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REVIEW ARTICLE

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# Oleaginous yeasts for biochemicals, biofuels and food from lignocellulose-hydrolysate and crude glycerol

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## Abstract

Microbial lipids produced from lignocellulose and crude glycerol (CG) can serve as sustainable alternatives to vegetable oils, whose production is, in many cases, accompanied by monocultures, land use changes or rain forest clearings. Our projects aim to understand the physiology of microbial lipid production by oleaginous yeasts, optimise the production and establish novel applications of microbial lipid compounds. We have established methods for fermentation and intracellular lipid quantification. Following the kinetics of lipid accumulation in different strains, we found high variability in lipid formation even between very closely related oleaginous yeast strains on both, wheat straw hydrolysate and CG. For example, on complete wheat straw hydrolysate, we saw that one *Rhodotorula glutinis* strain, when starting assimilating D-xylosealso assimilated the accumulated lipids, while a *Rhodotorula babjevae* strain could accumulate lipids on D-xylose. Two strains (*Rhodotorula toruloides* CBS 14 and *R. glutinis* CBS 3044) were found to be the best out of 27 tested to accumulate lipids on CG. Interestingly, the presence of hemicellulose hydrolysate stimulated glycerol assimilation in both strains. Apart from microbial oil, *R. toruloides* also produces carotenoids. The first attempts of extraction using the classical acetone-based method showed that β-carotene is the major carotenoid. However, there are indications that there are also substantial amounts of torulene and torularhodin, which have a very high potential as antioxidants.

KEYWORDS

biofuels, crude glycerol, green chemicals, lignocellulose, oleaginous yeasts

## 1 | INTRODUCTION

The current economy heavily depends on fossil resources. For example, in fuel production, many branches of the chemical and food industry rely heavily on petrol polymers. This is problematic due to the finite nature of fossil resources and their alarmingly strong negative influence on climate change. The major challenge for humankind is to change to a bio-based economy, using renewable resources in a sustainable way. Biomass is seen as a potential raw material to replace fossil resources (Yang et al., 2021). Strictly

speaking, fossil resources also derive from biomass origins but are highly reduced in oxygen and stored for a very long time (Sato, 1990). When utilising fossil fuels, the stored carbon is released into the atmosphere as carbon dioxide, whereas new biomass binds carbon dioxide. Thus using new biomass will lead to a more balanced carbon cycle (Spagnuolo et al., 2019).

However, the replacement of fossil resources by biomass can result in other problems, such as competition for arable land, land-use changes in natural ecosystems, and in some cases even increased GHG release when compared to fossil-based systems (Gontard et al., 2018). These challenges can at least partially be approached by the use of by-products, co-products and organic waste from agriculture and forestry, that is, nonedible lignocellulosic residues (Gontard et al., 2018; Passoth & Sandgren, 2019; Valentine et al., 2012). Lignocellulose is rich in oxygen and also has the potential to generate other chemicals than those generated from substrates of petrochemical origin. However, for fuel production or in the oleochemical industry, there is a need to have a reduced oxygen content in the feedstock (Demirbas, 2011).

Lipids are essential for all organisms and are produced for instance as membrane components, storage lipids or for regulatory functions (Eisenberg & Büttner, 2014; Lingwood & Simons, 2010; Sandager et al., 2002). The proportion of lipids on the cell dry mass (CDM) is usually around 7%–15% (Kaneko et al., 1976). Oleaginous yeasts are able to accumulate more than 20% of their CDM as lipids; lipid contents of more than 70% have been reported (Ochsenreither et al., 2016; Ratledge & Wynn, 2002). Oleaginous yeasts are able to convert carbon present in lignocellulose hydrolysate to lipids, as a potential resource for fuels, chemicals and even for food or feed production, due to their general similarity to vegetable oils (Bharathiraja et al., 2017; Blomqvist et al., 2018; Patel et al., 2016). They are very diverse, including both ascomycetes—for instance, *Yarrowia lipolytica*, *Lipomyces starkeyi*, *Lipomyces lipofer* or *Blastobotrys adeninivorans*—and *basidiomycetes*—for instance, *Rhodotorula toruloides*, *Rhodotorula glutinis*, *Rhodotorula babjevae*, *Cutaneotrichosporon curvatum (syn. Cryptococcus curvatus)* and *Cutaneotrichosporon oleaginosus* (Mota et al., 2022; Sanya et al., 2021). Many of these yeasts can grow on various carbon sources including sugars derived from cellulose and hemicellulose (D-glucose, D-xylose, L-arabinose, D-cellobiose), organic acids, aromatic compounds derived from lignin, and on glycerol, obtained as a by-product from biodiesel production (Chmielarz et al., 2021; C. Huang et al., 2013; Middelhoven, 1993; Qin et al., 2017; Sànchez i Nogué et al., 2018; Valdés et al., 2020).

This review highlights some recent developments in research on oleaginous yeasts, their physiology on various carbon sources, the application of yeast lipids as feed and the production of potential other products besides lipids.

**Take-away**

- Methods for production and rapid quantification of yeast lipids established.
- Huge diversity in sugar assimilation and lipid formation in oleaginous yeasts.
- Mixing with hemicellulose hydrolysate increased glycerol assimilation.
- Co-production of chemicals to improve the feasibility of lignocellulose conversion.

## 2 | PHYSIOLOGY OF LIPID ACCUMULATION IN OLEAGINOUS YEASTS

Oleaginous yeasts accumulate lipids as storage lipids, usually in the form of triacylglycerols (TAGs). However, also a substantial proportion of free fatty acids has been recently found in several oleaginous yeasts (Nagaraj et al., 2022; Shapaval et al., 2019). Lipid accumulation takes place when there is a surplus of a carbon source (D-glucose, etc.) combined with limited availability of nutrients, such as nitrogen, phosphorus, sulphur, and so forth (Granger et al., 1993). Under these conditions, carbon flux is directed toward lipid synthesis. Sugars are converted via glycolysis and pentose-phosphate pathway (PPP) to pyruvate, which is transported into the mitochondria and oxidatively decarboxylated to acetyl-CoA by pyruvate dehydrogenase. The acetyl-CoA is further metabolised via the tricarboxylic acid (TCA) cycle (Figure 1). Pentoses are metabolised via the nonoxidative PPP. The most abundant pentose xylose is, as in most fungi, first converted to xylitol with an NADPH-dependent xylose reductase, which is then re-oxidised by an $NAD^+$-dependent xylitol dehydrogenase to xylulose. Xylulose is converted by xylulokinase to the PPP-metabolite xylulose-5-P. However, xylulose-5-P can also be converted to glyceraldehyde-3-P and acetyl-CoA by a phosphoketolase reaction (Ratledge & Wynn, 2002). Recently, an alternative pathway of xylulose utilisation has been suggested in *R. toruloides*, where it is first reduced to D-arabitol, and then re-oxidised to ribulose, which can then be phosphorylated and metabolised via the PPP (Jagtap et al., 2021; Jagtap & Rao, 2018).

Nitrogen limitation is one of the best-studied inducers of lipid biosynthesis. To provide ammonium ions ($NH_4^+$) for cell maintenance, adenosine monophosphate (AMP) deaminase cleaves AMP into inosine monophosphate (IMP) and $NH_4^+$ to provide additional nitrogen. The associated decrease in intracellular AMP influences the activity of the TCA cycle in the mitochondria. Isocitrate dehydrogenase (ICDH) in oleaginous yeasts is allosterically activated by AMP, thus it is deactivated by the decrease of AMP. ICDH is transforming isocitrate into α-ketoglutarate. On losing ICDH-activity, isocitrate and thus also citrate accumulate, which are in equilibrium. On reaching a critical value, citrate is transported out of the mitochondria into the cytoplasm in an exchange with malate, presumably by a citrate/malate shuttle (Evans et al., 1983). Citrate is subsequently cleaved into acetyl-CoA and oxaloacetate by ATP-citrate lyase, a key enzyme present in the cytosol in oleaginous microorganisms. This is at the expense of ATP and the opposite reaction of citrate synthetase in the TCA cycle. The increasing amount of acetyl-CoA is further shuttled into fatty acid synthesis, which takes place in the cytoplasm. The formed oxaloacetate is

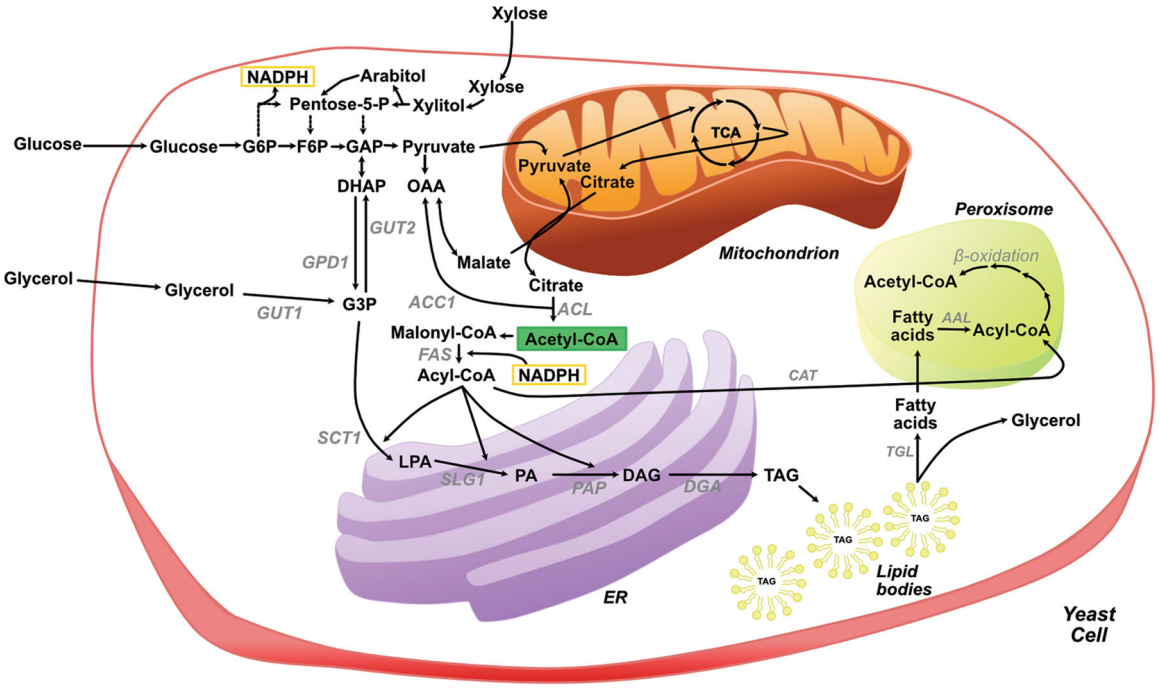


**FIGURE 1** Simplified scheme of lipid accumulation in oleaginous yeasts. Nitrogen and phosphate limitation results in the degradation of adenosine monophosphate, which is an essential cofactor of isocitrate dehydrogenase. Isocitrate is not further metabolised, accumulates and is converted to citrate, which is in equilibrium with isocitrate. Citrate is exported to cytosol with the citrate-malate shuttle. In the cytosol, citrate is degraded to acetyl-CoA and oxaloacetate. The latter is converted to malate and transported back to the mitochondria. Acetyl-CoA is the precursor of fatty acid (FA)-synthesis, which is finally converted to triacylglycerol (TAG) and stored in lipid droplets. Pentoses like xylose are converted to lipids via the pentose-phosphate pathway or a phosphoketolase reaction (see text, modified from Chmielarz [2021]).

converted via malate dehydrogenase to malate, which is used in countering the citrate efflux system (Donot et al., 2014; Papanikolaou & Aggelis, 2011; Ratledge & Wynn, 2002) (Figure 1). Under phosphate limitation, AMP is dephosphorylated to adenosine and phosphate, to provide phosphate for cellular processes such as nucleic acid synthesis. This also results in the inactivation of ICDH, export of citrate from the mitochondria and thus acetyl-CoA accumulation in the cytoplasm. However, during P-limitation, a lower flux through the PPP has been observed, resulting in a limitation of NADPH, which is required as an electron donor for lipid synthesis (Wang et al., 2018).

Acetyl-CoA is converted into malonyl-CoA by acetyl-CoA carboxylase. In the next step, the fatty acid synthase (FAS) complex forms acyl-CoA from acetyl-CoA and malonyl-CoA, where the acyl-CoA chain is prolonged by two C-atoms, and a $CO_2$ is released. For Acyl-CoA formation, NADPH is required. This in most cases is probably generated by D-glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in the PPP, or by a maleic enzyme (ME) (Ratledge, 2014). In some cases, for example, in *L. starkeyi*, ME uses $NAD^+$, not $NADP^+$ as a cofactor, and might, thus, not be involved in fatty acid synthesis (Tang et al., 2010). The acyl-CoA chains produced are transferred to the endoplasmic reticulum, where esterification with glycerol-3-phosphate (G-3-P) takes place to generate either structural lipids (such as phospholipids or glycolipids) or storage lipids in the form of TAGs (Fakas, 2017). Lipids are stored in intracellular lipid droplets (LD), also called lipid bodies. Their biogenesis starts between the two membrane leaflets of the endoplasmic reticulum. The mechanisms involved are not yet fully understood, but enzymes connected to the TAG synthesis have been found in these organelles. It has been proposed that LD grow by synthesising TAGs on their surface (Athenstaedt et al., 2006; Garay et al., 2014; Zanghellini et al., 2010).

The mechanism of fatty acid synthesis is conserved between eukaryotes and bacteria, the major difference is the structure of FAS. While in eukaryotes FAS is an enzyme complex with different activities, in bacteria, FAS consists of a variety of independent soluble enzymes. Bacterial fatty acids are shorter than yeast fatty acids, lack polyunsaturation and have their double bounds in different positions. Some bacteria form branched-chain fatty acids (Cronan & Thomas, 2009). Many bacteria accumulate polyhydroxyalkanoates as storage molecules, but in some actinomycetes, cyanobacteria and *Rhodococcus* species also TAGs are accumulated (Dourou et al., 2018).

## 3 | LIPID PRODUCTION FROM LIGNOCELLULOSIC RAW MATERIALS

Lignocellulose mainly consists of three organic polymers cellulose, hemicellulose and lignin. The composition of lignocellulose can vary depending on the plant species, but also on the growth conditions of

the respective plant. Apart from organic polymers, lignocellulose also contains inorganic compounds, which remain as ash after burning. The content varies, but for instance in rice straw, ash content can be up to 20%. The major element within the ash is Si, amorphous Si polymers have been observed to form incrustations in the epidermis, vascular bundle and other plant tissues. Si polymers may inhibit cellulase enzymes, case scaling or fouling of equipment (Satlewal et al., 2018).

D-Cellulose is a homo-polysaccharide composed of cellobiose subunits, that is, D-glucose linked by β−1,4-glycosidic bonds, packed tightly in cellulose fibres. It has a crystalline structure and is composed of several hundred to tens of thousands of glucose monomers (Nishiyama et al., 2003). Native cellulose has a high degree of polymerisation, is insoluble in water and is difficult to hydrolyse. Moreover, in most conditions cellulose is encased in hemicellulose and lignin (Isikgor & Becer, 2015).

Hemicellulose is a hetero-polysaccharide, which structure can vary between plants, plant tissues and even within molecules (Biely et al., 2016; Gírio et al., 2010). These polysaccharides have a lower degree of polymerisation, are often branched, and their side chains can be acetylated. Hemicelluloses are classified by their main sugar polymer backbone, for example, xylan, which is composed of β −1,4-linked D-xylose and may also contain some other sugars, such as L-arabinose, D-galactose, D-glucose and D-mannose. Hemicellulose of wheat straw, belonging to the grass family, is mainly composed of glucuronoarabinoxylan, whereas that of birch, belonging to hardwoods, is mostly composed of 4-O-methylglucuronoxylans, with both containing mainly D-xylose. Softwood instead contains more C-6 sugars, in this case, D-mannose in the form of galactoglucomannan. The hemicellulose structure is more easily hydrolysable than the cellulose fraction.

Lignin is a complex aromatic polymer composed of three different types of phenyl propane units, p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, connected by alkyl-aryl, alkyl-alkyl and aryl-aryl ether bonds. Lignin can form covalent bonds to hemicellulose and cover the cellulose structure, protecting it from microbial or chemical degradation (Cai et al., 2016; Jönsson & Martín, 2016; Jørgensen et al., 2019).

Lignocellulose has evolved to provide rigidity to plant cell walls and a barrier against infection by microbes (Gupta et al., 2016; Maity, 2015). This implies that energy-intensive pretreatment and enzymatic degradation of lignocellulose are required, to make the carbon sources bound in it available for the yeast. A variety of pretreatment methods has been developed. Pretreatment is out of the scope of this article, there are several excellent recent reviews dedicated to this topic (Andlar et al., 2018; Galbe & Wallberg, 2019). Apart from sugars, also inhibitors including organic acids (which also can be carbon sources for oleaginous yeasts), sugar anhydrides such as furfural and hydroxymethylfurfural and aromatic compounds are released during pretreatment (Jönsson & Martín, 2016), which have an impact on subsequent substrate conversions. Pretreatment is actually a major driver of processing costs in lignocellulose-based processes. The costs of palm oil are currently estimated at $901.50/t (https://www.indexmundi.com/commodities/?commodity=palm-oil%26months=60). Estimates of the minimal selling prices of lignocellulose-derived yeast oil range from $1800 to more than $10,000 per metric ton. To obtain a cost-competitive process, a biorefinery approach, valorising co-products of oil production is crucial (Parsons et al., 2020).

Combined production of biodiesel and biogas from *L. starkeyi* biomass grown on wheat straw hydrolysate, as well as using the residues for the production of electricity and heat was shown to result in a fossil fuel replacement potential of 5.74 MJ/kg straw. Utilising the lignin to obtain process energy and not returning it to the soil had the highest impact on greenhouse gas savings. Improved lipid extraction and shortening the energy-intensive aerobic fermentation process were identified as the major crucial factors for improving the energy output and greenhouse gas balance of biodiesel production based on microbial lipids. Utilisation of crude glycerol (CG) for biodiesel production was also an important factor (Karlsson et al., 2016, 2017).

Inhibitor tolerance would be one important factor to decrease fermentation times. A variety of detoxification procedures for lignocellulose hydrolysate has been developed, however, those steps should be minimised, as detoxification can represent up to 22% of second-generation production costs (Koppram et al., 2012). Substrate toxicity can also be overcome by isolating inhibitor-resistant mutants or adapting strains to high concentrations of inhibitors (Liu et al., 2021). Obtaining stable mutants and further development of genetic tools may identify genetic and metabolic networks crucial for inhibitor tolerance.

Diluting a substrate would also be an option for reducing the inhibitor concentrations, however, this would also dilute the carbon sources for lipid production. This can be avoided by running cultures in a fed-batch mode, which gives the cells the opportunity to adapt to increasing inhibitor concentrations. Those strategies have frequently been used for converting hemicellulose hydrolysate to lipids. Hemicellulose hydrolysates are frequently generated as a side product of the pulp- and paper industry. They can be used for steam and electricity production, but the heating value is relatively low (Helmerius et al., 2010; H.-J. Huang et al., 2010). Hemicellulose hydrolysate is also generated in acid pretreatment of lignocellulosic biomass, which is a quite common method for pretreatment (Galbe & Wallberg, 2019). Under acidic conditions, most of the hemicellulose polysaccharides are hydrolysed without any enzyme addition and are thus solubilised in the liquid phase of the biomass, while the solid phase contains most of the cellulose and the lignin. Since hemicellulose contains a large proportion of sugar monomers like D-xylose and arabinose, which are not fermentable by for instance the most commonly used ethanol-producing yeasts, it is an option to remove the hemicellulose phase after thermochemical pretreatment. However, hemicellulose hydrolysate also contains a high concentration of water-soluble inhibitors, including furfural and organic acids (Jönsson & Martín, 2016). For instance, a birch wood hydrolysate contained 45.06 ± 0.44 g/L D-xylose, 0.46 ± 0.60 g/L D-glucose, 13.07 ± 0.16 g/L acetic acid and 4.7 ± 0.04 g/L furfural (Brandenburg et al., 2016). *L. starkeyi* CBS 1807 was not able to grow in undiluted hydrolysate, however, when running the culture in different fed-batch-cultivations, it was possible to obtain growth and

lipid accumulation. Interestingly, acetic acid was preferably consumed, resulting in a pH-increase and growth-stop in an uncontrolled fermentation. This feature was used to establish a pH-regulated fed-batch cultivation, where feeding of hemicellulose hydrolysate was connected to pH-regulation. With this approach, a final lipid concentration of 8 g/L was reached after 7 days (lipid content 51.3% of CDM), with a yield of 0.1 g per g consumed carbon source (Brandenburg et al., 2016). From the hemicellulose fraction of sugarcane bagasse using a strain of *R. toruloides*, a lipid concentration of 3.7 g/L was reached within 4 days. These experiments were performed in a batch-mode, however, concentrations of sugars, acetic acid and probably also other inhibitors were by far lower than in the above-mentioned experiment with birch wood. In another experiment with sugar cane bagasse as substrate and *L. starkeyi*, a final lipid concentration of 3.1 g/L was reached (intracellular lipid content 27.3% of CDM), and the lipid yield in continuous fermentation was 0.18. Also, in this case, concentrations of sugars and inhibitors were comparatively low, about 33% of what has been reported above for birch wood hydrolysate (Xavier et al., 2017).

As mentioned above, pentoses from hemicellulose can be converted to their anhydride furfural, which is a fermentation inhibitor. On the other hand, furfural is classified as an important platform chemical, which is exclusively produced from plant biomass. Established methods for generating furfural from lignocelluloses damage the other polysaccharides in a way that it is almost impossible to further convert them by microbial conversion. However, a few years ago a novel catalytic method has been developed to produce furfural, leaving the cellulose fraction intact (Vedernikovs, 2020). The combined production of furfural and ethanol or lipids from wheat straw has been investigated. The cellulose hydrolysate had a relatively low inhibitory potential, no dilution or detoxification was required. Ethanol production resulted in

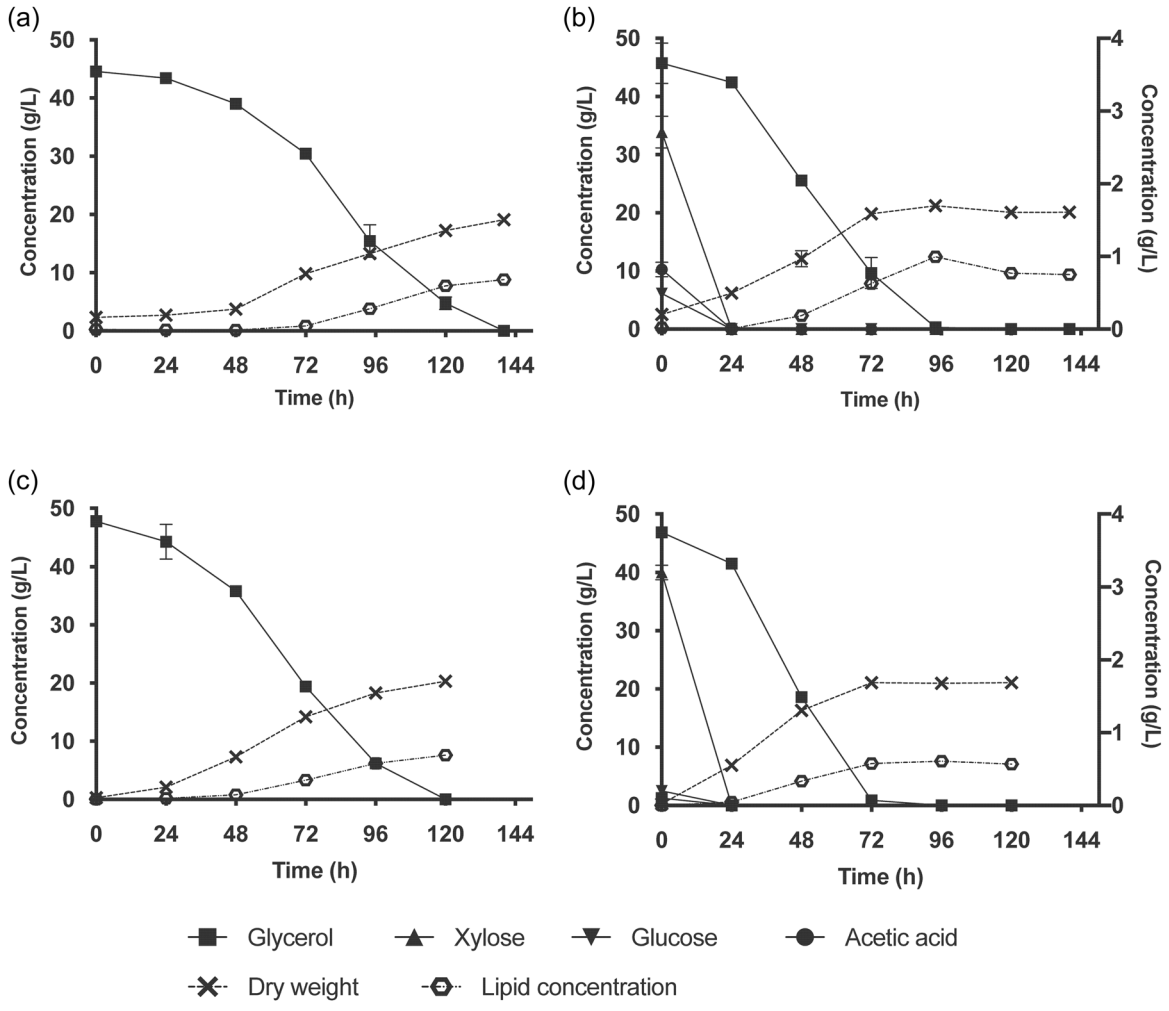


**FIGURE 2** Effect of hemicellulose addition on crude glycerol (CG) consumption in *Rhodotorula toruloides* and *Rhodotorula glutinis* (reproduced from Chmielarz et al. [2021]). (a) *R. toruloides* CBS 14 bioreactor cultivation with 55 g/L CG. (b) *R. toruloides* CBS 14 bioreactor cultivation in 55 g/L CG and 10% addition of hemicellulose hydrolysate; (c) *R. glutinis* CBS 3044 bioreactor cultivation in 55 g/L of CG. (d) *R. glutinis* CBS 3044 bioreactor cultivation in 55 g/L CG and 10% addition of hemicellulose hydrolysate; D-Glucose, D-xylose and acetic acid are shown on the right *y*-axis. The addition of hemicellulose hydrolysate (b and d) shortened fermentation by about 24 h, compared to cultures with pure CG (a and c).

a theoretical yield from the released sugar. In total, a production potential of 110 g furfural and 111 g ethanol or 33 g lipids from 1 kg of wheat straw was determined. Lipid production was tested with *L. starkeyi* CBS 1807 and *R. babjevae* DBVPG 8058. Interestingly, the two strains showed strong differences in their lipid production potential. *L. starkeyi* reached a lipid yield of 0.09 g per g consumed D-glucose, and a production rate of 0.08 g/l h; *R. babjevae* 0.17 g/g and 0.18 g/l h (Brandenburg et al., 2018). Similarly, furfural could also be produced from rapeseed oil, and ethanol production from the cellulose fraction was demonstrated (Rozenfelde et al., 2021).

Another opportunity for reducing the concentration of inhibitors would be mixing them with another low-value carbon source, for instance, CG. CG is a residue from the production of biodiesel, where the glycerol in the triglycerides is replaced by methanol, forming fatty acid methyl esters. CG contains several toxic components like soaps, ash and methanol, which can be removed by for instance distillation, ion exchange resin purification, membrane separation and acidification followed by neutralisation and solvent extraction. All of these methods result in loss of substrate, and some like distillation require high energy input (Abdul Raman et al., 2019). Utilising CG as a carbon source for the growth of microorganisms and especially for the production of lipids can be performed without extensive purification and has been tested previously (Gajdoš et al., 2020; Papanikolaou & Aggelis, 2009; Posada & Cardona, 2010). Mixing CG with lignocellulose hydrolysate, especially with the more inhibitory hemicellulose hydrolysate, may dilute the different inhibitors present in both residual compounds and enable growth at relatively high concentrations of CG. Interestingly, the addition of a comparatively low amount of hemicellulose hydrolysate to CG activated the glycerol metabolism in *R. toruloides* CBS 14 and *R. glutinis* CBS 3044, resulting in fermentation times shortened by about 24 h (Figure 2). No stimulating effect was seen in *L. starkeyi*. The highest lipid yield for *R. toruloides* was 0.25 on a mixture of glycerol and hemicellulose hydrolysate, and 0.20 for glycerol alone. In *R. glutinis*, the lipid yield was not significantly different on a mixed or pure substrate (0.15–0.16) (Chmielarz et al., 2021).

The positive effects of mixing CG with other residual products have also been described in other studies. This for instance includes lipid production by *Trichosporon fermentans*, when CG was mixed with hydrolysate of sweet potato vines (the bulk of the above-ground parts of sweet potato), where a more than fourfold higher lipid concentration was obtained after 14 days of fermentation (Shen et al., 2013). Higher lipid concentration and yield were obtained with *C. curvatum* when mixing corn stover hydrolysate with CG, compared to a culture with pure corn stover as a carbon source: 10.8 g/L, 0.18 vs. 4.6 g/L, 0.16, respectively (Gong et al., 2016). The physiological basis of metabolic activation of glycerol metabolism by adding ligno—especially hemicellulose hydrolysate is not completely clear. A small amount of easier available carbon sources like sugars or acetic acid would result in an initial increase in biomass, which would then result in higher volumetric consumption. However, at least for *R. toruloides* CBS 14 an increased specific glycerol uptake rate has been demonstrated (Chmielarz et al., 2021). This indicates that there are probably also other mechanisms responsible for the metabolic activation, for example, the availability of additional nitrogen sources in the hydrolysate or the activation of some stress response, which then might result in a higher substrate uptake (Chmielarz, 2021).

## 4 | METHODS FOR LIPID QUANTIFICATION

Probably the most commonly used methods for lipid quantification in oleaginous yeasts are based on the Folch or Bligh and Dyer extraction methods (Bligh & Dyer, 1959; Folch et al., 1957). Both methods have been designed for animal tissues and are based on extraction with organic solvents and final gravimetric lipid quantification. For microorganisms such as yeasts, the methods have to be adapted due to the complex and rigid cell wall and membrane structures (Jacob, 1992). Various mechanical, chemical or enzymatic pretreatments of cell biomass have been tested to disrupt the cells and make most of the intracellular lipids accessible. This includes, for example, acid-catalysed hot water treatment, autoclaving, bead beating, homogenisation, microwave radiation, ultrasonication or thermolysis (Bonturi et al., 2017; Brandenburg et al., 2018; Patel et al., 2019). However, these methods are time-consuming, require organic solvents, which are problematic from an environmental point of view and require a relatively large sample volume for accurate determination, especially at low lipid concentrations. Staining may provide a more rapid method for lipid quantification. Nile red is a lipophilic lysochrome dye that stains different types of lipids including neutral lipids inside cells. However, the method has limits when it comes to the quantification of intracellular lipids in oleaginous yeasts since the accuracy in-between different tested yeast strains was rather low. This method is quite sensitive to differences in cell culture preparation, strain variations and incubation time and to our experience not easily comparable between different experiments (unpublished results) (Kimura et al., 2004; Sitepu et al., 2012). On the other hand, this staining gives impressive results while using fluorescent microscopy for visualisation of intracellular lipid bodies and could be used in screening studies (Shi et al., 2015).

Spectroscopy is a nondestructive method that allows rapid and noninvasive investigation of biomolecules, for instance, proteins or lipids. Near infrared (NIR) or mid infrared (MIR) light is close to the visible light spectra, with wavenumbers between 12,500–4000 and 4000–400 $cm^{-1}$, respectively. MIR spectroscopy, commonly called IR spectroscopy, can be used to study the fundamental vibrations of molecules. With the NIR spectroscopy overtones and combination bands are detected. The advantage of MIR is that the absorption spectrum provides more clear peaks referring specifically to functional groups. With NIR the spectra are broader and more undefined but may hold more information. Fourier transform (FT)-NIR applies additional equipment, interferogram, which allows plotting light intensity as a function of time, which is then Fourier transformed to a frequency domain. FT-(N)IR is a much faster technique allowing to measure the whole wavelength range simultaneously, is more sensitive and has less

background interference when compared to (N)IR (Pandey et al., 2018; Raja & Barron, 2019).

The advantages of this type of spectroscopy are that almost no sample preparation is required, besides simple washing and drying, and results are given almost in real-time. It reduces costs, labour, analysis time and a lot of solvent usage. In addition, the amount of needed sample volume can be decreased tremendously, making it possible to work in small-scale experiments for screening or lipid kinetics studies (Ami et al., 2014; Brandenburg et al., 2021; Chmielarz et al., 2021; Forfang et al., 2017; Kosa et al., 2017).

Quantitative measurements with FT-(N)IR must always be calibrated against a set of reference values, determined by classical lipid extraction and analysis, creating a prediction model. As water molecule vibrations cause huge responses in FT-IR and -NIR spectroscopy, it strongly interferes with the lipid prediction models. Therefore, it is important to dry the cells before measurement. Prediction models can also be influenced by the yeast strain used in measurements. FT-NIR models for the prediction of total lipids were established for *L. starkeyi* and *R. toruloides*. The models had a high accuracy with $R^2$-values of 96% and 98%, respectively. The *L. starkeyi* model enabled predicting the lipid content in *L. starkeyi* and *Y. lipolytica* grown under several conditions, but not that of strains of pigmented yeasts, that is, *Rhodotorula* spec., similarly, the *Rhodotorula* model was not appropriate to predict nonpigmented strains. A combined model was established by including strains of *L. starkeyi*, *Y. lipolytica*, *R. toruloides*. For testing the models, also strains of *R. glutinis* and *R. babjevae* were included. This model enabled the prediction of the lipid content in all yeast strains but with lower accuracy than the specific models (Figure 3).

In another approach, an FT-IR method was established, based on 13 oleaginous yeast strains. Different spectral regions and several spectral processing methods were investigated to build up robust and accurate prediction models for total lipid amount and lipid profile. Spectral region evaluation showed that the spectral range for 3100–2800 $cm^{-1}$ was most reliable for the prediction of total lipids, saturated, mono-unsaturated and polyunsaturated fatty acids; =C—H stretching; C—H asymmetric stretching of $CH_3$; asymmetric stretching >$CH_2$ of acyl chains; symmetric stretching of $CH_2$ of acyl chains. In addition, the spectral region 1800–700 $cm^{-1}$ was also used for total lipid determination, including the peak for C=O stretching and regions that refer to proteins, $CH_2$ deforming, $CH_3$ bending, C—O—C stretching, carbohydrates and polyphosphates (Figure 4). For some strains, a peak at 1710 indicates the presence of free fatty acids. This was confirmed by thin-layer chromatography (Shapaval et al., 2019). In some strains, free fatty acids were the second most abundant class of lipids, which was also confirmed in a recent study in *R. toruloides* CBS 14 (Nagaraj et al., 2022).

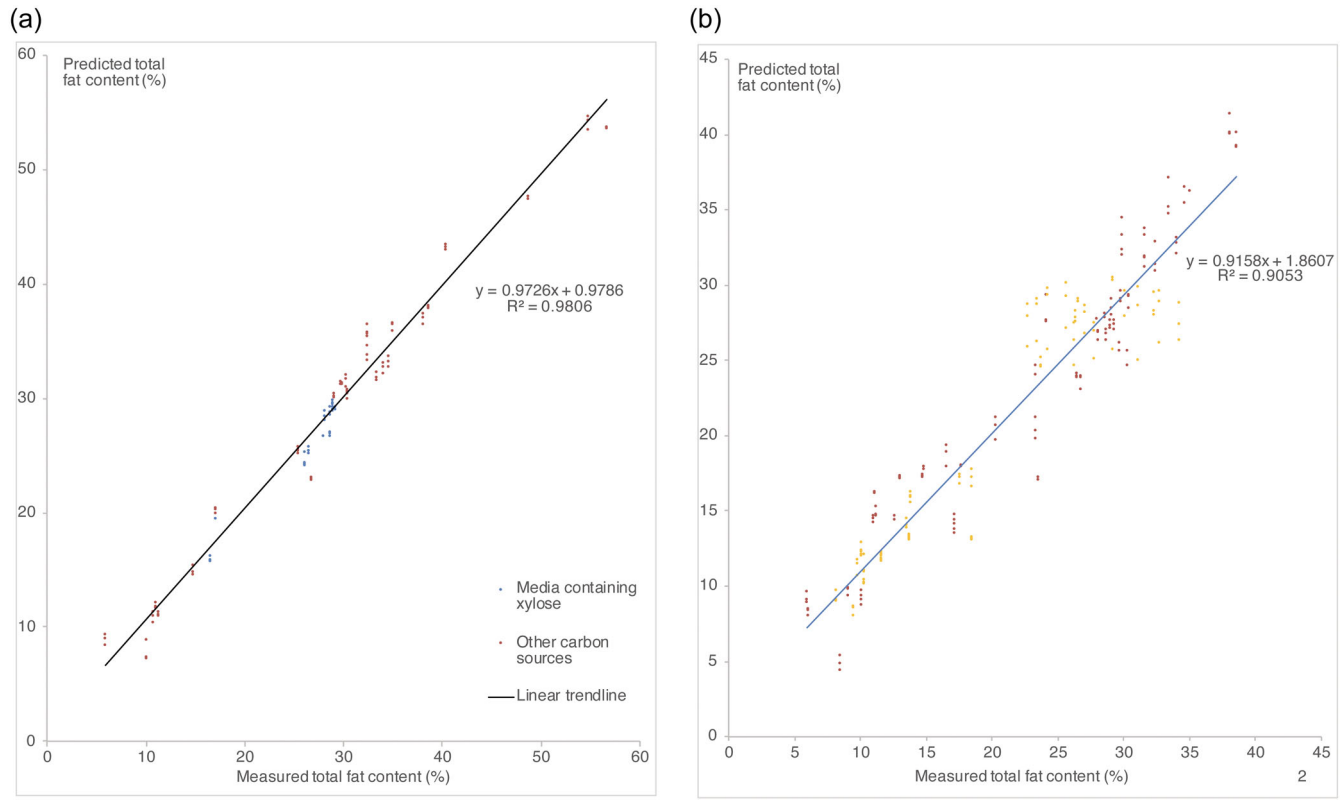


**FIGURE 3** Models for prediction of total lipid content in yeast cells by FT-NIR. (a) Test set validation of total fat content in *Rhodotorula toruloides* CBS 14. (b) Cross-validation of a combined model of *R. toruloides*, *Lipomyces starkeyi* and *Yarrowia lipolytica*. Red dots represent red yeast, yellow dots represent *L. starkeyi* and *Y. lipolytica* samples. Figures reprinted from Chmielarz et al. (2019).

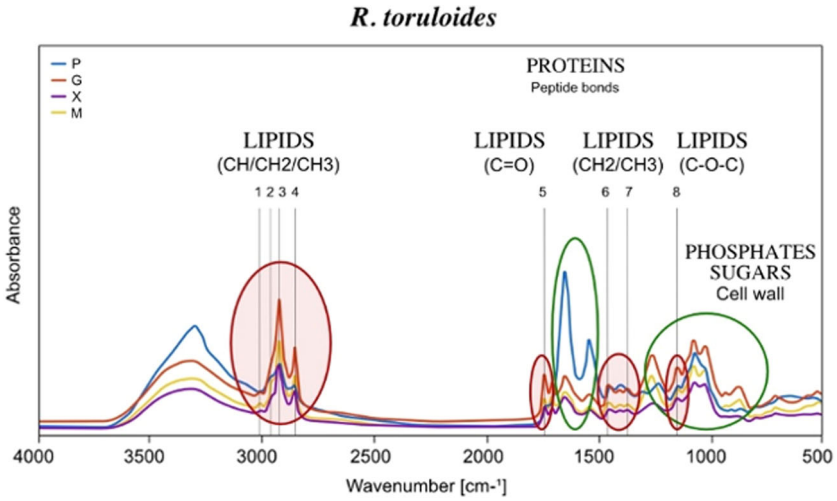


**FIGURE 4** FT-IR spectrum of *Rhodotorula toruloides* grown on yeast extract peptone dextrose medium (P), nitrogen-limited medium (NLM) containing D-glucose (G), NLM containing D-xylose (X), NLM containing D-glucose and D-xylose (M). Indicated are Lipid relevant peaks corresponding to 1, C—H stretching; 2, C—H asymmetric stretching of $CH_3$; 3, asymmetric stretching $CH_2$ of acyl chains; 4, symmetric stretching of $CH_2$ of acyl chains; 5, C=O stretching; 6, $CH_2$ deforming; 7, $CH_3$ bending; 8, C—O—C stretching. Modified from Shapaval et al. (2019).

## 5 | STRAIN DIFFERENCES IN THE ABILITY TO ACCUMULATE LIPIDS

Using the above-described spectroscopic methods, a variety of oleaginous strains were tested on D-glucose, D-xylose, CG and lignocellulose hydrolysate. A huge physiological diversity even between strains belonging to the same or closely related species was observed (Brandenburg et al., 2021; Chmielarz et al., 2021).

Among 13 tested strains of *Lipomyces* spp., four were not able to grow on D-xylose (Brandenburg et al., 2021), although these species are usually characterised by a good ability to grow on this substrate (Kurtzman et al., 2011; Sitepu et al., 2014) (https://theyeasts.org/, accessed 31/10/2022). Among the 16 tested *Rhodotorula* strains, four were not able to grow on D-xylose. The ability of lipid accumulation was also very diverse between strains. The final cell density of *Rhodotorula*-strains was lower on D-xylose than on D-glucose (Figure 5). Some strains that were not able to grow on D-xylose as the sole carbon source nevertheless consumed it when growing on a mixture of D-glucose and D-xylose. In general, *Rhodotorula*-strains reached their maximum cell density faster than *Lipomyces* spp. Since shortening fermentation time is one of the major factors for making yeast-based oil production sustainable (see above, Karlsson et al., 2016), this finding may indicate that the red yeasts are generally more appropriate for developing biotechnological processes based on yeast single-cell oil. On the other hand, the *Rhodotorula*-strains required additional nitrogen sources, in contrast to *Lipomyces* strains.

Five of the well-performing strains were then investigated further in a bioreactor with wheat straw hydrolysate, following the lipid formation kinetics by FT-IR. Two *Rhodotorula* strains, *R. babjevae* DBVPG 8058 and *R. toruloides* CBS 14, were superior in growth and lipid accumulation capacity compared to the others. They reached maximum lipid concentrations of 18.1 and 11.7 g/L, respectively (representing respective yields of 0.24 and 0.15) within 96 and 91 h, respectively. *L. starkeyi* CBS 1807 and 7544 reached 7.3 and 11.9 g/L, respectively (yields 0.09 and 0.16) within 126 and 138 h, respectively. Interestingly, *R. glutinis* CBS 2367 only reached a final lipid concentration of 2.15 g/L after 168 h. Lipid accumulation kinetics of this strain showed that it was accumulating lipids when consuming glucose and acetic acid, but it was consuming the intracellular lipid reserves when it was assimilating D-xylose. This was not expected because the strain was accumulating lipids from both D-glucose and D-xylose when growing in the model substrate. These results show that especially for the conversion of D-xylose to lipids in yeasts, some factors are still unknown (Brandenburg et al., 2021). Tiukova et al. (2019) demonstrated expression of proteins involved in β-oxidation in *R. toruloides*, that is, cells were also activating the pathway for lipid degradation on this sugar, even when still accumulating lipids. Cells also induced proteins involved in the reaction to oxidative stress. Possibly, there is a narrow balance between lipid accumulation and –consumption, when growing on D-xylose, and stress factors such as inhibitors in the substrate may shift the balance towards lipid consumption.

CG has been tested as a substrate for oleaginous yeasts in a variety of studies (e.g., Chatzifragkou et al., 2011; Diamantopoulou et al., 2020; Papanikolaou & Aggelis, 2009; Posada & Cardona, 2010). Chmielarz et al. (2021) tested 27 strains for growth on this substrate. Interestingly, only 11 of them were able to grow on the tested CG (obtained from Perstorp AB, Sweden), using FT-NIR for following lipid accumulation over time. Out of the 11 tested *Lipomyces*-strains, only *L. starkeyi* CBS 7786 was able to grow on CG. However, it was growing slower than the identified positive *Rhodotorula* strains and was strongly inhibited at CG concentrations of 60 g/L. Out of the 16 tested *Rhodotorula* strains, 10 were able to grow on CG. All of these strains were able to grow up to CG concentrations of 60 g/L, although with different growth rates. As mentioned above, mixing the CG with hemicellulose hydrolysate had a positive effect on growth and lipid accumulation by *Rhodotorula* strains, but not by *L. starkeyi*.

The above-mentioned results show that there is a huge diversity in lipid accumulation between strains even of closely related species. Understanding the physiological basis of these differences would enable the identification of crucial steps in lipid production and thus

| Strain | Growth OD G | Growth OD X | Growth OD M | Lipid % G | Lipid % X | Lipid % M |
|---|---|---|---|---|---|---|
| *Lipomyces starkeyi* CBS 1807 | | | | | | |
| *Lipomyces starkeyi* CBS 1809 | | | | | | |
| *Lipomyces starkeyi* CBS 2512 | | | | n.d. | n.d. | n.d. |
| *Lipomyces starkeyi* CBS 6047 | | | | | | |
| *Lipomyces starkeyi* CBS 7536 | | | | n.d. | n.d. | n.d. |
| *Lipomyces starkeyi* CBS 7537 | | | | n.d. | n.d. | n.d. |
| *Lipomyces starkeyi* CBS 7544 | | | | | | |
| *Lipomyces starkeyi* CBS 7545 | | | | | | |
| *Lipomyces starkeyi* CBS 7786 | | | | n.d. | n.d. | n.d. |
| *Lipomyces starkeyi* CBS 7851 | | | | n.d. | n.d. | n.d. |
| *Lipomyces starkeyi* CBS 7852 | | | | | | |
| *Lipomyces lipofer* CBS 944 | | | | | | |
| *Lipomyces lipofer* CBS 5842 | | | | | | |
| *Rhodotorula babjevae* DVBPG 8058 | | | | | | |
| *Rhodotorula babjevae* CBS 7808 | | | | | | |
| *Rhodotorula babjevae* CBS 7809 | | | | n.d. | n.d. | n.d. |
| *Rhodotorula glutinis* CBS 20 | | | | n.d. | n.d. | n.d. |
| *Rhodotorula glutinis* CBS 2203 | | | | n.d. | n.d. | n.d. |
| *Rhodotorula glutinis* CBS 2367 | | | | | | |
| *Rhodotorula glutinis* CBS 2889 | | | | | | |
| *Rhodotorula glutinis* CBS 2890 | | | | n.d. | n.d. | n.d. |
| *Rhodotorula glutinis* CBS 3044 | | | | n.d. | n.d. | n.d. |
| *Rhodotorula glutinis* CBS 5182 | | | | n.d. | n.d. | n.d. |
| *Rhodotorula glutinis* CBS 5805 | | | | | | |
| *Rhodotorula glutinis* CBS 7538 | | | | | | |
| *Rhodotorula glutinis* CBS 7796 | | | | | | |
| *Rhodotorula glutinis* CBS 9477 | | | | n.d. | n.d. | n.d. |
| *Rhodutorula graminis* CBS 3043 | | | | | | |
| *Rhodutorula toroluides* CBS 14 | | | | | | |

| OD(600nm) | Lipid % of CDW |
|---|---|
| <10 | <20% |
| 10-40 | 20-30% |
| 40-80 | 30-40% |
| 80-120 | 40-50% |
| >120 | >50% |

**FIGURE 5** Growth and lipid content of *Lipomyces* and *Rhodotorula* strains grown in medium containing D-glucose (G), D-xylose (X) or a D-glucose/D-xylose mixture (M). The cultivations were done in shake flasks at 25°C, 130 rpm and performed in duplicates. Dark green represents strong growth and high lipid content, light green/white little or no growth and low lipid content. n.d., not determined due to no or very poor growth. Reprinted from Brandenburg et al. (2021).

theory-based optimisation of lipid accumulation. Recent investigations of two strains of *R. babjevae* demonstrated that the genomes of two strains (type strain 7808 and DBVPG 8058) had almost as big differences to each other as to strains of *Rhodotorula graminis* and *R. glutinis* (Martín-Hernández et al., 2022). There is obviously a huge diversity of the genomes of different *Rhodotorula* strains, even if they only differ by a few nucleotides in the D1D1- and the ITS region, which are usually used as standard sequences for species identification (Kurtzman et al., 2011).

## 6 | CONCLUSIONS AND OUTLOOK

Oleaginous yeasts have great potential to replace vegetable oils in a variety of applications. Yeast oil has a similar composition to plant oil. Apart from producing biodiesel from it, it can also be used as a feed additive (Blomqvist et al., 2018; Brunel et al., 2022), or even as food (Lundin, 1950). Carotenoids are formed by red yeasts and have great potential to be used in food and feed, and high-value chemicals (Rapoport et al., 2021). Especially the yeast-specific carotenoids torulene and torularhodin are of interest because they have a higher antioxidant potential than the most commonly used β-carotene (Ungureanu & Ferdes, 2012). There are reports about exopolysaccharides and glycolipids formed by *Rhodotorula* strains (Byrtusová et al., 2021). We also found exopolysaccharides and polyol esters of hydroxy-fatty acids formation in several strains (unpublished results). These findings indicate a great biotechnological potential especially in the basidiomycetous red yeasts, also because they showed more rapid growth and lipid formation compared to the ascomycetous strains. A variety of efforts have been taken to improve the potential of oleaginous yeasts for lipid production, including optimisation of culture conditions, engineering metabolic pathways involved in producing building blocks for lipid synthesis and TAG assembly, decreasing the activity of lipid degradation pathways and other pathways competing with lipid syntheses, such as polysaccharide accumulation or secretion of small molecules such as citric acid or polyols (Dourou et al., 2018). However, we are still far away from understanding the physiology of lipid accumulation. There is a big need to understand the genome organisation of *Rhodotorula* yeasts, especially since it is different from ascomycetous model organisms in terms of having more introns and regulatory phenomena such as alternative splicing and antisense transcripts (Martín-Hernández et al., 2021). Understanding different levels of regulation will open up novel ways to improve the production of lipids and other advanced molecules. Genetic tools are available for some *Rhodotorula* strains, but they are by far less developed compared to the ascomycetous oleaginous model yeast *Y. lipolytica* (Ledesma-Amaro & Nicaud, 2016; Zhao et al., 2021). Nevertheless, with awareness of the huge diversity of oleaginous yeasts and improved techniques for cultivation, analyses and metabolic engineering, those yeasts have a great potential to play a role in establishing future biotechnology as a basis of a circular economy.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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