Y. lipolytica*, a few works have been described using digital image analysis. Kawasse et al. [122] used this technique to analyze dimorphism under thermal and oxidative stress. Braga et al. [123] evaluated the cells stained with safranin to study aroma production in SmF. Later, the same group reported a morphology study of the same strain without safranin staining in bright field microscopy [123]. In a more recent publication, Botelho et al. [124] studied *Y. lipolytica* adhesion onto polystyrene, PET, teflon, and glass. The authors quantified the area colonized by the cells using strains IMUFRJ 50682 and W29 and associated the higher adhesion to polystyrene with the more hydrophobic characteristic of this material. In SSF, Peclat [118] developed a protocol for biomass sampling after fermentation and was able to verify the evolution of *Y. lipolytica* population obtained in solid-state fermentation in bright field microscopy, but with safranin staining, it being a promising tool for better understanding of this type of process. A limitation found in the latter is the manual choice of threshold limit for segmentation.

Images have also been used for filamentous fungi quantification without performing cell extraction. In this case, images of the fermented solid are made and directly analyzed. Duan et al. [125] extracted the contours of the material and used fractal geometry to quantify over time the changes in the solid fermented by the *Penicillium decumbens* system, as it is promising as a non-destructive method of analysis and online cell monitoring of solid-state fermentation. This idea of whole-material analysis was also studied in fermentation with *Rhizopus oryzae* NRRL 195 and *Aspergillus awamori* with sugarcane bagasse and industrial wheat residues [126], although in this case, the authors used the fungi color development to extract biomass information. They also point out that a natural limitation to this method may exist if the fungal colony does not differ from the solid matrix considerably in color.

5. Conclusions

Due to their ability to act on insoluble substrates in aqueous and non-aqueous media, lipases have versatility for potential applications in the food, detergent, pharmaceutical, leather, textile, cosmetic, and paper industries.

Yarrowia lipolytica, a non-conventional yeast, is a microbial model suitable to produce large amounts of lipases, mainly due to the process's metabolic characteristics and operational conditions that corroborate its viability. In addition, it can be produced through solid-state fermentation that offers high volumetric productivity, target-concentrated compounds, tolerance of high substrate concentration, and less wastewater generation.

Another advantage that supports the use of *Yarrowia lipolytica* for lipase production is the possibility of using agro-industrial residues as a substrate, since these residues are generated in large quantities during the processing of raw materials, making it extremely necessary to adopt strategies for the integral use of residues or, even, for conversion into higher value-added products.

Nevertheless, there are some gaps herein reported for the adequate scheduling of the lipase production process by *Yarrowia lipolytica*, especially regarding the monitoring of biomass and the type of agro-industrial residue, leading to the appropriate cell-substrate binomial.

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