Strategies for increasing lipid accumulation and recovery from Y. lipolytica: A review

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Abstract – Microbial-based biodiesel is produced by transesterification of lipids extracted from microbial cells, and is considered as a potential replacement of fossil fuel due to its advantages in reducing greenhouse gas emissions. Yarrowia lipolytica is one of the most studied oleaginous yeasts able to produce lipids under some fermentation conditions and is considered as a potential industrial host for biodiesel production. Several approaches have been evaluated to increase the economical attraction of biodiesel production from Y. lipolytica lipids. In this review, we highlighted the different strategies reported in the literature, allowing this yeast to achieve high lipid accumulation. These include metabolic engineering strategies, the use of low-cost effective substrates, and the optimization of the cultivation conditions for higher lipid productivity and less operating cost. We also summarized the most effective cell disruption technologies that improve the extraction efficiencies of lipids from Y. lipolytica.

Keywords: Y. lipolytica / microbial oils / lipid accumulation / lipid extraction / biodiesel

Résumé – Les stratégies adoptées pour améliorer l’accumulation et l’extraction des lipides à partir de Y. lipolytica. Le biodiesel d’origine microbienne est produit par transestérification de lipides extraits de cellules microbienues et est considéré comme un substitut potentiel des combustibles fossiles en raison de ses avantages dans la réduction des émissions de gaz à effet de serre. Yarrowia lipolytica est l’une des levures oléagineuses les plus étudiées capables de produire des lipides dans certaines conditions de fermentation et est considérée comme un hôte industriel potentiel pour la production de biodiesel. Plusieurs approches ont été évaluées pour augmenter l’attrait économique de la production de biodiesel à partir des lipides de Y. lipolytica. Dans cette revue, nous avons mis en évidence les différentes stratégies rapportées dans la littérature, permettant à cette levure d’atteindre une accumulation élevéée de lipides. Ceux-ci incluent des stratégies d’ingénierie métabolique, l’utilisation de substrats carbonés et azotés à faible coût et l’optimisation des conditions de culture pour une productivité lipidique plus élevéée et des coûts d’exploitation plus faibles. Nous avons également résumé les technologies de lyse cellulaire les plus efficaces qui améliorent l’efficacité d’extraction des lipides de Y. lipolytica.

Mots clés : Y. lipolytica / huiles microbienues / accumulation des lipides / extraction des lipides / biodiesel

Highlights
- The potential use of Y. lipolytica as a promising host for biodiesel production is highlighted.
- Biosynthesis and degradation pathways of lipids in Y. lipolytica are described.

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1 Introduction

Fossil fuels are commonly used as a primary source of non-renewable energy. Their combustion releases large amounts of greenhouse gases, which negatively contributes to global warming. Concerns about climate change and the quick exhaustion of fossil fuel in the near future have driven research on the production of renewable biofuels as
alternatives (Mat Aron et al., 2020). First, second and third generation biofuels are obtained depending on the raw material and the technology used for their production (Singh et al., 2011). Ethanol produced by fermenting sugar extracted from edible feedstocks or biodiesel obtained by transesterification of vegetable oils extracted from plants are examples of first generation biofuels (Tan et al., 2010; Singh et al., 2011; Xie, 2017). The main limitation of first generation biofuels is the use of edible feedstocks such as sugarcane and corn starch that can present a serious conflict with food industries (Datta et al., 2019). Besides competition with food crops, the cultivation of biofuel crops requires large amounts of land area, fertilizers and water, which result in a high production cost (Pascalidou et al., 2016; Enamala et al., 2018). Therefore, the use of non-edible biomass such as agricultural lignocellulosic biomass is favored for the production of second-generation biofuels. However, the production of lignocellulosic biofuels requires complicated pretreatment of the biomass to obtain fermentable sugars, consequently compromising its economic feasibility (Pascalidou et al., 2016; Ali et al., 2020). Consequently, third generation biofuels obtained from microorganisms such as bacteria, yeasts or microalgae have received increasing attention as sustainable and renewable alternatives to plant-based oils (Abghari and Chen, 2014). Microalgae have shown a promise potential to be used as a source for biofuels production. However, their usage is still limited due to the high cost of cultivation, low biomass concentration and the lack of genetic engineering and transformation tools to create dependable models of algal strains (Li et al., 2008; Gimpel et al., 2015; Xue et al., 2021). Compared to algae, bacteria have a higher growth rate and their cultivation is easier but they accumulate lipids in their membrane which make them difficult to extract (Meng et al., 2009). Therefore, among the oleaginous microorganisms, yeasts are the most effective in terms of lipid accumulation (Darvishi et al., 2017).

*Yarrowia lipolytica* is a non-conventional yeast which has received particular interest for single cell oil production because of its easily manipulated genome and its extensively studied metabolism which permit the engineering of high lipid accumulation species (Ledesma-Amaro et al., 2015). *Y. lipolytica* belongs to the Ascomycota phylum. It was previously named as *Candida, Endomycopsis* or *Saccharomyces lipolytica*. The name “*Yarrowia*” suggested in 1980, refers to the researcher David Yarrow who has identified this genus. The species name “*lipolytica*” is related to the ability of the yeast to hydrolyze lipids (Zieniuk and Fabiszewska, 2019). *Y. lipolytica* strains are isolated from lipid or protein-rich environments such as dairy products and sewage (Carsanba et al., 2018). *Y. lipolytica* as Generally Recognized As Safe (GRAS) is a non-pathogenic yeast which has the ability to assimilate hydrophilic and hydrophobic substrates to produce value-added byproducts such as long chain dicarboxylic acids mainly used in the synthesis of polyesters and polylamides (Abghari et al., 2017), organic acids (Papanikolaou et al., 2002b) and lipids through de novo and ex novo pathways (Hardevi, 2014; Brabender et al., 2018; Amalia et al., 2020).

Cellular lipid production in *Y. lipolytica* depends on the genetic background of the strain, the type of substrates used in the culture media and the cultivation conditions. For example, some wild-type strains of *Y. lipolytica* can accumulate respectively up to 40% lipids using volatile fatty acids (Fontanille et al., 2012) and 58.5% lipids using sugar cane bagasse hydrolysate (Tsigie et al., 2011). Moreover, lipid accumulation can exceed 80% in obese *Y. lipolytica* strains through genetic modifications (Nicot, 2012). Concerning growth conditions, nitrogen starvation is known to favor the accumulation of lipids in this yeast. However, most of the wild-type strains cultivated on non-fatty substrates such as glucose or glycerol are not capable to accumulate high lipid levels even under nitrogen limited conditions, since those are degraded to the benefit of organic acids production (Papanikolaou et al., 2002a, 2009). Using a double nitrogen and magnesium limited-media containing glucose or glycerol as substrates, allowed an increase in lipid accumulation while organic acid production was low (Bellou et al., 2016). Lipids produced by *Y. lipolytica* are accumulated in subcellular compartments called lipid bodies. These lipids consist mainly of triacylglycerols (TAG) and sterol esters and comprise of up to 80% of unsaturated fatty acids with a high proportion of linoleic acid which gave them an important added value in terms of biofuel production (Gálvez-López et al., 2019). Interestingly, the lipid profile of *Y. lipolytica* oil can be modified by using low-cost fatty substrates to provide tailor-made lipids (Papanikolaou and Aggelis, 2010). For example, *Y. lipolytica* cultivated on stearin produced lipids with significant amounts of stearic acid and low amounts of unsaturated fatty acids which gave them a composition similar to that of cocoa butter (Papanikolaou et al., 2001, 2003).

The cost of oil and biodiesel production from *Y. lipolytica* could be extrapolated from the estimated numbers presented in the study of Koutinas et al. (2014). They found that the cost of biodiesel production from 10 000 ton of microbial oil/year is relatively high. About M$ 34.1/year are needed for lipid production using glucose ($ 400/ton) as a substrate (Koutinas et al., 2014). Therefore, one of the main challenges for industrial lipid production from *Y. lipolytica* is to achieve high lipid yields and productivity especially in the engineered strains that use low-cost substrates. Moreover, the improvement of the fermentation and downstream processes represent another challenge to decrease the cost of the overall process (Abghari and Chen, 2014). In this regard, the first part of this review summarizes the strategies applied to boost lipid production in the oleaginous yeast *Y. lipolytica*. These include metabolic engineering strategies, controlling fermentation conditions (e.g., agitation, pH, etc.), and the use of low-cost effective substrates. The second part provides an overview of the cell disruption technologies that have been used to increase lipid recovery from *Y. lipolytica*.

2 Biosynthesis and degradation of lipids in *Y. lipolytica*

*Y. lipolytica* stores its synthesized fatty acids (FAs) in lipid bodies which mainly consist of about 85% TAG and 8% sterol esters (SE) (Xie, 2017). It can synthesize TAG via two pathways. The first one is the de novo pathway, which requires hydrophilic substrates (e.g., sugars, organic acids, alcohols, etc.) to produce FAs from the precursor acetyl-CoA, and assemble them into TAG. The second one is the ex novo pathway, which requires the hydrolysis of hydrophobic substrates (e.g., alkanes, TAG, etc.) into FAs and glycerol.
De novo lipid synthesis is activated under nitrogen-limited conditions and requires carbon substrates such as sugars and alcohols (Mathiazhakan et al., 2016). The decrease in nitrogen amount improves the activity of the enzyme adenosine monophosphate deaminase 1 (AMPD1) which changes adenosine monophosphate (AMP) into inosine monophosphate (IMP) and ammonia. The decrease in AMP concentrations inhibits the action of the enzyme isocitrate dehydrogenase (IDH) responsible for the catalysis of the oxidative decarboxylation reaction of isocitrate, giving α-ketoglutarate and carbon dioxide. As a result, the tricarboxylic acid (TCA) cycle is downregulated and the accumulated citrate is transported from the mitochondria into the cytosol. Citrate is then cleaved by ATP-citrate lyase (ACL1 and ACL2) to obtain cytosolic acetyl-CoA, the starting material for lipid synthesis (Lazar et al., 2018; Wang et al., 2020). Several reactions take place in the cytosol to produce FAs from acetyl-CoA substrate. First, acetyl-CoA carboxylase (ACC1) catalyzes the conversion of acetyl-CoA into malonyl-CoA. Then the FA synthase complex (FAS1 and FAS2) allows the transformation of malonyl-CoA into acyl-CoA, and the elongation of acyl-CoA to 16 or 18 carbon atoms. In a next step, the obtained C16:0 and C18:0 molecules are transported to the endoplasmic reticulum for further elongation and desaturation (Ledesma-Amaro et al., 2016).

The formation of TAG from three FAs and one glycerol-3-phosphate (G3P) follows the Kennedy pathway (Dulermo and Nicaud, 2011). G3P is converted to lysocephosphatic acid (LPA) under the action of G3P acyltransferase (SCT1). LPA is subsequently transformed to phosphatidic acid (PA) by LPA acyltransferase (SLC1). Diacylglycerol (DAG) is then generated through the dephosphorylation of PA by PA phosphatase (PAP). In a final step, TAG can be synthesized by 2 different reactions. The first one is catalyzed by DAG acyltransferase (DGA1 or DGA2) and uses acyl-CoA as the final acyl group donor. The second one is independent from acyl-CoA and uses glycerophospholipid as the acyl group donor under the action of phospholipid DAG acyltransferase (LRO1) (Athenstaedt et al., 2006; Lazar et al., 2018; Wang et al., 2020).
Y. lipolytica can also synthesize lipids through the ex novo pathway using hydrophobic substrates such as TAG, alkanes and free fatty acids (FFAs) (Lopes et al., 2018). Therefore, many studies have investigated the potential use of this yeast for the valorization of waste and by-products. Animal fats such as tallow (Papanikolaou et al., 2007) and pork fat (Patrignani et al., 2011), vegetable (Louhasakul et al., 2020), cooking (Lopes et al., 2019) and fish (Fabiszewska et al., 2021) oils waste have been used as substrates by Y. lipolytica to produce single cell oil via ex novo pathway. However, some hydrophobic substrates may be toxic to the cells according to their chain length. For example, no growth was observed when alkanes and fatty acids shorter than C9 are used as carbon sources. Moreover, Y. lipolytica which could use a large variety of fatty acids with a chain length over C9 at concentrations above 3% is not able to grow on some type of C9 fatty acids such as nonanoic acid at a concentration higher than 0.02% (Moreno, 2018). Similarly, Y. lipolytica exhibited partiality to short chain FAs (C12:0, C14:0 and C16:0) which were totally consumed in a fermentation media containing glucose, glycerol and stearin (Papanikolaou et al., 2003). The hydrolysis of TAG substrates into glycerol and FFAs is regulated by extracellular lipases (Donot et al., 2014). The hydroxylation of alkanes entering the cells into fatty alcohols occurs in the endoplasmic reticulum via the cytochrome P450-dependent alkane monooxygenases. Fatty alcohols are then oxidized to fatty aldehydes and further to FAs respectively by fatty alcohol and fatty aldehyde dehydrogenases (Fukuda, 2013). The obtained FAs are converted to acyl-CoA, the substrate for TAG synthesis, through the acyl-CoA synthetase (FAA1) (Wang et al., 2020).

When carbon is insufficient, TAG degradation occurs to maintain cellular metabolism. In a first step, the intracellular lipases TGL3 and TGL4 degrade TAG into FFAs (Dulermo et al., 2013). Subsequently, the enzyme FAA1 generates acyl-CoA from the released FAs. Four reactions ensure the cleavage of the long acyl-CoA chains in the peroxisome through the β-oxidation pathway to obtain acetyl-CoA. The first reaction is catalyzed by six acyl-CoA oxidase (POX1-6) (Beopoulos et al., 2008). The second and third reactions occur under the action of a multifunctional enzyme (MFE1) and the last reaction is catalyzed by 3-ketoacyl-CoA thiolase (POT1) (Blazeck et al., 2014; Lazar et al., 2018).

FAs profile of Y. lipolytica’s oil depends on the substrate used in the fermentation media. When tallow, rich in saturated fatty acids, was used as a carbon source, the oil produced by Y. lipolytica was characterized by a high content in saturated FAs (78% of C18:0 and 17% of C16:0) and only small amounts of unsaturated FA (5% of Δ9C18:1) (Papanikolaou et al., 2007). Likewise, when fish oil was used as carbon source, the content of lipids accumulated in the cells was comparable to the substrate and was characterized by the presence of very long chain FAs (Fabiszewska et al., 2021). Similarly, when stearin and hydrolyzed oleic rapeseed oil were used as carbon sources, saturated FAs were the most prevalent and their percentage proportionally increased with that of stearin. This is due to the fact that unsaturated FAs (oleic acid) are utilized for growth needs, while saturated FAs are used for lipid storage (Papanikolaou et al., 2001). However, the use of glucose as co-substrate with stearin favored the production of unsaturated FAs due to the novo FAs biosynthesis (Papanikolaou et al., 2003). Some studies have also investigated the effect of cultivation conditions (pH, aeration, nitrogen depletion) on FA profile of cellular lipids (Fabiszewska et al., 2021). For example, higher quantity of oleic acid was obtained when Y. lipolytica was cultivated in highly aerated bioreactor compared to flask trials (Papanikolaou et al., 2007).

3 Enhancing lipid accumulation in Y. lipolytica

Various research studies have worked on the improvement of lipid accumulation in Y. lipolytica, by upregulating lipid synthesis or knocking out lipid degradation pathways and modifying the fermentation conditions (e.g., carbon sources, pH, aeration, fermentation mode, etc.). Table 1 summarizes the different strategies and approaches applied to enhance lipid accumulation in Y. lipolytica.

3.1 Metabolic engineering strategies

Since wild-type strains of Y. lipolytica are not the most efficient for lipid production (Lazar et al., 2018), researchers have used various metabolic engineering approaches to improve lipid productivity. The enzymes targeted in metabolic engineering strategies are shown in Figure 1.

3.1.1 Modulating endogenous gene expression

The potential of Y. lipolytica to accumulate lipids can be improved by adjusting the expression of native genes related to lipid biosynthesis and degradation. Blazeck et al. (2014) adopted a combinatorial strategy to simultaneously over-express and delete multiple key genes associated to lipid productivity in Y. lipolytica, which generated 57 different genotypes. The most advantageous engineered strain was obtained by eliminating both MFE1 and peroxisome biogenesis (PEX10) genes and overexpressing the DGA1 gene. Compared to the wild-type strain, the engineered strain represented 60-fold improvement in terms of lipid production in the optimized bioreactor conditions (Blazeck et al., 2014). Silverman et al. (2016) studied the effect of overexpressing 44 native genes on lipid biosynthesis in Y. lipolytica. The highest increase in lipid yield (246%) was obtained in the strain that overexpressed the DGA2 gene when glucose was used as a substrate (Silverman et al., 2016). Sagnak et al. (2018) evaluated the overexpression of DGA2 gene simultaneously with glycerol-3-phosphate dehydrogenase 1 (GPD1) gene. The resulting strain showed a 4-fold increase in lipid content compared to the control (Sagnak et al., 2018). Similarly, Amalia et al. (2020) overexpressed the LRO1 gene which catalyzes the binding of an acyl group into DAG for the formation of TAG. As a result, the lipid content increased from 7.5% (wild-type strain) to 12% (Amalia et al., 2020). Tai and Stephanopoulos (2013) used a push-and-pull strategy by overexpressing simultaneously ACC1 (under the hp4d promoter) and DGA1 (under the TEFin promoter) involved respectively in the first step of FAs synthesis and last step of TAG synthesis pathway. The lipid content increased from 8.7% in the control to 41% in the engineered strain that produced 0.143 g of lipids/L of culture/hour in a 2 L bioreactor (Tai and
| Carbon source | Scale          | Y. lipolytica strain | Modification                | Lipid content % (grams of lipids per gram dry weight) | Lipid titer g/L (grams of lipids per liter of culture) | Lipid yield g/g (grams of lipid per gram of carbon substrate consumed) | Productivity g/L/h (grams per lipid per liter of culture per hour) | References |
|---------------|----------------|----------------------|-----------------------------|--------------------------------------------------------|--------------------------------------------------------|------------------------------------------------------------------------|---------------------------------------------------|----------------|
| Glucose       | Bioreactor     | Po1f                 | Δmfe1, Δpex10, DGA1          | 71.0                                                   | 25.30                                                  | –                                                                      | 0.210                                             | (Blazeck et al., 2014) |
| Glucose       | Flask          | Po1g                 | DGA2                        | –                                                     | 2.4                                                    | 0.55                                                                  | –                                                 | (Silverman et al., 2016) |
| Glucose       | Bioreactor     | JMY3501              | GPID1, DGA2                  | 55.0                                                   | –                                                      | 0.140                                                                 | –                                                 | (Sagnak et al., 2018) |
| Glucose       | Bioreactor     | Po1g                 | hpl4d-ACC1, TEFm-DGA1       | 61.7                                                   | –                                                      | 0.195                                                                 | 0.143                                              | (Tai and Stephanopoulos, 2013) |
| Glucose       | Bioreactor     | Po1g                 | TEFm-ACC1, TEFm-DGA1,      | –                                                      | 55.00                                                   | 0.234                                                                  | 0.707                                              | (Qiao et al., 2015) |
| Glycerol      | Flask          | Po1d                 | Δgsy1                       | 52.0                                                   | –                                                      | –                                                                      | –                                                 | (Bhutada et al., 2017) |
| Glucose       | Flask          | Po1g                 | Hpl4d-LRO1                  | 12.0                                                   | 1.30                                                   | –                                                                      | –                                                 | (Amalia et al., 2020) |
| Glucose       | Flask          | ATCC 20362           | ΔΔm1                        | 30.0                                                   | –                                                      | –                                                                      | –                                                 | (Seip et al., 2013) |
| Glycerol      | Bioreactor     | H222                 | Δmex1, DGA1, DGA2           | 53.0                                                   | 4.80                                                   | –                                                                      | 0.070                                              | (Abghari and Chen, 2017) |
| Glucose       | Flask          | Po1f                 | EMS mutagenesis             | 87.0                                                   | 38.9                                                   | 0.243                                                                  | 0.509                                              | (Liu et al., 2015a, b) |
| Glucose       | Bioreactor     | Po1h                 | Δmga2                       | –                                                      | 25.00                                                   | 0.213                                                                  | 0.145                                              | (Liu et al., 2015b) |
| Glycerol      | Flask          | AACA-DC 50109        | Δmhy1                       | 43.1                                                   | –                                                      | –                                                                      | –                                                 | (Wang et al., 2018) |
| Glucose       | Bioreactor     | Po1f                 | ACC1, DGA1, SCD, Δpex10,   | 77.8                                                   | 50.00                                                   | –                                                                      | –                                                 | (Yan et al., 2020) |
| Glucose       | Bioreactor     | NS18                 | DGA1 (from Rhodosporidium torulosum), DGA2 (from Claviceps purpurea), ΔmG1 | 77.0                                                   | 85.00                                                   | 0.210                                                                  | 0.730                                              | (Friedlander et al., 2016) |
| Glycerol      | Flask          | Po1h                 | ACL (from mus musculus)     | 23.1                                                   | –                                                      | –                                                                      | –                                                 | (Zhang et al., 2014) |
| Glucose       | Bioreactor     | Po1h                 | Haemoglobin (from Vitreocilla) | 14.5                                                   | –                                                      | –                                                                      | –                                                 | (Zhang et al., 2019a) |
| Glucose       | Bioreactor     | Po1g                 | perCAT2 (from S. cerevisiae), ACC1, DGA1 | –                                                      | 66.40                                                   | 0.229                                                                  | 0.565                                              | (Xia et al., 2016) |
| Xylose        | Bioreactor     | Po1d                 | DGA2, GPD1, XDH, XR, XK, XPKA (from Aspergillus nidaunis), ACK (from Aspergillus nidaunis), Δpox1-6, ΔmG1 | 67.0                                                   | 16.50                                                   | –                                                                      | 1.850                                              | (Niehus et al., 2018) |
| Glucose/xylose| Bioreactor     | Po1f                 | XK, DGA1, Δpex10            | 56.7                                                   | 13.50                                                   | –                                                                      | –                                                 | (Yook et al., 2020) |
| Glucose       | Bioreactor     | Po1g                 | ACC1, DGA1, MCE2 (from Mucor circinelloides), GAPC (from Clostridium acetobutylicum) | 66.70                                                   | 99.3                                                   | 0.279                                                                  | 1.200                                              | (Qiao et al., 2017) |
| Glucose       | Bioreactor     | Po1g                 | GSR, yIGPO, ScZw f, ScALDH E, TSTR 5151 | 82.5                                                   | 72.70                                                   | 0.252                                                                  | 0.970                                              | (Xu et al., 2017) |
| Crude glycerol| Flask          | TISTR 5151           | –                          | 64.0                                                   | –                                                      | –                                                                      | –                                                 | (Cheirsilp and Louhasakul, 2013) |
| Crude glycerol| Flask          | A101                 | –                          | 25.0                                                   | 1.69                                                   | –                                                                      | –                                                 | (Dobrzenski et al., 2016) |
| Crude glycerol| Flask          | QU21                 | –                          | 18.9                                                   | 1.27                                                   | 0.060                                                                  | –                                                 | (Poli et al., 2014) |

Table 1. Summary of strategies for increasing lipid accumulation in Y. lipolytica. The symbol Δ represents a gene knockout and gene expression is represented by all uppercase letters.
Table 1. (continued).

| Carbon source | Scale          | Y. lipolytica strain | Modification                                                                 | Lipid content % (grams of lipids per gram dry weight) | Lipid titer g/L (grams of lipids per liter of culture) | Lipid yield g/g (grams of lipid per gram of carbon substrate consumed) | Productivity g/L/h (grams per lipid per liter of culture per hour) | References                  |
|---------------|----------------|----------------------|------------------------------------------------------------------------------|--------------------------------------------------------|----------------------------------------------------------|------------------------------------------------------------------------|---------------------------------------------------------------------|-----------------------------|
| Crude glycerol| Bioreactor     | SKY7                 | Purification of crude glycerol using phosphoric acid                          | 7.21                                                   | 19.47                                                    | –                                                                      | –                                                     | (Kumar et al., 2020)       |
| Purified glycerol| Bioreactor     | –                    | –                                                                             | –                                                      | 7.21                                                    | –                                                                      | –                                                     |                             |
| Crude glycerol| Flask          | SM7                  | Addition of olive oil                                                         | 35.80                                                  | 6.13                                                    | –                                                                      | –                                                     | (Magdouli et al., 2017)    |
| Palm oil mill effluent | Flask | TISTR 5151 | Addition of surfactant                                                        | 20.40                                                  | 6.13                                                    | –                                                                      | –                                                     | (Louhasakul et al., 2020)  |
| Acetic acid   | Flask          | CICC 31596           | Maintain an alkaline pH                                                       | 27.22                                                  | 10.11                                                   | 0.144                                                                  | –                                                     | (Gao et al., 2020)         |
| Glucose      | Culture tubes  | Po1f                 | Using urea as a nitrogen source                                               | 20.40                                                  | 6.13                                                    | –                                                                      | –                                                     | (Brabender et al., 2018)   |
| Glucose      | Bioreactor     | ACA-DC50109          | Double nitrogen and magnesium limitation                                      | 47.5                                                   | 5.80                                                    | –                                                                      | –                                                     | (Bellou et al., 2016)      |
| Crude glycerol| Bioreactor     | SKY7                 | pH controlled at 6.5                                                          | 45.4                                                   | 7.78                                                    | –                                                                      | –                                                     | (Kuttiraja et al., 2018)    |
| Glycerol     | Flask          | W 29                 | pH 5.5                                                                        | 45.4                                                   | 7.78                                                    | –                                                                      | –                                                     | (Sekova et al., 2019)       |
| Glucose      | Flask          | ATCC 20460           | Increase in OTR factor                                                        | –                                                      | 0.023                                                   | –                                                                      | –                                                     | (Lopes et al., 2018)       |
| Glucose      | Bioreactor     | H222                 | Drop of oxygen concentration from 50% to 1% after 20 h cultivation           | –                                                      | 0.65                                                    | –                                                                      | –                                                     | (Kavicek et al., 2015)      |
| Glycerol     | Bioreactor     | SM7                  | Two-stage cultivation with a dissolved oxygen shift from 60% to 30%         | 52.7                                                   | 13.60                                                   | –                                                                      | –                                                     | (Sara et al., 2016)        |
| Glucose and | Bioreactor     | MUCL 28849           | Two-stage fed-batch fermentation                                              | 40.7                                                   | 12.36                                                   | 1.030                                                                  | 0.160                                                               | (Fontanille et al., 2012)   |
| acetic acid  | Bioreactor     | S47                  | Two-stage fed-batch fermentation                                              | 57.3                                                   | 12.20                                                   | –                                                                      | –                                                     | (Wang et al., 2019)        |
| Pretreated cane molasses | Bioreactor | –                    | –                                                                             | –                                                      | –                                                        | –                                                                      | –                                                     |                             |
| Glycerol     | Bioreactor     | LGAM S(7)1           | Continuous fermentation                                                       | 43.0                                                   | –                                                      | 1.200                                                                  | 0.430                                                               | (Papanikolaou and Aggelis, 2002) |
| Glycerol     | Chemostat      | JMY4086              | Continuous fermentation                                                       | –                                                      | 24.20                                                   | 0.100                                                                  | 0.430                                                               | (Rakicka et al., 2015)      |
3.1.2 Modulating metabolic regulators expression

Stephanopoulos, 2013). Qiao et al. (2015) upregulated the simultaneous expression of ACC1, DGA1 and stearoyl-CoA desaturase (SCD) genes in Y. lipolytica. Total lipid yield increased by 2.93-fold in the engineered strain that showed a maximal lipid productivity close to 1 g/L/h after 78 h of fermentation in a 1.5 L bioreactor. The improvement in lipid yield in the engineered strain is due to the upregulated activity of the SCD enzyme that catalyzes the Δ9-desaturation of saturated FAs to mono-unsaturated FAs. Therefore, the possible allosteric inhibition of ACC1 by saturated FAs could be released, which improves the FAs biosynthetic flux (Qiao et al., 2015). Lipid transporters have also been regulated to increase lipid accumulation in Y. lipolytica. Dulermo et al. (2014) found that Ylfat1 protein is responsible of the export of FAs from lipid bodies. Therefore, ΔYlfat1 strain accumulated 37% of lipids compared to 25% for the parent strain (Dulermo et al., 2014). The knockout of pxa1 transporter of fatty acyl-CoA peroxisome in addition to other fatty acyl-CoA oxidase genes has also resulted in 11-fold improvement of FAs titer compared to the wild-type strain (Ghogare et al., 2020). Finally, redirecting the metabolic flux toward lipid synthesis has effectively enhanced lipid accumulation in Y. lipolytica. Bhutada et al. (2017) deleted the glycogen synthase gene (GSY1) involved in glycogen synthesis in Y. lipolytica strain engineered for high lipid accumulation. They observed a 60% increase in TAG accumulation with up to 2-fold improvement of desaturated C18 FAs compared to the control strain. They concluded that glycogen synthesis pathway competes with TAG accumulation and the elimination of GSY1 gene redirected the cellular carbon flux from glycogen to TAG synthesis (Bhutada et al., 2017). In another study, Liu et al. (2015a) created spontaneous mutations in the Y. lipolytica strain Po1f Δpex10 Δmfe1 and studied the strain E26 that presented the highest lipid content. The results of genome sequencing of E26 revealed a mutation in the open reading frame UGA2 which encodes for a succinate semialdehyde dehydrogenase involved in the γ-aminobutyric acid (GABA) assimilation pathway. They concluded that a decrease in GABA assimilation pathway could reduce the flux through the TCA cycle, thus redirecting more carbon towards the lipogenic pathway (Liu et al., 2015a).

3.1.3 Heterologous gene expression

Several studies evaluated the expression of heterogeneous genes to improve lipid accumulation in Y. lipolytica. Yan et al. (2020) combined the overexpression of native genes (SCD, Δ-12 desaturase (Δ-12D), ACC1, DGA1) and heterologous Δ-15 desaturase (Δ-15D) gene from flux with the deletion of the endogenous PEX10 and MFE1. The resulting strain showed a maximal lipid content of 77.8% with a lipid yield of 50 g/L (Yan et al., 2020). Friedlander et al. (2016) applied similar modifications by overexpressing DGA1 and DGA2, respectively from Rhodosporidium toruloides and Claviceps purpurea, and deleting the endogenous TGL3 involved in lipid degradation, and demonstrated that these modifications resulted in a lipid yield of 85 g/L (Friedlander et al., 2016). The overexpression of ACL gene involved in the production of acetyl-CoA from Mus musculus increased the lipid content in Y. lipolytica from 7.3% to 23% without a significant change in the FAs profile (Zhang et al., 2014). Moreover, the overexpression of Vitreoscilla haemoglobin in Y. lipolytica for improving O2 consumption during fermentation, increased the total FAs content by 40% compared to the wild-type strain when the dissolved oxygen concentration was measured at 30% (Zhang et al., 2019a).

3.1.4 Engineering alternative pathways

3.1.4.1 Alternative acetyl-CoA pathways

In Y. lipolytica, the ACL provides acetyl-CoA from citrate when the TCA cycle is repressed under nitrogen depletion conditions. To decouple acetyl-CoA production from nitrogen depletion and increase the amount of acetyl-CoA, Xua et al. (2016) overexpressed the peroxisomal carnitine acetyltransferase (perCAT2) from S. cerevisiae and the endogenous genes (ACC1, DGA1). They observed an increase in lipid content and titer compared to the strain that only overexpressed ACC1 and DGA1 genes (Xua et al., 2016). In another study, Niehus et al. (2018) engineered a new strain of Y. lipolytica that can produce lipids using xylose as a carbon substrate. They overexpressed native xyitol dehydrogenase (XDH), xylose reductase (XR), and xylulose kinase (XK) in the Po1d strain, to allow growth on xylose. They also designed two efficient pathways for the production of acetyl-CoA from xylulose-5-P.

In the first pathway, they overexpressed phosphoketolase (XPKA) from Aspergillus nidulans that converts D-xylose-5-P to acetyl-P and phosphotransacetylase (PTA) from Bacillus subtilis that changes acetyl-P to acetyl-CoA. In the second pathway, they overexpressed the XPKA and acetate kinase (ACK) enzymes from Aspergillus nidulans to obtain acetate to 60.2% (Wang et al., 2013). Liu et al. (2015b) studied the role of mga2 as a regulator of desaturase gene expression in Y. lipolytica. They showed that mutating or deleting the mga2 gene allowed the overexpression of Δ-9-desaturase (OLE1) and resulted in the increase of the metabolism of unsaturated FAs (notably C16:1 and C18:1) (Liu et al., 2015b). Wang et al. (2018) evaluated the regulatory function of MHY1 gene in lipid synthesis. The deletion of this gene regulated the expression of 1567 genes involved in lipid biosynthesis in Y. lipolytica and increased lipid content from 30.2% (wild-type strain) to 43.1% (Δmhy1 strain) (Wang et al., 2018).
| Y. lipolytica strain | Technology | Extraction process | Lipid yield | Advantages | Disadvantages | References |
|---------------------|------------|--------------------|-------------|------------|--------------|------------|
| ATCC 20460          | Conventional solvent extraction | 3 g of wet yeast biomass with 10 mL chloroform:methanol (1:2, v/v); 30 min maceration, constant stirring at room temperature | 6.23 ± 0.51 g/100 g of dry weight (dw) | Easy to conduct; Reproducible; Easy to scale up | Limited diffusion due to strong cell walls; Lower extraction yields compared to the other treatments | (Meullemiestre et al., 2016) |
|                     | Ultrasound assisted-extraction | 10 g of wet biomass with 50 mL chloroform:methanol (1:2, v/v) placed in a double jacket reactor; 30 min sonication at 300 W, 20 °C | 8.10 ± 0.24 g/100 g of dw | Higher extraction yields compared to conventional solvent extraction | Degradation of DAG into FFAs; Change in FAs profile: higher proportion of palmitic acid C16 compared to the other methods | |
|                     | Bead milling | 3 g of wet biomass with 10 mL chloroform:methanol (1:2, v/v) placed in 20 mL tube with 20 g ceramic beads; 30 min treatment | 13.16 ± 0.68 g/100 g of dw | Efficient for cell disruption; Higher extraction yields compared to conventional solvent extraction | – | |
|                     | Microwave-assisted extraction | 10 g of wet biomass with 50 mL chloroform:methanol (1:2, v/v) placed in a Teflon microwave reactor; 30 min treatment at 100 W, 110 °C | 7.13 ± 0.45 g/100 g of dw | Higher extraction yields compared to solvent extraction | Degradation of DAG into FFAs | |
|                     | Cold drying under reduced pressure pretreatment | Biomass placed in a reactor; 48 h, −80 °C, −20 mbar. Then, maceration in chloroform: methanol (1:2, v/v) | 15.56 ± 0.24 g/100 g of dw | – | Increase in processing time and energy; High emission of carbon dioxide | |
|                     | Freezing/defrosting pretreatment | Biomass frozen for 48 h at −20 °C then placed for 12 h at 4 °C. The process is repeated 3 times followed by oil extraction in chloroform: methanol (1:2, v/v) | 5.53 ± 0.43 g/100 g of dw | Decrease in the use of solvents | High energy consumption; High emission of carbon dioxide | |
|                     | Bead milling pretreatment | 3 g of wet biomass with 10 mL chloroform:methanol (1:2, v/v) placed in 20 mL tube with 20 g ceramic beads; 30 min treatment, 4000 rpm. Then, oil extraction in chloroform: methanol (1:2, v/v) | 12.73 ± 0.41 g/100 g of dw | Decrease in the use of solvents; Low energy consumption | – | |
|                     | Microwave pretreatment | 10 g of biomass placed in a Teflon microwave reactor and heated; 15 min, 45 °C, 20 W. | 8.18 ± 0.67 g/100 g of dw | Decrease in the use of solvents | – |
| Y. lipolytica strain | Technology                        | Extraction process                                                                 | Lipid yield | Advantages                  | Disadvantages                                           | References                        |
|---------------------|----------------------------------|-------------------------------------------------------------------------------------|-------------|-----------------------------|---------------------------------------------------------|-----------------------------------|
| JMY 5289            | High pressure homogenization (HPH) pretreatment | 1 kg of 15% DM cell suspension preteated with HPH (5 passes, 1500 bar). Then, oil extraction from dry biomass (after lyophilization) in n-hexane, at room temperature, 1 h, liquid/solid ratio of 10:1 (v:w), agitation 700 rpm | 100%        | High extraction yields      | High energy consumption during the drying process         | (Drévillon et al., 2018)          |
| JMY 5578            | Mechanical expression pretreatment | 85 g yeast suspension placed in the pressing chamber; 45 min at 5.10^5 Pa            | ≈25 ± 0.5%  | --                          | Lowest extraction yields compared to the tested pretreatment techniques | (Drévillon et al., 2019)          |
|                     | High pulsed electric field pretreatment | 200 g of yeast cell suspension (15% DM) placed in treatment chamber (500 pulses, 20 Kv/cm); lyophilization; oil extraction in n-hexane (ratio 1:10, w/v), 1 h, at room temperature with agitation at 700 rpm | 29.4 ± 3%   | Additional release of oil compared to the untreated cells | --                                                      |                                   |
|                     | High voltage electrical discharges pretreatment | 200 g of yeast cell suspension (15% DM) placed in treatment chamber (500 pulses, 2 = 40 Kv/cm); lyophilization; oil extraction in n-hexane (ratio 1:10, w/v), 1 h, room temperature with agitation at 700 rpm | 31.7 ± 6.5% | Additional release of oil compared to the untreated cells | Degradation of the oil Significant changes in FAs composition compared to the other treatments except for C14:0, C18:0 and C18:2 |                                   |
|                     | Ultrasound pretreatment           | 300 g yeast cells (15% DM) placed in US reactor (1 h, 400 W, 293 ky); lyophilization; oil extraction in n-hexane (ratio 1:10, w/v), 1 h, room | 35.5 ± 6.1% | Increase in extraction yields compared to the untreated cells (control) | No significant change in FAs profile                   |                                   |
Table 2. (continued).

| Y. lipolytica strain | Technology | Extraction process | Lipid yield | Advantages | Disadvantages | References |
|---------------------|------------|--------------------|-------------|------------|--------------|------------|
|                     | HPH pretreatment | temperature with agitation at 700 rpm | 83.9 ± 4.8% | Highest extraction yields compared to the tested pretreatment techniques | Change in FAs profile | – | (Imatoukene et al., 2020) |
| JMY5289             | HPH and bead milling pretreatment | Cell suspension (600 g, 15% DM) pretreated with HPH (298 K, 20 passes, 1500 × 10^5 Pa) followed by bead milling in chloroform:methanol (2:1, v:v) (stainless steel beads of 4.9 mm, and during 3 × 30 s) | 99.6% | Available for large scale processing | – | – | (Poli et al., 2013) |
| QU21                | Liquid nitrogen with sonication | Liquid nitrogen added to biomass, then sonication 10 times for 30 s each in distilled water followed by maceration in 20 mL chloroform and methanol (2:1, v:v) for oil extraction | 26.5% | Increase in the extraction yields compared to conventional maceration (14.3%) | – | – | (Tsigie et al., 2012) |
| Po1g                | Sub-critical water treatment | 1 g biomass dissolved in 20 mL water and treated at 175 °C for 20 min | 42.69% | Environmentally friendly technique Very similar FAs profile from untreated and sub-critical water treated samples | – | – |
which is then converted to acetyl-CoA by the acetyl-CoA synthetase (ACS2) (Niehus et al., 2018). Similarly, Yook et al. (2020) introduced a xylose isomerase pathway in *Y. lipolytica*. They combined the overexpression of xylose isomerase gene *XylA* from *S. cerevisiae* and the endogenous XK and DGA1 genes with the deletion of PEX10 gene to prevent lipid degradation and improve lipid production from xylose substrate (Yook et al., 2020).

### 3.1.4.2 Cytosolic NADPH pathways

TAG synthesis involves large amounts of NADPH in both elongation step and desaturation reaction (Ratledge, 2014). Previous studies showed that malic enzyme (MAE) has a key role in providing NADPH in oleaginous yeasts and its overexpression resulted in a 2.5-fold increase in lipid content in both *Mucor circinelloides* and *Mortierella alpine* (Zhang et al., 2007). To understand the source of NADPH in *Y. lipolytica* during lipid accumulation, Wasylenko et al. (2015) used 13C-Metabolic Flux Analysis of a *Y. lipolytica* engineered strain that overexpresses ACC1 and DGA1 for a high lipid production from glucose substrate. The comparison of the flux between the wild-type and the engineered strains showed that the oxidative pentose phosphate pathway (oxPPP) is the main source of lipogenic NADPH in *Y. lipolytica* (Wasylenko et al., 2015). Qiao et al. (2017) adopted several strategies to increase NADPH supply in *Y. lipolytica*. They activated the pyruvate/oxaloacetate/malate cycle which can convert one molecule of NADH to NADPH by the presence of high potassium concentration in crude glycerol used as carbon sources for yeast lipid production (Hapeta et al., 2017; Qin et al., 2017). Several studies investigated the efficiency of low-cost substrates such as industrial by-products or lignocellulosic biomass for effective lipid production in *Y. lipolytica*. Crude glycerol has many advantages including low cost, high availability and less CO₂ emission during fermentation (Qin et al., 2017). Previous studies have shown the ability of *Y. lipolytica* to convert crude glycerol coming from industrial by-products into single cell oils (Cheirsilp and Louhasakul, 2013; Dobrovolski et al., 2016). Poli et al. (2014) observed no significant difference in lipid content when they replaced pure glycerol (1.48 g/L) with crude glycerol (1.27 g/L) in a medium containing ammonium sulfate as a nitrogen source (Poli et al., 2014). However, the presence of impurities in crude glycerol could negatively affect lipid production. Kumar et al. (2020) purified crude glycerol to use it as a carbon source for lipid production in *Y. lipolytica*. The cultures with purified glycerol presented higher lipid yields (19.47 g/L) compared to the cultures with crude glycerol (7.21 g/L). This difference in lipid content could be explained by the presence of high potassium concentration in crude glycerol that may inhibit the ACL enzyme responsible for the conversion of citrate into acetyl-CoA. The accumulated citrate is therefore secreted out of the cell rather than converted into lipids (Kumar et al., 2020). The addition of fat such as olive oil to crude glycerol improved lipid accumulation in *Y. lipolytica* due to lipase activation (Magdouli et al., 2017; Ayadi et al., 2018). Krzyczkowska and Kozłowska (2017) found that the addition of almond oil as a carbon source stimulated the activity of lipase enzyme, and resulted in the highest lipid yield after 48 h of culture (Krzyczkowska and Kozłowska, 2017). Louhasakul et al. (2020) highlighted the effect of adding biosurfactants to lipid productivity. *Y. lipolytica* cultivated in palm oil mill effluent with 2% crude glycerol presented more lipids in the presence of biosurfactants (Louhasakul et al., 2020). Therefore, the addition of surfactants to the fermentation media containing hydrophobic substrates, may increase the permeability of the cell membranes, leading to more nutrients uptake (Taoka et al., 2011) and lipase secretion (Deive et al., 2009). Gao et al. (2017) evaluated the growth and lipid accumulation of *Y. lipolytica* cultured on volatile fatty acids (VFAs) such as butyric, propionic and acetic acids. Among the tested VFAs, the yeast showed a fastest consumption rate of acetate that could be directly cleaved into acetyl-CoA, unlike propionate and butyrate. The highest lipid yield coefficient (0.29 g lipid/g dry cell weight) was obtained with an initial concentration of 2.5 g/L of acetic acid at pH 6 (Gao et al., 2017). In a further study, Gao et al. (2020) improved lipid accumulation in *Y. lipolytica* cultivated on VFAs. They obtained the highest lipid concentration of 10.11 g/L from 70 g/L of acetic acid under alkaline conditions (optimal pH8) (Gao et al., 2020).

In addition to carbon, nitrogen is an essential component of fermentation media. Ammonium sulfate is generally used as a nitrogen source in the media for yeast fermentation. Brahender et al. (2018) conducted a study to evaluate the effect of synthetic urine (18 g/L urea) as an alternative low-cost nitrogen source for the accumulation of lipids in *Y. lipolytica* Δpex10 strain. The cultures containing synthetic urine and glucose as a carbon source (C:N≈240:1) accumulated

### 3.2 Modifying the medium composition

Multiple nutrients are essential for microbial growth and lipid production. Monosaccharides such as glucose, fructose, galactose, sucrose, pure glycerol and organic acids are usually used as carbon sources for yeast lipid production (Hapeta et al., 2017; Qin et al., 2017). Previous studies have shown the ability of *Y. lipolytica* to convert crude glycerol coming from industrial by-products into single cell oils (Cheirsilp and Louhasakul, 2013; Dobrovolski et al., 2016). Poli et al. (2014) observed no significant difference in lipid content when they replaced pure glycerol (1.48 g/L) with crude glycerol (1.27 g/L) in a medium containing ammonium sulfate as a nitrogen source (Poli et al., 2014). However, the presence of impurities in crude glycerol could negatively affect lipid production. Kumar et al. (2020) purified crude glycerol to use it as a carbon source for lipid production in *Y. lipolytica*. The cultures with purified glycerol presented higher lipid yields (19.47 g/L) compared to the cultures with crude glycerol (7.21 g/L). This difference in lipid content could be explained by the presence of high potassium concentration in crude glycerol that may inhibit the ACL enzyme responsible for the conversion of citrate into acetyl-CoA. The accumulated citrate is therefore secreted out of the cell rather than converted into lipids (Kumar et al., 2020). The addition of fat such as olive oil to crude glycerol improved lipid accumulation in *Y. lipolytica* due to lipase activation (Magdouli et al., 2017; Ayadi et al., 2018). Krzyczkowska and Kozłowska (2017) found that the addition of almond oil as a carbon source stimulated the activity of lipase enzyme, and resulted in the highest lipid yield after 48 h of culture (Krzyczkowska and Kozłowska, 2017). Louhasakul et al. (2020) highlighted the effect of adding biosurfactants to lipid productivity. *Y. lipolytica* cultivated in palm oil mill effluent with 2% crude glycerol presented more lipids in the presence of biosurfactants (Louhasakul et al., 2020). Therefore, the addition of surfactants to the fermentation media containing hydrophobic substrates, may increase the permeability of the cell membranes, leading to more nutrients uptake (Taoka et al., 2011) and lipase secretion (Deive et al., 2009). Gao et al. (2017) evaluated the growth and lipid accumulation of *Y. lipolytica* cultured on volatile fatty acids (VFAs) such as butyric, propionic and acetic acids. Among the tested VFAs, the yeast showed a fastest consumption rate of acetate that could be directly cleaved into acetyl-CoA, unlike propionate and butyrate. The highest lipid yield coefficient (0.29 g lipid/g dry cell weight) was obtained with an initial concentration of 2.5 g/L of acetic acid at pH 6 (Gao et al., 2017). In a further study, Gao et al. (2020) improved lipid accumulation in *Y. lipolytica* cultivated on VFAs. They obtained the highest lipid concentration of 10.11 g/L from 70 g/L of acetic acid under alkaline conditions (optimal pH8) (Gao et al., 2020).

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approximately 2 times more lipids compared to cultures containing ammonium sulfate as a nitrogen source (Brabender et al., 2018). This study showed the possibility of using natural waste product such as human urine as a nitrogen source to improve lipid production in yeast.

Bellou et al. (2016) studied the effect of nutrients limitation on lipogenesis. When glycerol was used as a carbon source, lipid content in Y. lipolytica increased from 12.8% (w/w) in the media with single nitrogen limitation to 23.5% w/w in the media with double nitrogen and magnesium limitation. A further improvement in lipid accumulation (47.5%, w/w) was observed when glycerol was replaced with glucose with double nitrogen and magnesium limitation (Bellou et al., 2016).

### 3.3 Regulating the physicochemical parameters

Changes in the cultivation parameters (e.g., pH, aeration, temperature, etc.) are needed to determine the optimal conditions for lipid accumulation. Kuttiraja et al. (2018) showed the effect of pH controlling on lipid production in Y. lipolytica. They observed a 15% increase in lipid production when the pH was maintained at 6.5 in glycerol-based fermentation media compared to pH-uncontrolled fermentation (Kuttiraja et al., 2018). In a separate study, Sekova et al. (2019) showed that a pH increase from 5.5 to 9, caused a decrease in TAG level in Y. lipolytica respectively from 63% to 37% (Sekova et al., 2019).

Studies have shown that the expression of genes involved in lipid synthesis was not pH dependent. However, some mitochondrial transporters are strongly expressed at pH 6 which results in an increase in citric acid transport into the cytoplasm and its subsequent conversion to FAs (Zhang et al., 2019b). Lopes et al. (2018) highlighted the influence of oxygen transfer rate (OTR) factor on lipid production in Y. lipolytica, cultivated on glucose media. They observed a 1.6-fold improvement in lipid content when the OTR increased from 96 mg/L/h to 192 mg/L/h (Lopes et al., 2018). Previous studies showed that the increase in dissolved oxygen concentration in the fermentation media upregulates the activities of ACL and malic enzymes involved in lipid synthesis (Bellou et al., 2014). By contrast, other authors observed a decrease in lipid accumulation in Y. lipolytica cultivated in glycerol media, when the dissolved oxygen concentration increased from 30% to 40% (Magdouli et al., 2018). Similarly, Papanikolau et al. (2002a) observed a remarkable growth of Y. lipolytica at an oxygen saturation of 60% but non-significant changes in lipid synthesis when the oxygen saturation varied between 5% and 15% (Papanikolau et al., 2002a).

The decrease in lipid accumulation in aerated cultures could be attributed to the increase in oil biodegradation (Martins et al., 2012; Magdouli et al., 2018). Therefore, it is important to find a suitable balance between oxygen needed for growth and lipid accumulation by the application of a two-stage cultivation strategy (Sara et al., 2016). Kavšek et al. (2015) decreased the dissolved oxygen concentration from 50% to 1% after 20 h of fermentation, which resulted in a 25-fold increase in lipid content in Y. lipolytica within 36 h (Kavšek et al., 2015).

### 3.4 Changing of the cultivation modes

Different operational modes can be applied for the cultivation of Y. lipolytica for lipid production. The batch mode is the most studied method for lipid production in oleaginous yeasts and it can be performed either in flasks or in bioreactors (Carsanba et al., 2020). It consists of a closed system where the cells and substrates are provided at the beginning and the fermentation occurs without any external intervention (Karamerou and Webb, 2019). However, in batch fermentation, a high C/N ratio is required which could lead to stressful conditions for yeast and result in the decrease of cell growth and lipid productivity (Christophe et al., 2012).

Therefore, fed-batch cultivation process has been applied to obtain high cell density and cellular lipid content in oleaginous yeasts. In the fed-batch cultivation, initial substrate inhibition can be avoided since nutrients are added intermittently or continuously to the culture vessel during fermentation (Poontawee and Limtong, 2020). The fed-batch fermentation using glucose and olive oil as substrates led to a 3-fold improvement in lipase production by Y. lipolytica compared to batch fermentation (Fickers et al., 2009). A two-stage fed-batch cultivation has also been applied for lipid production by Y. lipolytica. In a first stage, the cultivation of cells in a nutrient-rich medium resulted in a high cell concentration. Then, in a second stage, the addition of carbon source without auxiliary nutrients increased lipid productivity. A two-stage fed-batch cultivation of Y. lipolytica using glycerol and VFAs as carbon sources, resulted in a significant increase in lipid yields compared to batch fermentation with one carbon source (Fontanille et al., 2012). Moreover, Wang et al. (2019) applied a two-stage fed-batch fermentation using pretreated cane molasses as a carbon source for lipid production in Y. lipolytica. The fermentation process resulted in a lipid level of 12.2 g/L which is higher than that obtained at batch flask level (7.2 g/L) (Wang et al., 2019). Continuous fermentation has also been effective to increase lipid productivity in Y. lipolytica. In continuous operation, cultures are continuously supplied with substrates and the fermentation broth is withdrawn at the same rate to keep the volume constant in the fermentation system. Therefore, the C/N ratio could be maintained constant in the steady state and lipid accumulation would only depend on the dilution rate (Rakicka et al., 2015).

The effect of dilution rates on lipid synthesis by Y. lipolytica in continuous fermentation process was evaluated and the highest lipid content was obtained at a low dilution rate of 0.03 h⁻¹ (Papanikolau et al., 2002a; Papanikolau and Aggelis, 2002). Rakicka et al. (2015) found that the continuous fermentation process was more effective than the batch-fermentation for lipid synthesis in Y. lipolytica. The yeast produced 24.2 g/L of lipids in continuous cultures when the dilution rate was maintained at 0.01 h⁻¹ compared to 22.6 g/L in the fed-batch cultivation process (Rakicka et al., 2015).

### 3.5 In silico modeling

New strains of Y. lipolytica with high lipid accumulation have been engineered using in silico modeling. Wei et al. (2017) have developed a genome scale metabolic model (GEM) of Y. lipolytica to predict metabolic engineering strategies that improve lipid accumulation. The simulation of overexpressing 6 genes enhanced the availability of acetyl-CoA and malonyl-CoA and resulted in a 34.1% improvement of TAG production. Further simulation predicted that the
addition of L-threonine or L-aspartate amino acids could lead to a 55.5% improvement in TAG synthesis rate (Wei et al., 2017). Kim et al. (2019) also used a computational method to predict the overexpression or knockout targets on lipid accumulation in *Y. lipolytica*. One of the knockout targets implicated in one-carbon/methionine metabolism has been reconstructed and showed a 45% increase in lipid accumulation compared to the wild-type strain (Kim et al., 2019). GEM was also used to predict the effect of nitrogen and oxygen depletion on lipid accumulation in *Y. lipolytica*. The model was validated experimentally using fed-batch fermentation (Kavšek et al., 2015). Therefore, GEMs are powerful tools to understand and predict the effects of genetic modifications or cultivation conditions on lipid accumulation in *Y. lipolytica*.

4 Enhancing lipid extraction from *Y. lipolytica*

Chloroform and methanol mixtures are commonly used to extract lipids from oleaginous yeasts according to the conventional Bligh and Dyer and the Folch methods (Breil et al., 2017). The efficiency of Folch method for lipid extraction depends on the yeast strain. For example, Folch method was efficient for lipid extraction from *Y. lipolytica* (Sarantou et al., 2021) while an acid digestion method (2.5 M, 100 °C, 30 min) was required prior to Folch method for lipid recovery from *Lipomyces starkeyi* (Tchakouteu et al., 2015). However, *Y. lipolytica* cell wall is characterized by a high chitin content (7% of wall mass) which is responsible to the strength and resistance of the wall and could limit the extraction of intracellular lipids (Khot et al., 2020). Therefore, after fermentation, the alteration of cell-wall permeability is required to improve and facilitate lipid extraction. For this purpose, several cell disruption technologies were investigated such as bead milling, high-pressure homogenization, ultrasound and microwave to intensify lipid extraction from *Y. lipolytica* (Tab. 2).

4.1 Bead milling

Bead milling is an efficient mechanical cell disruption method characterized by its high potential for industrial implementation. Generally, a bead miller involves a motor-driven central shaft that may carry different designs of agitators (e.g., concentric or eccentric disks) inside a grinding chamber filled with glass or ceramic beads. During the operation, the movement of the beads causes compaction, shear forces and energy transfer to the cells, which results in cell disruption such as grinding (Günerken et al., 2015). Meullemiestre et al. (2016) compared bead milling (4000 rpm, 25°C) to other technologies such as microwave (20 bar, 110°C) and ultrasound (300 W, 25°C) for the extraction of lipids from *Y. lipolytica*. Results have shown that bead milling was the most efficient cell disruption technique and increased the extracted lipid yields by two times compared to the conventional maceration in methanol-chloroform (2:1, v/v) (constant stirring, 25°C) (Meullemiestre et al., 2016).

4.2 High-pressure homogenization

High pressure homogenization (HPH) is a second mechanical process that aims to push a suspension to pass through a narrow gap at high pressures. During its passage, the suspension is subjected to multiple phenomena such as friction, cavitation and turbulence which results in obtaining a fine homogenate (Tribst et al., 2008). This technique was efficient for the recovery of lipids from algae (Yap et al., 2015) and from the oleaginous yeast *Cryptococcus curvatus* (Thiru et al., 2011). Drévillon et al. (2018) investigated the efficiency of HPH pre-treatment prior to high-speed maceration in n-hexane for the recovery of lipids from *Y. lipolytica*. They found the optimal HPH parameters of 5 passes and 1500 bar yielded a 100% recovery of lipids in dry route and 80% in wet route (Drévillon et al., 2018). In another study, the authors evaluated different cell disruption techniques prior to maceration in n-hexane for oil recovery from *Y. lipolytica*. Among mechanical expression (ME), moderate pulsed electric field assisted mechanical expression (MPEF-ME), high pulsed electric fields (HPEF), high voltage electrical discharges (HVED), ultrasound (US), and HPH pre-treatment techniques, HPH was the most efficient technique and allowed the extraction of 83.8 ± 4.8% of oil compared to 19.8 ± 0.5% with maceration in n-hexane without any pre-treatment (Drévillon et al., 2019). Imatoukene et al. (2020) combined HPH with bead milling pretreatment and evaluated the efficiency of several solvents for lipid recovery from *Y. lipolytica*. The highest lipid yield (99.6%) was obtained when bead milling was preceded by HPH and the oil extraction was performed in chloroform:methanol. Among the tested green solvents, isoamyl acetate allowed the extraction of 96.7% of lipids from pretreated biomass (Imatoukene et al., 2020).

4.3 Ultrasound

Ultrasound technology is one of the most widely used method to disrupt the cell wall of oleaginous microorganisms (Patel et al., 2018). It is a physical treatment based on the cavitation phenomenon. During the propagation of ultrasound waves in a liquid media, expansion (low pressure) and compression (high pressure) cycles are induced, involving the formation of cavitation bubbles, their growth and their implosion when their size reaches a critical value. The collapse of cavitation bubbles induces a local increase in temperature and pressure, respectively, up to around 5000 K and 50 to 1000 atm, which causes cell membrane disruption (Chemat et al., 2017). Previous studies evaluated the effect of ultrasound on the intensification of lipid extraction from oleaginous yeasts (Kumar et al., 2020) and microalgae (Araujo et al., 2013). Few reports are available on the extraction of lipids from *Y. lipolytica* using ultrasound treatment. Poli et al. (2013) studied the effect of sonication on the disruption of *Y. lipolytica* cell walls. The experiment consisted on adding liquid nitrogen to biomass followed by sonication 10 times for 30 s each, in a mixture of chloroform-methanol. Compared to dry maceration in chloroform-methanol which yielded 14.3% of oil, ultrasound extraction technique allowed an increase in oil recovery up to 26.5% (Poli et al., 2013).
4.4 Microwave

Microwave treatment was also used to enhance lipid extraction from oleaginous yeasts. Microwaves are electromagnetic radiations with a frequency ranging from 0.3 to 300 GHz. The energy produced during microwave treatment provokes a rapid and homogeneous heating of the solvent and the suspension, by dipole rotation and ionic conduction (Bouras et al., 2015). The cell disruption induced by microwave treatment increased the extraction rates of lipids from S. cerevisiae compared to the conventional maceration in methanol-chloroform (Khoornrungruang et al., 2013). Microwave treatment combined to acid-catalyzed transesterification was also efficient to extract and modify lipids from Rhodotorula glutinis in 30 s at 120 °C compared to 4 h by Soxhlet solvent extraction (Chuck et al., 2014). However, in the case of Y. lipolytica, the treatment of the biomass with microwaves (1000 W, 110 °C) slightly increased lipid yield to 7.13 g/100 g of DW compared to 6.53 g/100 g of DW with the conventional maceration in methanol-chloroform (2:1, v/v). The obtained result was attributed to the degradation of lipids during microwave-assisted extraction. To improve cell disruption by microwaves and avoid lipid degradation, the authors tested microwave pretreatment of biomass during 15 min at a power of 20 W and a constant temperature of 45 °C followed by two washing steps in chloroform:methanol (1:2, v/v) at room temperature. In these conditions, lipids recovery increased to 8.18 ± 0.67 g/100 g of DW (Meullemiestre et al., 2016). In the same study, cold drying (–80 °C, −20 mbar, 48 h), freezing/defrosting (−20 °C, 24 h/4°C, 12 h) and bead milling (4000 rpm, 30 min) were also tested as pretreatment techniques to improve lipids recovery from Y. lipolytica. The maximum lipid yield of 13.56 g/100 g of DW was obtained when cold drying under pressure was applied prior to the maceration step (Meullemiestre et al., 2016).

4.5 Sub-critical water

Sub-critical water (SCW) treatment was also efficient for the recovery of lipids from Y. lipolytica (Tsigie et al., 2012; Tran Nguyen et al., 2013). It consists of using water as a solvent at high temperatures (between 100 °C and 374 °C) under high pressure to keep water in the liquid state. Under these conditions, the dielectric constant of water decreases which improves the extraction of hydrophobic compounds without using organic solvents (Shitu et al., 2015). In a first study, Tsigie et al. (2012) applied SCW on Y. lipolytica biomass as a pretreatment step prior to maceration in a mixture of hexane:methanol (2:1, v/v) for 4 h. The amount of extracted neutral lipids increased from 23.21% w/w for untreated biomass to 42.69% w/w when the biomass was pretreated at 175 °C using 20 mL water for 20 min (Tsigie et al., 2012). In another study, the pretreatment of Y. lipolytica biomass with SCW for 15 min at 175 °C caused cell walls’ rupture and resulted in 10 times increase in the extractable neutral lipids compared to the untreated biomass (Tran Nguyen et al., 2013).

Further cell disruption technologies for oil recovery from oleaginous microorganisms were developed by Koubbaa et al. (2020). However, to date, there is no study regarding the membrane-based purification techniques of oil extracted from oleaginous yeasts including Y. lipolytica.

5 Conclusions

Single cell oils produced by oleaginous microorganisms are considered as potential alternatives to plant and animal oil for biodiesel production. The yeast Y. lipolytica presents a promising host for biodiesel production, due to its ability to accumulate high amounts of intracellular lipids under some cultivation conditions. The first crucial point for the commercial utilization of microbial lipids is the cost-effective production that depends on the yeast strain and the fermentation conditions. This review summarized the approaches that have been used to improve lipid production in Y. lipolytica. Higher lipid productivity in Y. lipolytica is achieved through metabolic engineering strategies including the regulation of endogenous genes and metabolic regulators, the expression of heterogeneous genes and the design and construction of alternative pathways. High lipid yields can also be obtained by using low-cost effective carbon and nitrogen sources and modifying fermentation conditions such as pH, temperature, aeration and cultivation mode. After the fermentation step, lipid extraction from biomass is a major issue that needs to be dealt with to improve the recovery yields. For this purpose, this review discussed the cell disruption technologies that have been used to optimize the recovery of lipids from Y. lipolytica.

Conflict of interest

The authors declare no conflict of interest.

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