



## REVIEW ARTICLES

# Biosynthesis of fatty acids and biosurfactants by the yeast *Yarrowia lipolytica* with emphasis on metabolic networks and bioinformatics

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### KEYWORDS

*Yarrowia lipolytica*;  
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Computational tools.

**Abstract:** *Yarrowia lipolytica* is a haploid, aerobic, and non-pathogenic yeast with biotechnological importance due to its lipolytic and proteolytic properties. It is capable of producing lipases, biosurfactants, long chain fatty acids, and also metabolizing different types of carbon sources such as hydrocarbons. Besides, *Y. lipolytica* also receives attention due to its ability to produce organic acids. In this review, we explore its metabolic abilities to produce fatty acids for the fine chemical industry (fatty acid-derived bio-based compounds) and also biosurfactants, important for the food and pharmaceutical industries and bioremediation. With the accelerated advance of 'omics' technologies, e.g., genomics, proteomics, metabolomics, genetic and metabolic engineering, combined with bioinformatics, substantial data are increasingly available, allowing to optimize biotechnological applications of this microorganism. Thus, this review covers the biosynthesis of fatty acids and biosurfactants by *Y. lipolytica*, exploring the current knowledge of how this yeast regulates the biosynthesis and accumulation of these compounds and the role of cutting-edge technologies in understanding and engineering its metabolism. Topics to be addressed in this review include the channeling of different carbon sources into lipid metabolism, the role of nitrogen and other growth factors in the metabolism of target compounds, besides fatty acid transport, regulatory networks, and the computational models available for metabolic engineering *Y. lipolytica*.

### Highlights

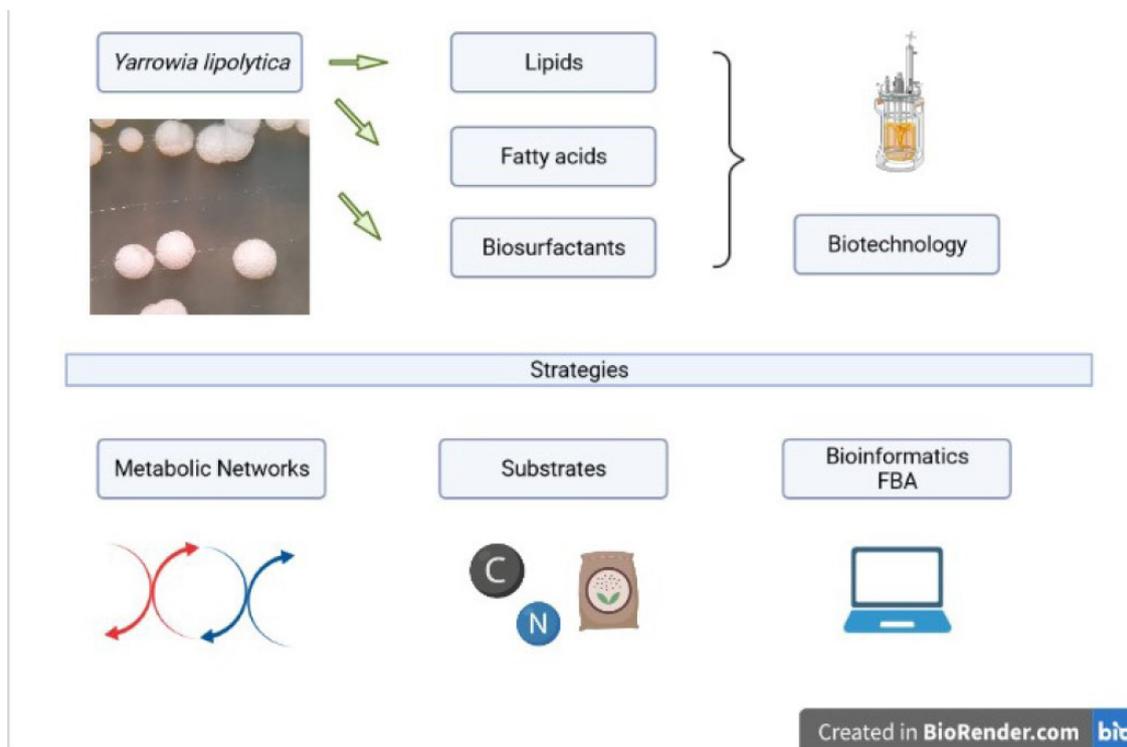
- *Yarrowia lipolytica* is a yeast with biotechnological importance due to its lipolytic properties;
- An overview of the fatty acid metabolism of this yeast is explored;
- The channeling of different carbon sources into lipid metabolism and the role of nitrogen is addressed;
- An overview of biosurfactants and emulsifiers produced by this yeast is presented;
- Computational models available for this yeast are discussed.

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## Graphical Abstract



## Introduction

The use of yeasts in biotechnology is not new, as they have been used in fermentation processes for over 8,000 years. This group of fungi is of great importance in food, biomedicine, biocontrol, environmental biotechnology, fundamental biological research, and heterologous protein production (Johnson & Echavarrri-Erasun, 2011). *Saccharomyces cerevisiae* has been the best-known and most-studied species. However, another group of yeasts, the non-conventional yeasts, has attracted attention in white biotechnology. The term “non-conventional” was created to differentiate this group of yeasts from conventional, widely studied species, such as *S. cerevisiae* and *Schizosaccharomyces pombe* (Barth & Gaillardin, 1997). Interest in non-conventional yeasts has grown over the past years, mainly because of their ease of cultivation and beneficial natural phenotypes that confer them several advantages over conventional yeasts, such as higher salt and other types of stress tolerance. In addition, non-conventional yeasts have a large number of applications, and a wide range of available molecular tools and techniques, with new developments in synthetic biology which eventually would facilitate their use (Navarrete & Martínez, 2020). In this sense, *Yarrowia lipolytica* is one of the most attractive non-conventional yeasts, currently exploited for several environmental, biotechnological, and industrial applications.

*Y. lipolytica* is an ascomycetous yeast (class: Saccharomycetes, order: Saccharomycetales) originally classified as *Endomycopsis lipolytica*, *Candida*, and *Saccharomycopsis lipolytica*. The name “Yarrowia” is in honor of David Yarrow, who identified this genus, and “lipolytica”

references the ability to hydrolyze lipids (Nicaud, 2012; Zieniuk & Fabiszewska, 2018). Presently, the genus *Yarrowia* comprises 13 species that are widespread geographically in different ecological niches: *Y. galli*, *Y. deformans*, *Y. bubula*, *Y. porcina*, *Y. alimentaria*, *Y. yakushimensis*, *Y. parophonii*, *Y. phangngensis*, *Y. keelungensis*, *Y. oslonensis*, *Y. divulgata*, *Y. hollandica*, and *Y. brassicae*. Like *Y. lipolytica*, all species in this genus are oleaginous and have potential biotechnological applications (Gaikwad et al., 2021). *Y. lipolytica* is the type species of the genus and, for a long time, it was the only known species. This species has a ubiquitous distribution. It has been found in marine environments, soil, sewage, oil-polluted waters, and substrates rich in lipids and proteins, such as meat and dairy products (Gaikwad et al., 2021; Sutherland et al., 2014; Groenewald et al., 2014; Fröhlich-Wyder et al., 2019; Paulino et al., 2017; Hassanshahian et al., 2012; Gonçalves et al., 2014). This is because of its capacity to metabolize a variety of carbon sources, such as organic acids, polyalcohols, and paraffins (Ledesma-Amaro & Nicaud, 2016a).

*Y. lipolytica* is a dimorphic heterothallic haploid yeast with mating types, i.e., Mat A and Mat B (Barth & Gaillardin, 1997; Gaikwad et al., 2021). This species is widely used industrially and has multiple applications in biofuel production, as a heterologous host for proteins, organic acids, and enzymes (Gaikwad et al., 2021; Gonçalves et al., 2014). It also has applications in bioremediation processes because of its versatile genomic and metabolic and physiological potential (Kurtzman, 2011). Moreover, it is well known as a great producer of biosurfactants, proteases, lipases, and long-chain fatty acids, as well as for its ability to consume hydrocarbons.

*Y. lipolytica* is a model organism for studies of dimorphism and metabolic pathways for lipid and fatty acid production. It has an efficient genetic engineering transformation system. Furthermore, its hyphal and yeast morphologies are easily distinguished depending on the environmental conditions. This can be extremely useful in bioprocess optimization, with practical importance in biotechnological applications (Xie, 2017).

*Y. lipolytica* is also considered a non-pathogenic microorganism, despite case reports of some patients with potential susceptibility to infection to it (Nicaud, 2012). It is classified as generally recognized as safe (GRAS) by the US Food and Drug Administration. Given its biotechnological and genetic importance, *Y. lipolytica*'s physiology and metabolism continue to arouse interest and, therefore, further studies are necessary. Besides, wild strains of this species are able to produce a variety of compounds of biotechnological interest like organic acids (i.e., citric acid), erythritol, proteins, and lipids.

This species is classified as an oleaginous yeast, i.e., organisms that accumulate more than 20% of their biomass as lipids (Ratledge & Wynn, 2002). Indeed, it has been reported that *Y. lipolytica* can store at least up to 70% of its biomass as lipids (Beopoulos et al., 2009b).

Several *Y. lipolytica* strains have been investigated in metabolic studies and Larroude et al. (2018) bring a list with the most common ones. Among them, E150 is a reference strain with its genome sequenced and annotated (Dujon et al., 2004). Additionally, the wild-type strains W29 (Pomraning & Baker, 2015; Magnan et al., 2016), H222 (Devillers & Neuvéglise, 2019), and CBS6124 (Barth & Gaillardin, 1997; Egermeier et al., 2017) gave origin to several inbred lines (Nicaud, 2012).

*Y. lipolytica* strains are more often isolated from substrates containing lipids or proteins than from substrates containing sugars (Barth & Gaillardin, 1996). Among sugars there is evidence that this species uses glucose (Papanikolaou et al., 2009), but preferably hydrophobic compounds, such as lipids, paraffins and alkanes, are catabolized (Papanikolaou et al., 2003).

The unique features of this species like its ability to grow on numerous complex compositions of nitrogen and carbon sources is also linked with sustainable processes, like bioremediation and waste biomass valorization.

In this review, we provide an overview of *Y. lipolytica* metabolic phenotypes under different carbon sources, the role of nitrogen and other growth conditions in the metabolism of target compounds of biotechnological interest, besides regulatory networks and computational models available for that species.

## ***Y. lipolytica* metabolism - a cell factory for lipid production and fatty acid-derived products**

*Y. lipolytica* is considered an 'oleaginous microorganism' and has attracted the attention of biotechnological studies that search for lipid hyperaccumulation. This species is considered an interesting biological model mainly because it has a metabolic chassis which is easy to manipulate in

laboratory, besides its plasticity in using different sources of substrates, directing the metabolism to the synthesis of reserve lipids and unusual fatty acids (Ledesma-Amaro et al., 2016). This phenotypic plasticity is due to its wide genome with several multigene families that regulate those metabolic pathways (Beopoulos et al., 2009a).

Comparatively, *Y. lipolytica* accumulates fewer lipids (~36% DW) than other species also called oil seeds, such as *Cryptococcus albidus* (~65% DW), *Rhizopus arrhizus* (~57% DW), and *Candida* sp. 107 (~42% DW) (Beopoulos et al., 2009a). Since lipids of biotechnological interest are the neutral ones and free fatty acids, the species does not accumulate free fatty acids in considerable amounts, with 80% of its lipid content directed to triacylglycerols - TAG (Athenstaedt et al., 2006). Neutral lipids are reserve molecules composed mainly by TAGs (85%) and steryl esters (SE - 8%). These compounds are basically fatty acids esterified with other molecules, mainly linoleic acid (18: 2 - 51%), oleic acid (18: 1 - 28%), and palmitic acid (16: 0 - 11%) (Beopoulos et al., 2009a). The proportion of TAGs and SE, as well as that of oleic acid, is highly influenced by the substrate used by the yeast culture. Thus, it has been shown that fatty acids as substrates have increased the proportion of oleic acid in lipid bodies, as well as the proportion of SE in relation to TAGs in certain *Y. lipolytica* strains (Athenstaedt et al., 2006). The rich profile in polyunsaturated fatty acids, such as linoleic acid, of that species makes it attractive for the production of a bio-oil with high commercial value.

## **De novo and ex novo lipid synthesis by *Y. lipolytica***

The search for lipid hyperaccumulation in *Y. lipolytica* is aimed at increasing its biosynthesis and it can follow two distinct pathways: *de novo* synthesis and *ex novo* synthesis, according to the substrate used by the yeast (Papanikolaou & Aggelis, 2011). *Ex novo* synthesis is distinguished from the *de novo* one in that it accumulates lipids simultaneously with its growth regardless of nitrogen depletion (Papanikolaou & Aggelis, 2011). Usually, under sugars and glycerol availability, *Y. lipolytica* accumulates lipids via *de novo* synthesis and when hydrophobic substrates are provided it efficiently uses an *ex novo* synthesis pathway.

*De novo* synthesis occurs when the available substrates are sugars or other carbon compounds. This pathway is elicited upon a depletion of nitrogenous sources in the environment, which leads to changes in metabolism, generating a series of regulatory events and altering the intracellular concentration of several metabolites, initiated by a decrease in the intracellular adenosine monophosphate (AMP) pool (Boulton & Ratledge, 1983). This alteration will result in the export of mitochondrial citrate to the cytosol, increasing the availability of lipogenic acetyl-CoA, a direct precursor of fatty acids, directing it to the generation of structural lipids and reservation. Reserve-directed lipids (TAGs) are incorporated into lipid bodies covered by a phospholipid monolayer with several proteins inserted with biochemical activities (Brown, 2001). The composition of this phospholipid layer is also variable depending on the carbon source available (Fujimoto et al., 2008).

The *ex novo* synthesis is restricted to microorganisms that can grow metabolizing fats or other hydrophobic compounds

as the only source of carbon and energy and, at the same time, accumulate significant amounts of lipids. To use these substrates, an active lipase enzyme system is required to hydrolyze them into free fatty acids and then be incorporated into the cell. These free fatty acids will be degraded into acyl-CoAs and acetyl-CoA and directed to the maintenance of metabolism and the formation of intermediate metabolites, which will be directed to the *de novo* synthesis for lipid production (Ledema-Amaro & Nicaud, 2016b).

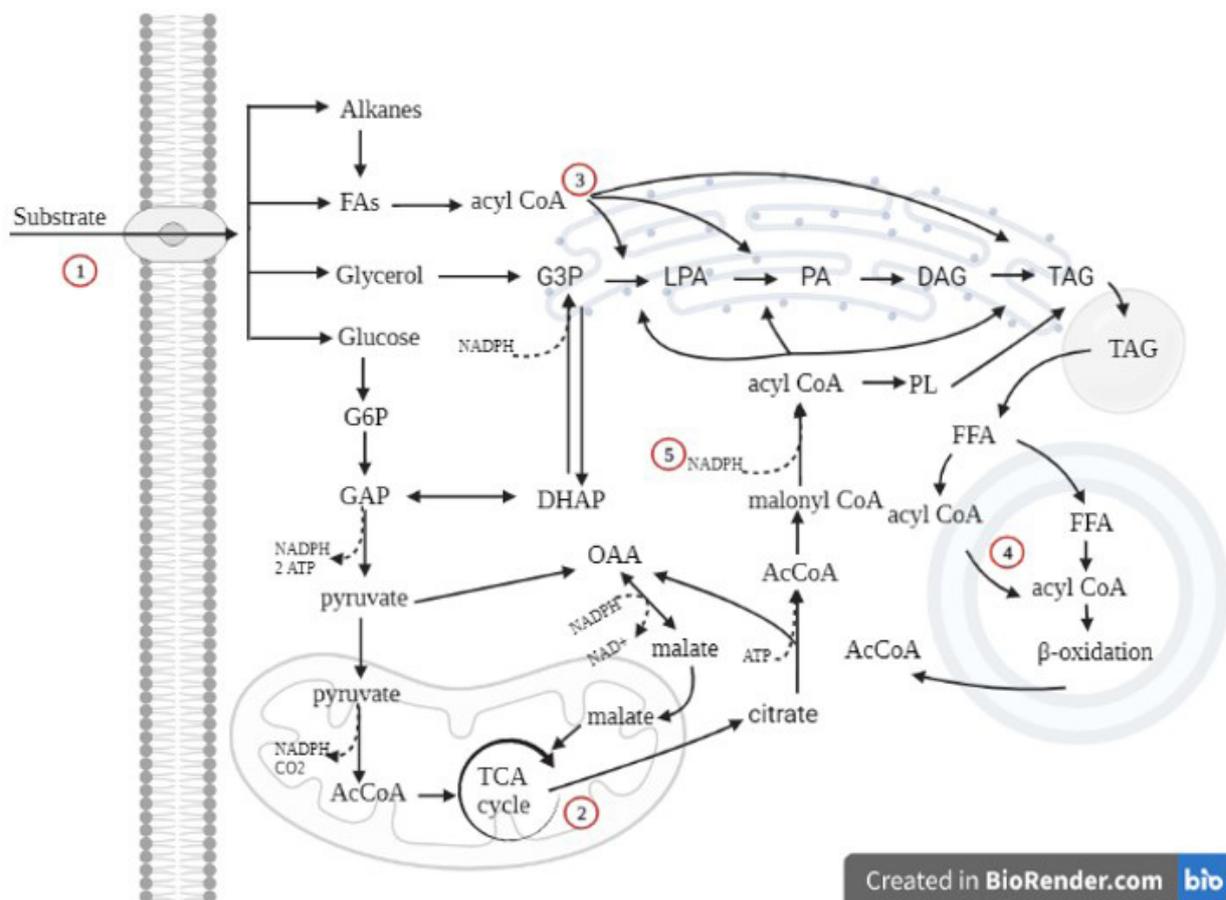
### Strategies to improve lipid biosynthesis

Recent research has focused on expanding the range of substrates used, e.g., glucose, glycerol, fatty acids, alkanes, waste leachate, in order to increase lipid production (Figure 1 - 1<sup>st</sup> strategy). The broadening of the range of substrates occurs because each culture medium is directed to one of the lipid synthesis pathways, and thus, the optimization of the process for the conversion of the lipid class (Johnravindar et al., 2018; Ledema-Amaro & Nicaud, 2016b; Dobrowolski et al., 2016; Sestric et al., 2014).

*Y. lipolytica* is not naturally a good biological converter of carbohydrates to lipids. This occurs by allosteric inhibition of fatty acid biosynthetic enzymes by saturated fatty acids (Goodridge, 1972). To overcome this limitation, Qiao et al. (2015) modified a strain to overexpress acetyl-CoA carboxylase (ACC1) and diacylglycerol acyl-transferase (DGA1). ACC1 carboxylates acetyl-CoA to malonyl-CoA, while DAG1 is directly involved in lipid accumulation. This super-producing mutant showed a yield of 55 g lipids/L and reached 84.7% of the maximum theoretical yield in the conversion of carbohydrates into lipids.

Another hypothesis for the increase in lipids is to consider the dynamics of cell wall biogenesis proteins as possible targets (Pomraning et al., 2015). This approach focuses on using a culture medium rich in glycerol and poor in amino acids, so the cell rapidly depletes the culture medium's amino acids content. When finally the intracellular amino acid concentration drops, an elicitation of the *de novo* pathway with high glucose levels occurs concomitantly with a lipogenesis process.

A second approach to improve lipid synthesis aims to promote mitochondrial acetyl-CoA downregulation and



**Figure 1.** Schematic representation of metabolic pathways leading to the production of lipids in *Yarrowia lipolytica*. The five strategic points for metabolic engineering are highlighted in red circles and are explored to a greater extent in the text. Abbreviations: TAG: triacylglycerol; FAs: fatty acids; G6P: glucose 6-phosphate; GAP: glyceraldehyde 3-phosphate; DHAP: dihydroxyacetone phosphate; LAP: lysophosphatidic acid; PA: phosphatidic acid; DAG: diacylglycerol; PL: phospholipid; FFA: free fatty acids; AcCoA: acetyl-CoA; ATP: adenosine triphosphate; NAD and NAD[P]H: Nicotinamide adenine dinucleotide [phosphate] (oxidized and reduced forms). Created with BioRender.com.

cytosolic acetyl-CoA upregulation (Figure 1 - 2<sup>nd</sup> strategy). This regulation occurs mainly upon nutrient depletion conditions, usually nitrate. With nutrient limitation, yeast metabolism activates AMP deaminase, reducing mitochondrial AMP concentration and activating citrate dehydrogenase. This inhibits the TCA cycle by increasing isocitrate. With this, citrate is released into the cytoplasm, which will be transformed into acetyl-CoA and then malonyl-CoA, an important fatty acid precursor (Gonçalves et al., 2014; Ledesma-Amaro & Nicaud, 2016b).

The lipid biosynthesis potential of *Y. lipolytica* is dependent on a high flux of acetyl-CoA, and unlike other yeasts that use acetyl-CoA synthase, ATP citrate lyase is preferred to provide acetyl-CoA in building blocks for the synthesis of fatty acids. In wild strains, ATP citrate lyase is only active when the TCA cycle is suppressed due to the lack of any nutrient. In order to uncouple nitrogen limitation and allow *Y. lipolytica* to produce large amounts of acetyl-CoA and thus accumulate lipids even in the presence of nitrogen, Xu et al. (2016) created five alternative pathways to increase cytosolic acetyl-CoA flux. With the expression of the CAT2 gene (carnitine acetyltransferase) which regulates the flux of acetyl-CoA mitochondria/cytosol, they were able to increase the production of lipids over the parental strain in 3.1-fold. In the end, manipulation of alternative cytosolic acetyl-CoA pathways partially uncoupled lipogenesis from nitrogen deprivation and released the lipogenic potential of *Y. lipolytica* (Xu et al., 2016).

In the past decades, genetic engineering techniques have driven the development of *Y. lipolytica* strains designed to accumulate lipids. With a total genome of 20.5 Mb and carrying 6 chromosomes with 49%-59% GC content in the coding sequence and with its cellular functions well compartmentalized, the species is an excellent model for genetic engineering (Barth & Gaillardin, 1997; Nicaud, 2012) and genome editing research such as CRISPR-Cas9 (Schwartz et al., 2016; Wong et al., 2017; Holkenbrink et al., 2018; Morse et al., 2018) and CRISPR-Cas12/cpf1 (Yang et al., 2019). These genome editing methods are advancing rapidly and a reissued strain for lipid accumulation in *Y. lipolytica* should soon emerge. Besides, following the genome sequence of that species, metabolic engineering approaches have been applied in different ways to improve its lipid biosynthesis and accumulation potential. For that, basically five main targets have been pursued by metabolic engineering yeast cells: *i*) designing new strains with overexpressing genes involved in the synthesis of TAG and other adjacent pathways such as leucine, and even adding heterologous genes in order to synthesize new compounds; *ii*) inhibiting  $\beta$  oxidation genes so as not to degrade FFA molecules; *iii*) promoting more cytosolic NADPH activating the pentose pathway (Figure 1); *iv*) modifying carbon incorporation by changing substrates and their absorption pathways; and *v*) minimizing the competition for intermediary molecules of lipid synthesis by inhibiting pathways such as glycogen and TCA. Some findings of such approaches are further presented and discussed.

A promising strategy was designed by Beopoulos et al. (2008) with the knockout of the GUT2 gene, followed by the suppression of the POX1 and POX6 genes. The initial strategy excluded the GUT2 gene that uses G3P to form DHAP, competing with the substrate for the formation

of lysophosphatidic acid (LPA), the first product of TAG formation. This strategy increased the lipid content of the species by 3-fold compared to the wild-type strain. Additionally, the researchers also suppressed the POX1 and POX6 genes, which are responsible for the limiting step of peroxisomal beta-oxidation controlled by acyl-CoA oxidase. Deletion of POX1 and POX6 genes in the transformed strain without GUT2 increased its lipid production by 4-fold. With these genetic changes, the yeast chassis directs all the G3P molecules to the synthesis of TAG and also inhibits the degradation processes of fatty acids in the peroxisomes. These results agree with previous report (Dulermo & Nicaud, 2011), where a genetically modified *Y. lipolytica* strain and its unique glycerol metabolism dedicated to G3P synthesis (and also TAG synthesis), competing for acyl-CoA-transferases consolidates them as a limiting factor in formation of TAG (Figure 1- 3<sup>rd</sup> and 4<sup>th</sup> strategy). Thus, the overexpression of genes that increase the targeting of acyl-CoA to chloroplasts with the knockdown of beta-oxidation genes will enable the increase in lipid production.

### Other strategies to facilitate lipid hyperaccumulation and downstream processes

In recent years, many researchers have focused on the increase of lipids in *Y. lipolytica*, however the downstream process that corresponds to lipid extraction is also considered a bottleneck. In this sense, the strategy of modulating metabolism to improve production and secretion of lipids in the culture medium has allowed to achieve best metabolic performance, with strains able to secrete up to 2.8 g/L (Ledesma-Amaro & Nicaud, 2016b). For that, two genetically engineered strains that include blocking concurrent pathways have been developed, increasing flow through selected pathways, and relocating key enzymes. The best yielding strain was designed with FAA1 gene deletion, with overexpression of diacylglycerol acyltransferase (DGA2) and TGL4 KITGL3 genes that activate intracellular lipases with the aim of lipid secretion. The FAA1 gene activates acyl-CoA synthetase, preventing free fatty acids from being reactivated or degraded by beta-oxidation, and overexpression of DGA2 promotes the formation of lipid bodies, as diacylglycerol (DAG) is an immediate precursor of TAG. These molecular alterations showed one of the best performances of the species, high lipid concentration and secreting floating solids in the culture medium, facilitating the downstream processes (Ledesma-Amaro & Nicaud, 2016b).

By genetically modifying *Y. lipolytica* strain simultaneously in five lipogenic targets (affecting three distinct metabolic pathways) a lipid-saturated cell phenotype was detected (Blazek et al., 2014). The best strategy presented was the deletion of *pex10* gene, overexpression of DGA1 or DGA2 genes, and restoration of a complete leucine biosynthetic pathway (leucine + genotype). The *pex10* gene is responsible for the activation of peroxisomal biogenesis, as DGA complex refers to diacylglycerol acyltransferases I and II isozymes that promote lipid bodies formation, and the restoration of the complete biosynthetic pathway of leucine, leading to increased amounts of lipids accumulated (Blazek et al., 2015). The final designed strain yielded 6.00 g.L<sup>-1</sup> lipids

with 74% lipid content, a ~15-fold improvement over control (0.41 g.L<sup>-1</sup> lipid and 16.8% lipid content).

Some *Y. lipolytica* strains have been more recently designed to produce non-species fatty acids and others organic compounds, such as conjugated linoleic acid (*trans*-10, *cis*-12-CLA) from glucose (Zhang et al., 2012, 2013), ricinoleic acid (RA; 12-hydroxy-octadeca-*cis*-9-enoic acid: C18: 1-OH) (Mutlu & Meier, 2010; Yazawa et al., 2013, 2014), eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA) (Xie et al., 2015; Hong et al., 2011; Xue et al., 2013), and also the organic compound itaconic acid (Blazek et al., 2015). Hong et al. (2011) were successful in the synthesis of EPA by using *Y. lipolytica* recombinant host cells, a technology that further became a licensed patent for a fermentation process and resulted in two commercial products, e.g., New Harvest™ EPA oil and Verlasso® salmon (Ledesma-Amaro et al., 2016).

Blocking  $\beta$ -oxidation is a strategy that has always been considered in metabolic engineering processes for lipid hyperaccumulation.  $\beta$ -oxidation step is the metabolic process that takes place in peroxisomes, where fatty acid molecules undergo two carbon shortenings to generate acetyl-CoA (Figure 1- 4<sup>th</sup> strategy). The idea is that with the inhibition of  $\beta$ -oxidation the cell will accumulate more free fatty acids (Ledesma-Amaro et al., 2016). Metabolic engineering targets to inhibit this process focus on the knockdown of one or more of the four reactions in the pathway. The first reaction is performed with acyl-CoA oxidase POX genes, a block with 6 genes, and the knockdown of all genes generates hyperaccumulation of lipids, but it is complex due to the amount of knockdown needed (Xue et al., 2013; Wang et al., 1999; Mlikova et al., 2004a, b; Beopoulos et al., 2008). The second and third metabolic steps are catalyzed by the multifunctional enzyme MFE with only one responsible gene, being the simplest strategy and indicated to generate high yields of lipid accumulation (Dulermo & Nicaud, 2011; Blazek et al., 2014). The last step is performed by thiolase *POT1* gene. *POT1* was suppressed by Berninger et al. (1993) and caused the yeast inability to grow in the presence of oleate, suggesting the catabolic function of *POT1* and the absence of additional catabolic thiolases. Each  $\beta$ -oxidation step shortens two acyl-CoA carbon atoms starting with POX genes, i.e., to avoid TAG degradation in industrial producer strains, eventually leading to hyperaccumulation of lipids (Zheng et al., 2019).

Lipids are molecules extremely reduced and require a high rate of reducing equivalents in the form of NADPH. The production of more cytosolic NADPH is a well-known bottleneck in metabolic engineering (Figure 1- 5<sup>th</sup> strategy). The supply of NADPH is carried out via the pentose pathway or by the malic enzyme in the pyruvate/OAA/malate transhydrogenase cycle (Tai & Stephanopoulos, 2013). Studies that overexpressed the malic enzyme were not successful in increasing lipid synthesis, showing that the primary source of reducers for lipid formation is the pentose phosphate pathway (Beopoulos et al., 2011; Blank et al., 2005).

The use of redox engineering to improve the productivity and yields of lipids in *Y. lipolytica* strains was adopted by Qiao et al. (2017), with the best-designed strain achieving productivity of 1.2 g/L/h using pure glucose (90 g/L, 1.3 g/L/h) as carbon source (Qiao et al., 2017; Davis et al., 2013).

## Carbon sources and the role of nitrogen for lipid accumulation in *Y. lipolytica*

Wild strains of *Y. lipolytica* are able to utilize sugars such as glucose, fructose, and mannose (Papanikolaou et al., 2009; Tsigie et al., 2011), hydrophobic substrates, e.g., *n*-alkanes, fats, oils, and fatty acids, among other carbon sources including acetate, glycerol and other waste materials.

### Glucose

Although *Y. lipolytica* is able to use glucose as a carbon source, in general this sugar is far from being the best substrate for lipid production. Lipid accumulation and citric acid production are intrinsically related in *Y. lipolytica*. In that sense, glucose as a carbon source plays a key role by defining which pathway the cell is going to choose. How does citric acid production relate to *Y. lipolytica*'s lipid and glucose metabolism?

Several studies have reported citric acid biosynthesis concomitantly with fatty acids or other compounds. When a hydrophilic carbon source such as glucose is used as substrate for *Y. lipolytica*, substantial citric acid production has been observed in nitrogen-limiting fermentation conditions and/or intracellular nitrogen exhaustion (Anastassiadis et al., 2002; Moeller et al., 2007). According to Anastassiadis et al. (2002), citric acid production is triggered by a decrease of intracellular nitrogen followed by a high concentration of NH<sub>4</sub><sup>+</sup>. According to Ratledge (1994), upon nitrogen depletion, AMP is broken down in inosine monophosphate and NH<sub>4</sub><sup>+</sup> and, because AMP activates the enzyme isocitrate dehydrogenase, isocitrate accumulates and consequently citrate as well. *Y. lipolytica* produces citrate as an overflow metabolite, which can be excreted from the cell or used in lipid production (Kerkhoven et al., 2016). Citrate, as a direct product of glucose metabolism, and because glucose assimilation continues despite nitrogen depletion, is the immediate precursor of cellular lipid accumulation in oleaginous microorganisms (Ratledge, 1994; Beopoulos et al., 2009a).

Papanikolaou et al. (2009) did not detect significant lipid amounts in *Y. lipolytica* cultured cells grown under glucose and nitrogen limiting conditions neither *de novo* accumulation of lipids, with maximum 0.2 g lipids/g dry matter, mainly composed of C16 and C18 fatty acids. Nonetheless, by increasing glucose content in culture medium induced to non-negligible increase in total lipid content in either wild-type and genetically modified strains for decreased expression of acyl-CoA oxidases (Papanikolaou et al., 2009). Besides, Tai & Stephanopoulos (2013) achieved lipid contents of 2-fold (17.9% DW) and 4-fold (33.8% DW) higher by engineered strains of *Y. lipolytica* overexpressing acetyl-CoA carboxylase and diacylglycerol acyltransferase respectively, grown under glucose as single carbon source in culture medium.

As lipid accumulation follows starvation of determined nutrients in the medium, usually nitrogen, which not rarely leads to a reduced cell growth; the challenge remains in increasing the yeast cells' lipid content maintaining adequate cell growth (Robles-Rodríguez et al., 2018). Robles-Rodríguez et al. (2018), by mathematically modeling the lipid accumulation in *Y. lipolytica* cells cultured under glucose with nitrogen depletion conditions, defined quotas

and indicators such as minimum nitrogen amount necessary to cell growth, maximum lipid production rate and lipid content, lipid inhibition by citric acid, and a minimum glucose uptake rate that triggers overflow metabolism.

Another possible explanation for citric acid production and overflow metabolism is the surface-to-volume ( $S/V$ ) ratio. At high growth rates cells tend to be bigger in size reducing the  $S/V$  ratio. With less surface area per volume the capacity of the electron transport chain to recycle  $NAD^+$  pool becomes impaired, leading to a toxic concentration of NADH. To overcome this, cells may choose to use the fermentation process to avoid excess of NADH production by TCA cycle, diverting glucose to overflow metabolism of citric acid (Szenk et al., 2017).

Repression of lipase production by glucose as unique carbon source was detected in certain *Y. lipolytica* strains, contrasting its stimulation by hydrophobic compounds such as oleic acid and TAGs (Pereira-Meirelles et al., 1997), probably due to a mechanism known as carbon catabolite repression (Fickers et al., 2005b). Interestingly, glucose grown cells exhibited lower hydrophobicity than cells grown under hydrophobic compounds (Zinjarde & Pant, 2002).

### Hydrophobic compounds

Lipid accumulation by *Y. lipolytica* from fat materials is a completely different process from that which occurs under sugars and from the *de novo* lipid accumulation process (Papanikolaou et al., 2009). Hydrophobic compounds as TAGs and fatty acids have largely been reported as inducers for lipid and lipase activity in *Y. lipolytica*. The hydrophobization of the cell membrane and the production of biosurfactants are some of the physiological responses of the cells to the presence of hydrophobic compounds. Hydrocarbons also have an effect in citric acid production, usually obtained by carbohydrate-based fermentation, and also as a substrate for lipid biosynthesis. Crolla & Kennedy (2004) optimized *Y. lipolytica* cultures under *n*-paraffin to produce citric acid up to 1.0 g citric acid/g *n*-paraffin. Indeed, one of the main contributions of these classes of compounds as substrates in *Y. lipolytica* metabolism is for lipid, lipases, and biosurfactants biosynthesis.

### Waste materials

Industrial waste such as olive oil mill waste, animal fats, and sugarcane bagasse are of interest for the industry due to their composition as a good source of carbon, nitrogen and other nutrient compounds and because these waste materials are readily available and usually cheap. However, to understand how *Y. lipolytica* can metabolize these materials demands much more studies, because of the complex chemical composition and variability of these sources. For example, olive oil mill waste contains polyphenols, oils, water, sugars, and salts like phosphates, beside the fact that these materials do not always have a favorable C/N ratio (Aly et al., 2014). Thus, it is expected to find distinct and peculiar yeast metabolic profiles according to the carbon source to be catabolized, with effects on the cell's lipid accumulation and yield.

*Y. lipolytica* is able to grow on diverse types of waste materials, e.g., stickwaters, fat-rich wastes from meat-processing facilities and used-cooked oils (Vasiliadou et al., 2018). One of the great challenges in utilizing waste materials, along with understanding the interactions with its varied and complex chemical composition, is probably to neutralize inhibitory effects of substrate contaminants (Spagnuolo et al., 2018).

Some studies (Ruiz-Herrera & Sentandreu, 2002; Zhao et al., 2015; Larroude et al., 2019) suggest wild strains of *Y. lipolytica* are unable to naturally grow on xylose, a sugar mostly present in waste biomass rich in lignocellulose. However, recently Ryu et al. (2015) confirmed putative xylose-degrading enzymes with transcriptomic and bioinformatics analyzes and adapted an *Y. lipolytica* strain to grow under xylose as sole carbon source in order to activate this catabolic pathway. After the adaptation period, no more than 15 generations, *Y. lipolytica* was able to consume about 2.23 g/L xylose in 72 h (Ryu et al., 2015). Tsigie et al. (2011) were able to obtain a lipid yield of 6.68 g/L with *Y. lipolytica* strain Po1g grown under sugarcane bagasse hydrolysate, with peptone as nitrogen source. Their findings indicate that xylose uptake may be competitively inhibited by the presence of glucose. Besides, it seems *Y. lipolytica* ability to uptake xylose may also be strain dependent due to the great metabolic differences reported regarding xylose uptake (Madzak, 2021).

Vasiliadou et al. (2018) showed that lipid turnover in *Y. lipolytica* could be prevented with the addition of carbon sources such as glycerol, glucose and olive oil, at various phases of lipid degradation, suggesting lipid accumulation is highly dependent on carbon source inputs.

### Glycerol

Many studies can be found reporting glycerol as a carbon source for *Y. lipolytica* growth, because of its relatively low-cost as an industrial effluent. Furthermore, acetate mutants of *Y. lipolytica* can synthesize single cell oil (SCO) and organic acids, as citric acid, in culture media containing glycerol as a carbon source (Rywińska & Rymowicz, 2010). In addition to lipids, citric acid is also of great importance in the pharmaceutical and food industries as an acidulant. To produce an alkaline lipase, Lee et al. (2007) verified that glycerol and glucose were the most efficient carbon sources for production by *Y. lipolytica* strain NRRL Y-2178. Also, significant biomass and single cell oil production can be achieved with an initial concentration of 89 g.L<sup>-1</sup> of crude glycerol (Sara et al., 2016).

### Ethanol

Regarding the provision of reducing compounds, ethanol is an efficient carbon source for lipid production in yeasts due to its chemically reduced nature that provides NADH equivalents concomitantly with acetyl-CoA and still, because of that, dispenses the need of the key enzyme ATP citrate lyase (Ratledge, 1994). Nonetheless, ethanol has been a substrate mostly used for organic acids production such as  $\alpha$ -ketoglutarate acid (Chernyavskaya et al., 2000; Finogenova et al., 2005; Kamzolova et al., 2012), citric and

isocitric acids (Arzumanov et al., 2000; Finogenova et al., 2002; Kamzolova et al., 2018), and succinic one (Kamzolova et al., 2009).

### Acetate

In addition to being an affordable low-cost substrate for the industry, acetate metabolism naturally flows to acetyl-CoA which is required for lipid biosynthesis (Spagnuolo et al., 2018). Liu et al. (2016) demonstrated that acetate metabolism for lipid production involves the glyoxylate shunt pathway, gluconeogenesis in determined conditions (growth phase and strain) and that lipogenic NADPH is provided by the pentose phosphate pathway. According to these authors, the constant draining of acetyl-CoA for lipid biosynthesis together with NADPH production decreases carbon availability to TCA cycle affecting energy production by the cell, a problem that still needs to be resolved through metabolic engineering when using acetate for lipid production (Liu et al., 2016).

### Oxygen

Despite the fact that oleaginous microorganisms are aerobic, lipid biosynthesis is independent of oxygen. However, to keep cells alive a minimum of oxygen is required which is more critical in high cell density cultures (Ratledge, 1994). Magdouli et al. (2018) showed that the availability of oxygen can indeed interfere with lipid biosynthesis by *Y. lipolytica*, as for maximum lipid yield (44.8% w/w) the yeast required a concentration of 30% dissolved oxygen. Similarly, Amaral et al. (2006) detected lipase production 23-fold higher in relation to control in a *Y. lipolytica* strain facilitating oxygen mass transfer using perfluorocarbon as a carrier of that nutrient.

### The role of nitrogen and C/N ratio

Several studies suggest the existence of a fine tune between the amount of carbon and nitrogen available to yeast metabolism and the consequent use of those nutrients either for growth as for lipid biosynthesis, as well as for the accumulation of organic acids like citrate. Thus, the growth rate of an organism is one of the main influences on its cell composition (Ratledge, 1994) and not rarely lipid accumulation occurs at the expenses of growth. One reason for that may be, that if other nutrients than nitrogen are used as growth-limiting factors, e.g., phosphate, magnesium, iron or sulphate, reactions other than synthesis of nucleic acids and proteins may be affected (Ratledge, 1994). Besides, lipid accumulation is inversely proportional to growth rate and growth rates lower than 0.1 per hour are normally required because cells need to have a minimum residence time to convert available carbon into lipids (Ratledge, 1994). Furthermore, when oleaginous microorganisms have exhausted the carbon supply their cells begin to consume the lipid reserves (Holdsworth & Ratledge, 1988).

Papanikolaou et al. (2013) have defined three metabolic states for *Y. lipolytica* as a function of C/N ratio: *i*) under low carbon flux than that required for growth, cells consume storage lipids, *ii*) under an optimal C/N ratio for growth, cells acquire maximal biomass production and *iii*) in excess of

carbon and limited nitrogen, cells can shift their metabolism to produce organic acids like citrate and/or lipids.

According Robles-Rodríguez et al. (2018), upon an excessive carbon substrate supply, when carbon uptake for lipids and biomass production encounters a saturation point, metabolism triggers citric acid overflow. Critical values for C/N ratio are intrinsically related to this, because overflow only occurs in nitrogen depleted conditions or when a critical intracellular nitrogen quota is reached. Nonetheless, somewhere in between these critical values for nitrogen limitation, lipid accumulation is affected.

In fact, in oleaginous microorganisms the trigger to lipid accumulation is usually the exhaustion of nitrogen, when the cells cease to proliferate because nitrogen is essential for nucleic acids and protein biosynthesis, combined with high availability of carbon which is then converted into fatty acids and triacylglycerols (Ratledge, 1994). Under limited nitrogen, cells may cleave adenosine monophosphate (AMP) to gain more nitrogen and the resulting decrease in AMP contents may cease tricarboxylic acid (TCA) cycle accumulating organic acids like citrate, which through the action of ATP citrate lyase (ACL) provides more acetyl-CoA for lipid biosynthesis (Papanikolaou et al., 2013). If the necessary nutrients including carbon are supplied, the accumulation of lipids continues until high levels of these metabolites have been reached (Ratledge, 1994). Additionally, *Y. lipolytica* cells can switch to citrate excretion namely under nitrogen deprivation, which may or not be desirable, and the exact conditions that make metabolism switch to one or another way are not clear. For instance, to control citric acid production under glucose supply and avoid the impairing of lipid production, Ochoa-Estopier & Guillouet (2014) showed the importance of maintaining the C/N ratio below 47.6 for *Y. lipolytica* W29. This threshold value was even more increased in another study (Sagnak et al., 2018), reaching 55.5-58.8-fold with another *Y. lipolytica* engineered strain, revealing greater flexibility in targeting metabolism to lipid production.

Usually, a C/N ratio of 30-50: 1 is used for lipid accumulation in continuous *Y. lipolytica* cell cultures (Ratledge, 1994). Gropoșila-Constantinescu et al. (2015) reported good results for lipid accumulation (up to 50% lipids of dry biomass) with a C/N ratio of 50. However, even if a high C/N ratio exerts positive influence on lipid accumulation, there is no consensus as to whether there is any optimal value or range for this parameter, probably because other parameters in the culture influence the carbon and nitrogen uptake by the cell. Kuttiraja et al. (2016b) investigated the effect of four different C/N ratios (25, 50, 100, 150; 100 g/L glycerol) balanced with yeast extract for nitrogen supply and found the best biomass and lipid concentration with a C/N ratio of 100 on *Y. lipolytica* SKY7 strain, i.e., 14.8 g/L and 43.8% (w/w) respectively. In another study (Kuttiraja et al., 2016a), C/N ratio was tested with glycerol (75-100 g/L) balanced with  $(\text{NH}_4)_2(\text{SO})_4$  as the sole source of nitrogen. The authors found the best results for lipid production (29.5 g/L) in the concentrations of 82.5 g/L glycerol and 75 C/N ratio. Papanikolaou et al. (2003) obtained maximum concentration of lipids similar to cocoa-butter (3.4 g/L) in high C/N ratio (175) for *Y. lipolytica* grown under stearin, glucose, and glycerol. Kerkhoven et al. (2016) increased total lipid content from 40.5 mg/g DW (C/N = 110) to 197 mg/g DW (C/N = 2.2).

If lipogenesis is dependent on the C/N ratio in limited nitrogen conditions and nitrogen starvation is related to greater availability of acetyl-CoA for lipid synthesis, then providing alternative ways to supply acetyl-CoA for lipogenesis can help decoupling nitrogen starvation from lipid biosynthesis (Xu et al., 2016).

Papanikolaou et al. (2013) engineered a *Y. lipolytica* strain by inactivating 2-methyl-citrate dehydratase and were able to produce significant quantities of lipid (31% w/w dry weight), 1.6-fold more when compared with equivalent nitrogen-limited cultures.

## Biosurfactants and emulsifiers

Studies show that compounds like biosurfactants are most likely synthesized in nitrogen depleted conditions. Nitrogen sources that exert a positive effect on biosurfactant activity are ammonium sulfate and potassium nitrate (Albuquerque et al., 2006). Zinjarde & Pant (2002) utilized the *Y. lipolytica* strain NCIM 3589 to produce an emulsifier and found this compound to be associated with the yeast's cell wall and composed of lipids, carbohydrates and proteins, under supplementation of ammonium sulfate (5g/L) and hexadecane (10g/L) to the culture medium. Interestingly, with good results, i.e., 3 units/mL emulsification activity and 3 mg/mL of biomass were detected with that source of nitrogen, contrasting with 1 unit/mL emulsification activity and 0.9 mg/mL of biomass when urea was supplied to the culture medium. Biosurfactants are predominantly glycolipids, but other structures can also be found. Yansan (Souza et al., 2012) and Liposan (Cirigliano & Carman, 1984), for example, are among the biosurfactants isolated and characterized from *Y. lipolytica* as further described.

Emulsifiers are present as part of the cell wall, facilitating the alkane uptake by the cells (Zinjarde & Pant, 2002). Biosurfactants activity can be seen as having preferentially emulsifying or surface tension reducing activity properties which can vary according to the culture medium composition (Souza et al., 2012; Cirigliano & Carman, 1984). Zinjarde & Pant (2002) observed an enhanced hydrophobic cell surface at the early stages of *Y. lipolytica* cell growth, but no extracellular emulsifier activity, being the later observed at the stationary phase under nitrogen limitation.

Liposan, a biosurfactant produced by a *Y. lipolytica* strain using diesel oil as substrate has been tested as to its activity on surface tension and emulsification. It was found a correlation between its low surface tension and higher emulsification properties (Souza et al., 2012). The production of biosurfactants in the presence of sea water enables this yeast species to be used in bioremediation process of oil spills. Liposan (Cirigliano & Carman, 1984), isolated from *Y. lipolytica* grown in hexadecane, paraffin, and vegetable oils is a water-soluble biosurfactant and showed to be stable up to 70°C/1h, being primarily composed of carbohydrates.

## Glucose as a substrate for biosurfactant production

In 1984, Cirigliano & Carman (1984) isolated a bioemulsifier from *Y. lipolytica*, at that time called *Candida lipolytica*,

primarily composed of carbohydrate, which they called *Liposan*. The authors found negligible emulsification activity when the yeast was grown in glucose as a carbon source, contrasting with maximum emulsification activity when hexadecane replaced glucose in the culture medium as carbon source. Later (Cirigliano & Carman, 1985), *Liposan* was further characterized as a glycoprotein, being composed of 83% carbohydrate and 17% protein.

In general, no emulsification activity is found when *Y. lipolytica* is grown under glucose as a carbon source and/or in the absence of water-immiscible compounds (Pareilleux, 1979). For example, the study of Zinjarde & Pant (2002) did not show emulsifier production under glucose, alcohol, sodium acetate, and glycerol as carbon sources in the culture medium. However, a bioemulsifier composed by 47% protein, 45% carbohydrate, and 5% lipids has been reported by growing *Y. lipolytica* cells under glucose (Sarubbo et al., 2001) in the absence of hydrocarbons, suggesting that the biosynthesis of those compounds may not uniquely result from the catabolism of hydrocarbons by the yeast cells. On the other hand, studies have pointed out that usually stronger emulsification activity is found when *Y. lipolytica* is grown under petroleum, hexadecane, paraffin, motor oil, soybean oil, canola oil, olive oil, corn oil, glycerol, and cottonseed oil (Cirigliano & Carman, 1984; Rufino et al., 2008, 2009; Silva et al., 2020). Higher emulsification activity of *Y. lipolytica* was also observed when growing cells in culture medium supplemented with soybean oil refinery residue associated with glutamic acid (1%) (Rufino et al., 2014). The authors have demonstrated to be the bioemulsifier a lipoprotein composed of 50% protein, 20% lipid, and 8% carbohydrate. Similarly, a bioemulsifier consisting of 70% lipids and 30% carbohydrates was recovered from *Y. lipolytica* cell cultures grown under 5% animal fat and 2.5% corn steep liquor (Santos et al., 2013), revealing that the chemical composition of biosurfactants may vary according to the carbon sources added to the culture medium. Taken together, these findings suggest that more detailed investigations on the *Y. lipolytica*'s biosynthetic pathways and networks associated to those metabolites are necessary for a better comprehension of the effects of carbon sources on its biochemical phenotypes.

## Lipases

Lipases hydrolyze TAGs and generate free fatty acids, mono and diglycerols, being a class of biocatalysts of great interest for the pharmaceutical, oleochemical, and detergents industries due to their wide range of actions, e.g., alkaline lipases for detergent additives, acidic lipases for foods and long-chain specific lipases for biofuels production (Syal & Gupta, 2017). Importantly, lipase activity in yeast species lacks information because studies have shown to exist a lot of variability between strains regarding their catalytic potential.

Fickers et al. (2005a) identified and characterized two genes, *LIP7* and *LIP8*, encoding active secreted extracellular lipases in *Y. lipolytica* with their maximum activities when supplementing culture medium with caproate and caprate, respectively. Syal & Gupta (2017) expressed five lipase genes from *Y. lipolytica* and all of them showed to be more active in neutral to alkaline medium and one of them, *YLIP13*, exhibited long-chain specificities under palmitate supplementation.

Dulermo et al. (2013) characterized two genes (*YITGL3* and *YITGL4*) that encode for intracellular lipases involved in TAG degradation localized in lipid bodies. Authors found that their spatial organization depends on the culture medium composition and cell growth phase, as inactivation of one or both genes enhances lipid accumulation.

Lipases are intimately related with lipids and surfactants. The addition of lipids such as vegetable oils (olive, sunflower, e.g.) to the culture medium have enhanced lipase activity (Dominguez et al., 2003; Deive Herva et al., 2009), an effect that seems to be dependent on the type of the vegetable oil. In fact, Darvishi et al. (2009) tested 10 vegetable oils and 11 nitrogen sources for lipase activity on *Y. lipolytica* DSM 3286 and found that olive oil was the best one for lipase production (34.6 U/mL). Kamzolova et al. (2005) achieved significant lipase secretion (2760 U/mL) under rapeseed oil contrasting animal fat. Sasarman et al. (2007) obtained 20.93 U/mL lipase content under sunflower oil and ammonium phosphate. Magdouli et al. (2017) enhanced lipase activity (25 U/mL) with olive oil and increased lipase activity even more (38 U/mL) with crustacean waste in *Y. lipolytica* SM7. Imandi et al. (2010) obtained maximum lipase activity (18.58 U/mL) with Palm Kernel cake, a waste product from the palm oil industry. Another study (Yan et al., 2018) tested low cost agro-industrial wastes such as sugarcane molasses, waste cooking oil, and crude glycerol from biodiesel production as substrates for production of lipase and single cell proteins (SCP) in *Y. lipolytica* cell cultures. The results showed that sugarcane molasses was the most effective carbon source, as peptone showed to be better than inorganic forms of nitrogen for lipase production (Pereira-Meirelles et al., 1997).

On the other hand, the addition of surfactants in the culture medium can enhance extracellular lipase activity in some cases, but it may be dependent on other variables, such as the yeast strain, for instance. Indeed, certain *Y. lipolytica* strains show the opposite phenotype following the addition of surfactants to the culture medium as no enhance of lipase activity is detected (Dominguez et al., 2003). Magdouli et al. (2017) enhanced lipid content and lipase activity up to 5.94 g/L and 15.10 U/mL, respectively, upon addition of the Tween 80 surfactant to the culture medium.

## Flux Balance Analysis (FBA) to elucidate *Y. lipolytica* metabolism

FBA (Orth et al. 2010) is a powerful tool in biotechnology, since it enables the prediction of metabolic responses of a given species regarding interesting compounds under genetic modifications and/or environmental conditions. It also saves resources and time because metabolic simulations can be done almost totally *in silico*, taking advantage of the amount of data available in literature or even with little experimental information. FBA is a genome scale-based computational approach and is possible due to dedicated software such as COBRA (Heirendt et al., 2019) and Optflux (Rocha et al., 2010), for instance.

Regarding *Y. lipolytica*, complete genome sequences for strains are available (Dujon et al., 2004; Magnan et al., 2016) and the quality of genome sequence annotations is crucial to

the constructions of accurate genome scale metabolic models (GEMs). At least six GEMs are available for *Y. lipolytica*: iNL895 (Loira et al., 2012), iYL619\_PCP (Pan & Hua, 2012), iMK735 (Kavšček et al., 2015), iYali4 (Kerkhoven et al., 2016), iYL\_2.0 (Wei et al., 2017), and iYLI647 (Mishra et al., 2018). Detailed descriptions and comparison of these GEMs can be found in the literature (Larroude et al., 2018; Xu et al., 2020). Some of them use *Saccharomyces cerevisiae* model as scaffold (e.g., iNL895 (Loira et al., 2012) and iMK735 (Kavšček et al., 2015), while others are based on genome annotation and biochemical databases (iYL619\_PCP (Pan & Hua, 2012) and some are built on other GEMs and or include reactions of other GEMs (iYali4 (Kerkhoven et al., 2016), iYLI647 (Mishra et al., 2018), yli v1.7 (Nambou et al., 2015) and iYL\_2.0 (Wei et al., 2017) (Larroude et al., 2018).

Kavšček et al. (2015) optimized lipid production in *Y. lipolytica* H222 wild-type strain using FBA and validated some hypotheses experimentally. The reduced aeration, i.e., limiting oxygen, can increase lipid yield, as citrate excretion by this species is a metabolic strategy to eliminate the excess of this compound. Besides, upon nitrogen-limited conditions the cells continue the uptake of glucose, producing citrate that in excess is excreted. Interestingly, authors were able to constrain glucose uptake and limit citrate production without affecting lipid content. These strategies, i.e., limiting aeration and glucose uptake without impairing final lipid content is crucial for industrial applications by lowering the costs.

To gain insights into TAG production by *Y. lipolytica*, Wei et al. (2017) developed a new GEM, iYL\_2.0, and used the model to simulate the utilization of 29 different carbon sources, being that except for hexadecane, 28 of them were consistent with experimental results. Further *in vivo* experiments revealed that hexadecane had a positive effect on *Y. lipolytica* growth, which was incorrectly predicted by the *in silico* model and attributed to the absence of the corresponding regulatory mechanism (Wei et al., 2017). By this model, iYL\_2.0, the authors identified knockouts for TAG production, e.g. genes for carbonate hydrolase, D-glyceraldehyde-3-phosphate, *O*-acyl-transferase, as well as genes responsible for glutamate degradation that might increase up to 55.5% the TAGs biosynthesis (Wei et al., 2017). Wei et al. (2017) also added two compartments to the model, peroxisome, associated to glyoxylate and dicarboxylate metabolism, and the endoplasmic reticulum, related to glycerolipid and glycerophospholipid metabolism and analyzed gene essentiality for *Y. lipolytica* growth. Gene essentiality showed to be variable according to the culture medium. These authors found that part of the genes related to amino acid metabolism are essential in minimal medium growth, but only 3% of that part are essential when *Y. lipolytica* grows under yeast extract rich in amino acids (Wei et al., 2017).

By the GEM iYali4 (Kerkhoven et al., 2016), using data of *Y. lipolytica* chemostats cultures in carbon and nitrogen depletion, lipid accumulation resulting from nitrogen limiting conditions was related to amino acid metabolism. At the level of regulation, Kerkhoven et al. (2016) verified poorly transcript levels of lipid metabolism, despite increased lipid content in nitrogen limitation have been detected, indicating, therefore, that lipid metabolism is not regulated

at the transcriptional level. These authors identified downregulation of amino acid metabolism and of their metabolic fluxes, leading to a redirection of metabolism to lipid production in a scenario where leucine is possibly a key regulator in this process (Kerkhoven et al., 2016). These findings suggest that *Y. lipolytica* senses the increase in leucine level and downregulates amino acid biosynthesis (Blazeck et al., 2014; Kerkhoven et al., 2016). According to Kerkhoven et al. (2016), lipid accumulation under nitrogen limitation resembles more an overflow metabolism than a regulation at the transcriptional factors level.

The model iYLI647 (Mishra et al., 2018) was designed and added with reactions of  $\beta$ - and  $\omega$ -oxidation to encompass degradation of fatty acids, included with an acetyl-CoA producing leucine degradation pathway and remodeling of biomass equation under limited carbon and nitrogen conditions to production of dicarboxylic acids.

## Conclusion

*Yarrowia lipolytica* is a non-pathogenic yeast with biotechnological importance, being capable of metabolizing different types of carbon sources and producing biosurfactants and lipids of industrial interest. The correct understanding of the key points that regulate the metabolism of target compounds in cell cultures of this yeast, in this case lipids and biosurfactants, seems to be crucial to obtain robust results and high-yield profiles, so that oleaginous strain cultures might be scaled up for their industrial production and utilization. Increases in production of target compounds can be achieved by metabolic engineering strains in an attempt to direct the carbon and nitrogen fluxes to target compounds, acting at various key points of metabolic networks predictively identified by the *in silico* models. Importantly, the computational models of metabolically engineered *Y. lipolytica* strains shall be experimentally validated, as a way to guarantee a rational basis for further developments in scaling up strain cultures with high yields of target compounds in bioreactors with large working volumes.

## Conflict of interests

The authors have no conflicts of interest to declare.

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