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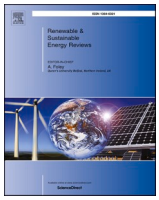

# Factors affecting the optimisation and scale-up of lipid accumulation in oleaginous yeasts for sustainable biofuels production

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ABSTRACT

The recent unprecedented increase in energy demand has led to a growing interest in emerging alternatives such as the production of microbial lipids with high energy density and environmentally-friendly characteristics. Oleaginous yeasts represent a versatile and attractive tool for the accumulation of such lipids, also known as single cell oils (SCOs), used to manufacture biofuels (e.g., biodiesel, aviation fuel) and bioproducts. This review provides an overview of the most common oleaginous species, analysing the viability of typical feedstocks and their effect on lipid accumulation. The best results in terms of lipid content using glucose, glycerol, lignocellulose, or acetic acid as substrates are 81.4, 70, 68.2 and 73.4% (w/w), respectively. Besides, an analysis of the parameters that can affect lipid production is also presented. For instance, the optimum conditions for lipid accumulation are usually a C/N ratio between 100 and 200, pH between 5 and 6 (being more alkaline if acids are used as substrates) and temperature around 30 °C. Besides, genetic modifications generally allow to increase the lipid yield, even by up to 400%. Finally, some cost analysis is provided for scaling-up, with feedstock costs estimated at 50–80%, followed by fermenter costs, and downstream costs estimated at around 13%.

## 1. Introduction

Biofuels have been developed and are being used due to concerns about energy supply and possible shortages, and environmental issues associated with fossil fuels [1]. Global energy demand is continuously increasing because of the fast-growing economies of some countries, such as China. Unfortunately, recent events have shown that, in a globalised world, energy supply shortages may become a severe problem due to unpredicted pandemic situations or geopolitical conflicts. Moreover, the exponential increase in cryptocurrency adoption over the past year has led to another unprecedentedly large energy consumption by bitcoin mining, currently consuming 204.5 TWh of energy per year, equivalent to the energy consumed by Thailand annually or 174% of the energy used by the Netherlands last year [2]. Those situations are triggering an unprecedented rise in energy prices, and therefore the search for other, non-conventional, or renewable energy sources has become more critical than ever.

Lipid-derived biodiesel and jet fuels have recently gained much interest as sustainable alternative fuels due to their high energy density, clean-burning characteristics, environmental friendliness, and biodegradability [3]. In addition to the reasons mentioned above, the world's population is expanding rapidly, leading to a higher demand for fuels than ever before, which could partly be covered by such biofuels [4]. Currently, lipid oils produced on an industrial scale are obtained from plants and animals. However, in the last decade, there has been an exponential increase in studies focusing on microorganisms capable of accumulating lipids. These microbe-derived lipids have comparable composition to those found in animals or plants. One of their natural benefits is that they are not affected by climate, seasonality or ubication, with the additional main advantage of reduced land requirements [5].

Due to rising demand for lipids and limits in their production from conventional sources, biotechnological techniques utilising oleaginous microorganisms have recently been considered attractive alternatives for producing lipids or microbial oils. A wide range of microorganisms, ranging from bacteria to yeasts, fungi, and microalgae, may be used as cell factories to generate a variety of bioproducts, with lipids being among the most attractive ones [6]. Among the above-mentioned

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**Abbreviations**

| | |
|---|---|
| ACC | acetyl-CoA carboxylase |
| ACL | ATP-citrate lyase |
| AD | anaerobic digestion |
| AMP | adenosine monophosphate |
| AMPD | adenosine monophosphate deaminase |
| C/N | Carbon/Nitrogen ratio |
| DAG | diacylglycerol |
| DCW | dry cell weight |
| DGAT | diacylglycerol acyltransferase |
| DHAP | dihydroxyacetone phosphate |
| DO | dissolved oxygen |
| ER | endoplasmic reticulum |
| F6P | fructose-6-phosphate |
| FA | fatty acid |
| FAS | FA synthase |
| FFA | free fatty acids |
| G3P | glycerol-3-phosphate |
| G6P | glucose-6-phosphate |
| GAP | glyceraldehyde |
| GHG | Greenhouse gas |
| Glu | glucose |
| ICCs | inhibitory chemical compounds |
| IDH | isocitrate dehydrogenase |
| IMP | inosine monophosphate |
| LCA | life cycle analysis |
| LPA | lysophosphatidic acid |
| NADPH | nicotinamide adenine dinucleotide phosphate dehydrogenase |
| N.D | no data |
| OAA | oxaloacetate |
| OD | optical density |
| OECD | Organisation for Economic Cooperation and Development |
| PA | phosphatidic acid |
| PAP | phosphatidic acid phosphatase |
| PUFAs | polyunsaturated fatty acids |
| Ref | reference |
| SCO | Single cell oil |
| SCT1 | G3P acyltransferase |
| SLC1 | 1-acyl-*sn*-glycerol-3-phosphate acyltransferase |
| TAG | triacylglyceride |
| TCA | tricarboxylic acid cycle |
| VFA | volatile fatty acid |
| WLP | Wood–Ljungdahl pathway |
| w/w | weight/weight |
| Xyl | xylose |
| YPD | Yeast extract-Peptone-Dextrose |

oleaginous microorganisms, yeasts present several advantages over the others. This is due to their relatively fast unicellular growth rate and capacity to accumulate large amounts of lipids [7]. The term "oleaginous" originates from the ability of these microorganisms to accumulate lipids at concentrations exceeding 20% (w/w) of their dry weight. In addition, oleaginous yeasts can generate additional "rare" lipids, such as cocoa butter (saturated lipids) and other forms of saturated exotic fats, which may be used to substitute high-value and expensive lipids found only in plants and animals [8].

The present review comprehensively describes the current state-of-the-art and addresses aspects not thoroughly reviewed elsewhere. For example, Chattopadhyay et al. review current developments in genetic engineering of oleaginous yeasts but do not go into detail on other experimental parameters that influence lipid accumulation [9]. Szczepańska and colleagues describe different products and compounds obtained from oleaginous yeasts, but do not mention costs and process challenges [10]. Caporusso and co-workers review the different parameters that can affect lipid accumulation in oleaginous yeasts, but do not address issues such as genetic modifications or process costs [11]. On the other hand, authors such as Karamerou et al. or Parsons et al. carry out an in-depth techno-economic analysis of microbial oil production, but do not assess, for example, how each feedstock affects lipid accumulation [12,13]. Abeln and colleagues provide a comprehensive review on the history, current status and perspectives of oleaginous yeasts, but address only superficially how different experimental parameters affect final lipid accumulation, e.g., it does not review the use of emerging substrates such acetic acid as a carbon source for oleaginous yeasts, while it is one of the most promising current substrates due to its favourable cost/performance characteristics [14].

This review provides an overview of the state-of-the-art process of microbial lipids production by oleaginous yeasts studying the influence of the substrate along with other operating parameters that may affect lipid yields. For instance, it reviews the possibility of using carboxylic acids as substrates for lipid production and even the indirect use of $CO_2$, as recent and innovative alternatives due to their low price. The industrial scale-up of this process is also a challenge; therefore, advances in techniques to reduce costs are analysed. Finally, a series of conclusions and future perspectives related to the implementation of this process on a large scale are also provided.

## 2. Oleaginous yeasts as a cell factory

For years, it has been known that oleaginous microorganisms, particularly some forms of oleaginous yeasts, may accumulate lipids as single cell oils (SCOs). It is also worth noting that not all microorganisms can store lipids as triacylglycerol (TAG). In recent years, the method by which these microbes gather this type of lipid has been studied, and better understood [15]. The mechanisms through which oleaginous yeasts can produce TAGs through the conversion of a given substrate are essentially the following two: *de novo* synthesis and *ex novo* synthesis [16]. When the microorganism is under stress, presenting a high C/N ratio with a lack of nitrogen, *de novo* synthesis occurs [17]. It is aided by a metabolic imbalance in the presence of a critical nutrient deficiency in the growth medium, causing a metabolic transition in which growth is halted, and the lipogenic phase is favoured. *Ex novo* synthesis, on the other hand, entails the hydrolysis of hydrophobic substrates (such as TAGs, alkanes and free fatty acids) to yield fatty acids (FAs) and glycerol which are then transported inside the cell to be reassembled into TAGs [16].

### 2.1. *De novo synthesis*

The nitrogen depletion process comprises several central metabolism-related regulatory processes. It is the most efficient means of initiating lipogenesis, resulting in increased substrate-to-lipid conversion yields and lipid content as internal biomass [18]. Nitrogen starvation activates the activity of adenosine monophosphate (AMP) deaminase (AMPD), which converts AMP to inosine monophosphate (IMP) and ammonia, is a result of nitrogen deficiency [19] (Fig. 1). As a result, the cell receives some nitrogen supply. The Krebs cycle, which pauses at the level of isocitrate, is negatively impacted by the following drop-in AMP concentration. Because it is allosterically triggered by intracellular AMP, isocitrate dehydrogenase (IDH), the enzyme that converts isocitrate into alpha-ketoglutarate, loses its activity (in the tricarboxylic acid cycle) [15]. This causes a build-up of mitochondrial isocitrate, which is subsequently balanced with citrate by the enzyme

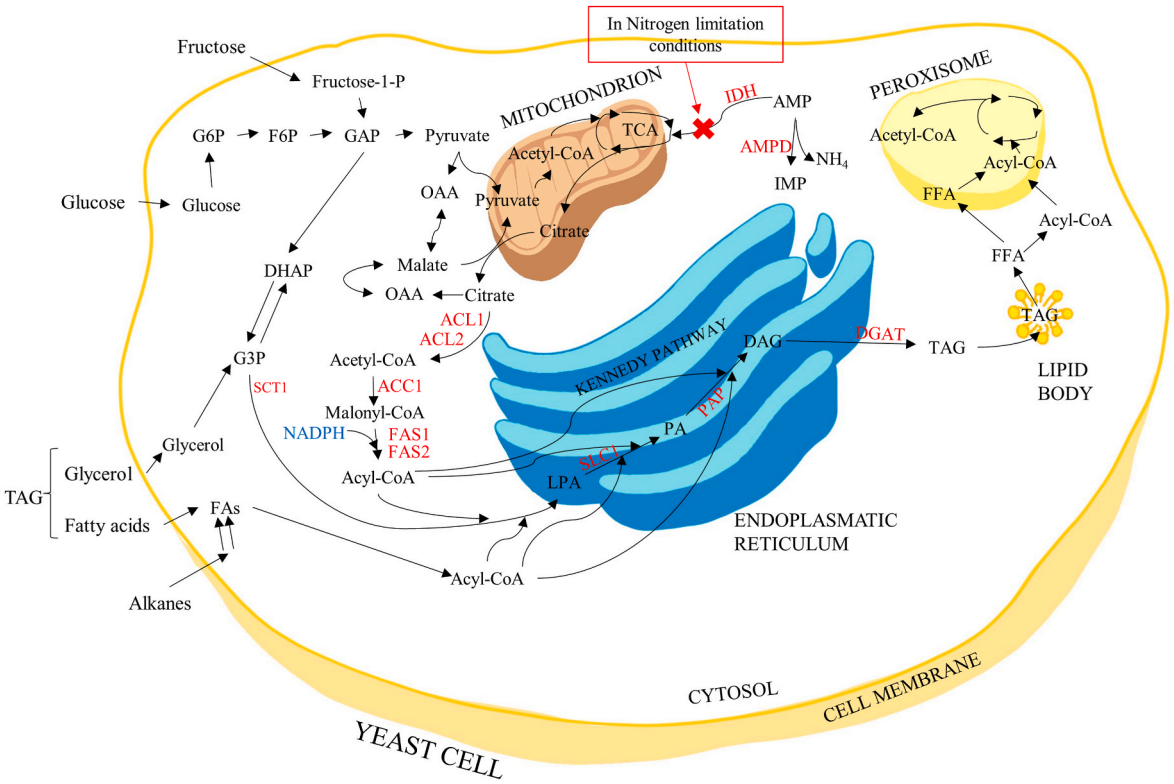


**Fig. 1.** Synthesis and degradation of TAGs in a yeast cell. Abbreviations: TAG, triacylglycerol; FAs, fatty acids; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; GAP, glyceraldehyde; DHAP, dihydroxyacetone phosphate; G3P, glycerol-3-phosphate; OAA, oxaloacetate; TCA, tricarboxylic acid cycle; IDH, isocitrate dehydrogenase; AMP, adenosine monophosphate; IMP, inosine monophosphate; LPA, lysophosphatidic acid; PA, phosphatidic acid; DAG, diacylglycerol; TAG, triacylglyceride; FFA, free fatty acids; NADPH, nicotinamide adenine dinucleotide phosphate dehydrogenase; IDH, isocitrate dehydrogenase; AMPD, adenosine monophosphate deaminase; ACL, ATP-citrate lyase; ACC, acetyl-CoA carboxylase; SCT1, G3P acyltransferase; FAS, FA synthase; SLC1, 1-acyl-*sn*-glycerol-3-phosphate acyltransferase; PAP, phosphatidic acid phosphatase; DGAT, diacylglycerol acyltransferase.

aconitase before being exported outside the mitochondria via the malate/citrate cycle. Citrate is then cleaved into acetyl-CoA and oxaloacetate (OAA) by the ATP-citrate lyase (ACL) enzyme, with ATP consumption [20]. The ACL enzyme is crucial for the lipogenesis phase exclusively found in oleaginous yeasts, and its absence restricts the flow of carbon to fatty acid synthesis [15] (Fig. 1).

A sequence of processes in the cytosol transforms the precursor acetyl-CoA into long-chain fatty acids, resulting in FA biosynthesis. Acetyl-CoA carboxylase converts acetyl-CoA to malonyl-CoA by first condensing it with bicarbonate (ACC1) [21]. Under the action of the enzyme FA synthase complex (FAS), acetyl-CoA is then reduced to acyl-CoA. Condensation, reduction, and cyclic dehydration processes create FAs. The reducing cofactor for FA synthase is NADPH, and each step in the acyl-CoA chain elongation needs two NADPH molecules. A typical chain length for spontaneously produced acyl-CoA is 16 or 18 carbon atoms, and these C16:0 and C18:0 molecules are then transported to the endoplasmic reticulum (ER), where they are further elongated and desaturated [22] (Fig. 1).

The Kennedy route produces TAGs by starting with glycerol-3-phosphate (G3P), which is derived from glycolysis and acts as the glycerol backbone. G3P 1-acyltransferase (by the gene SCT1) catalyses the conversion of G3P to lysophosphatidic acid (LPA), which is the first step in TAG formation [23]. LPA is subsequently acylated further by LPA acyltransferase (by the gene SLC1), resulting in phosphatidic acid (PA). Phosphatidic acid phosphatase (PAP) then dephosphorylates PA to generate diacylglycerol (DAG). Lastly, diacylglycerol acyltransferase (DGAT) or phospholipid diacylglycerol acyltransferase acylates the DAG to create TAGs that are stored as lipid droplets (lipid body) (Fig. 1). These lipid droplets' size, shape, and quantity differ significantly across the different types of genera and closely species [24].

### 2.2. *Ex novo* synthesis

When hydrophobic substrates are used, *ex novo* lipid synthesis occurs. These carbon sources, which include FAs and TAGs, are delivered into the cell and may either be utilised as an energy source or modified by enzymes [25]. Free FAs uptake selectivity and rate are typically unique for specific fatty acids, allowing for the change of FA profiles over time. Fat biomodification, also known as selective FAs usage, may be utilised to modify hydrophobic substrates' FA profiles into value-added oils [16]. Non-oleaginous microorganisms prefer to accumulate carbohydrates and generate secondary metabolites [25].

In terms of nitrogen dependence, *ex novo* lipid production differs from *de novo* lipid biosynthesis. Lipid build-up starts independently of nitrogen availability in the hydrophobic medium (i.e., waste cooking oils, industrial waste stream) for *ex novo* synthesis, and it occurs concurrently with cell development [26,27].

### 2.3. *Specifications of the primary species*

Around 70 yeast species out of about 1600 are known to be oleaginous and are then characterised by their ability to accumulate more than 20% internal lipids, and the number of species is continuously increasing. Of these 70 oleaginous yeasts, at least 25 species can accumulate up to 40% lipids as dry cell weight (DCW) [28]. The lipid content varies depending on the species, and it can reach up to 70–80% of DCW under certain nutritional conditions and with a carbon surplus, among others [29,30].

The oleaginous yeast divisions best known and able to accumulate the highest amount of lipids in the form of TAGs, classified according to their phylum, are the following two: *Basidiomycota* (e.g., *Rhodotorula*

*toruloides, Cutaneotrichosporon curvatus*) and *Ascomycota*; subdivision *Saccharomycotina* (e.g., *Yarrowia lipolytica, Lipomyces starkeyi*). To quantify the importance of research dedicated to oleaginous yeasts, Fig. 2 shows the total number of publications from 1950 till April 2022 related to studies on lipid production. The pie chart in Fig. 2 shows the percentage of each oleaginous yeast species studied with respect to the amount of publications available in the literature, while the bar chart quantifies the number of publications written and their popularity. Popularity is defined as those publications in which the yeast is mentioned even if it is only for comparison with another target yeast studied and described in the publication. The main characteristics of some of them will be described below.

*Rhodotorula toruloides* is a red yeast that was previously known as *Rhodosporidium toruloides*. It is capable of producing both lipids and carotenoids. *Torula rubescens* was the first name given to that yeast, originally isolated from the air in Dalian, China, in 1922 [29]. *R. toruloides* grows over a relatively wide range of temperatures, typically 10 to 30 °C [31], and initial pH values (from 3.0 to 10.0), generally with best results at pH 6.0 [32]. As for the carbon source used to feed this yeast, several sources have been described in the literature in recent years, e.g., hydrolysate from lignocellulose, crude glycerol, monosaccharides including hexoses and pentoses, organic acids such as acetic acid, or even longer chain FAs, with addition to D-galacturonic acid [33–37]. Regarding nitrogen sources, the most effective ones are ammonium, nitrate, amino acids, cadaverine and small peptides [38–40]. *R. toruloides'* robustness in terms of resistance to biomass-derived inhibitors has been established, among others observing good production of lipids and carotenoids when grown on biomass hydrolysates [41].

*Cutaneotrichosporon curvatus*, previously known as *Cryptococcus curvatus*, is one of the best-known and most performant oleaginous yeasts. In 2016, *C. curvatus'* first draft genome sequence was reported, allowing for significant advances in understanding this oleaginous yeast's metabolic behaviour and the creation of biomolecular tools for enhanced lipid synthesis [11]. This yeast may use lignocellulose, a mix of volatile fatty acids (VFAs), or chitin as carbon sources, reporting lipid production yields of more than 65% in terms of DCW [42–45]. The optimum incubation temperature at which this yeast grows is 28 ± 1 °C, and its preferred pH is around 6.0 [46].

*Yarrowia lipolytica* is another, non-conventional, yeast. According to Nicaud [47], the generic name "*Yarrowia*" honours David Yarrow's discovery of a new genus. *Y. lipolytica* is resistant to a wide range of physical conditions, including acidic and alkaline pH values (from 2.9 to 9.0), low temperatures (from 18 °C to 32 °C) and different salinities [48, 49]. Furthermore, *Y. lipolytica* can use a range of cheap renewable carbon sources and can handle large acetyl-CoA fluxes [50,51]. The literature's most widely reported carbon sources include lignocellulosic sugars, hydrophobic substrates, including TAGs and FAs derived from animal fats, alkanes, acetate, and even other volatile fatty acids from municipal, agricultural and industrial wastes [11]. Since the 1960s, *Y. lipolytica* has also been extensively studied to produce single cell proteins related to its ability to generate lipases and proteases [52]. This yeast can accumulate lipids at up to 65% content, with more than 90% of those lipids being stored as TAGs [53,54]. Furthermore, besides lipases, proteases and TAGs, organic acids such as isocitric, citric, ketoglutaric, itaconic, succinic and acetic acids can also be produced by *Y. lipolytica.* [11].

*Lipomyces starkeyi* was first characterised by Robert Starkey [55]. *L. starkeyi* can metabolise a wide variety of carbon and nitrogen sources: e.g., lignocellulosic sugars, glycerol, cellobiose, paper mill waste and acetic acid, among others, as carbon sources [11,56]. *L. starkeyi* can also metabolise inhibitors found in cellulosic hydrolysates, and it tolerates moderately acidic pH values between 5.0 and 6.5 [57]. Recommended agitation speeds and temperatures for healthy *L. starkeyi* fermentation have been reported to be 150–400 rpm and 28–30 °C, respectively [58]. With up to 70% lipid content as DCW, this yeast can accumulate large quantities of TAGs, similar in composition to palm oil [11].

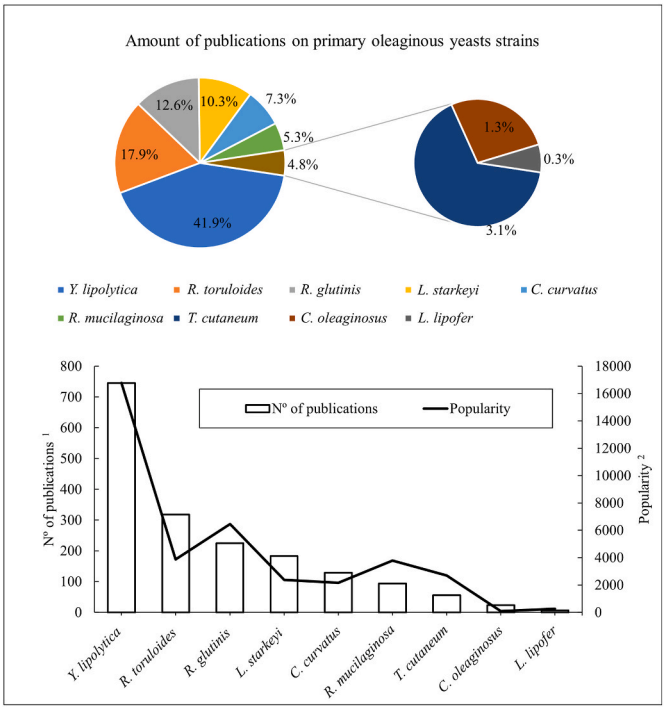


**Fig. 2.** Number of publications and popularity of the primary oleaginous yeast strains, from 1950, focused on lipids or biodiesel production. Updates of the yeast names have been considered. Example of search performed: ("R. toruloides OR "*Rhodotorula toruloides*" OR "*Rhodosporidium toruloides*") AND ("lipid*" OR "biofuel" OR "biodiesel" or FAME*"). Data obtained from dimensions. ai, the world's largest research database, accessed April 2022.1.- Number of publications that contain the name of the yeast in the title and/or abstract. 2.- Number of publications that contain the name of the yeast in the full text.

## 2.4. Global demand and market trend

The pandemic conditions resulting from the COVID-19 situation limited people's travels over the period 2020–2021 and, consequently, during that same period, the world's consumption of transportation fuels decreased [59]. On the other side, over the last 30 years, the production of fats and oils has increased by 167%, from 83.5 to 223 Mtonnes, and it is expected to increase further in the coming decades [14]. The market for fossil fuels and biofuels rebounded in 2021 as the economy started to recover and limitations of movements were gradually lifted. The biodiesel market grew due to increased blending regulations, direct subsidies, tax incentives, and market-wide decarbonisation measures. The medium-term growth of the biofuels industry is anticipated to be led by developing nations, according to the Organisation for Economic Co-operation and Development (OECD). They will enact blending laws and providing subsidies to encourage local production and the use of blended fuels. The loss of fossil fuels and fewer legislative incentives will, however, limit the growth of biofuels production in already established nations like the EU [59].

The resurgence of the demand for fossil fuels was favoured by the recovery of the world's economy and the relaxation of transportation limitations, which had a favourable impact on the biofuels market. In only one year, the usage of biodiesel surged by 55 billion litres in 2021 (IEA, 2022) [60]. The production margins for biofuels were impacted by increasing feedstock and producing costs notwithstanding the recovery, which had a detrimental effect on the production of biofuels in certain key producing nations. For instance, Argentina reduced its biodiesel blend rates in 2021 due to rising production costs and higher vegetable

oil prices. Nevertheless, a few nations, like Indonesia and India continue to subsidise, tax-credit, and demand increasing biofuel production [59]. The high cost of fossil fuels has, in some ways, given the biofuels business greater clout. The nominal price of biodiesel was historically high in the last year due to a rebound in demand and increased feedstock costs.

Over 2022–2031, international biofuel prices are expected to decrease in real terms while being stable in nominal ones. The pricing of feedstocks, crude oil, and distribution expenses, as well as consumers' disposable income and consumption choices, only partly represent the underlying drivers of the biofuel market. Policies that tie biofuel usage, such as domestic governmental support, also tend to influence price trends over time [59].

### 2.5. Commercial applications of lipids synthesised from oleaginous yeast

There are currently different commercial applications for the use of lipids synthesised from oleaginous yeasts. The most common use is in energy, specifically in the production of biofuels, but there are also other applications, e.g., in the food industry.

As for the application in the biodiesel industry, today, approximately 75% of biodiesel comes from first generation processes (vegetable oils) [59]. There are transport sectors, such as short-haul aviation, where the biofuel used is mainly based on Hydro-processed Esters and Fatty Acids (HEFA), produced from vegetable oils, waste lipids and animal fats [61]. More than 450,000 flights have used different aviation biofuel mixes [62]. Although there have not yet been any flights with biofuel produced from microorganisms, these data show that a future may be in the offing. Due to high prices, limited fuel supply, retrofitting and bunkering procedures, and institutional permission, fewer than 1% of marine transport employs biofuels, mostly in inland or short-sea transportation [63]. The most significant mode of international cargo transportation, merchant shipping, still uses fossil fuels. The use of third-generation biodiesel in road transport is expected to be the most common sector where it will be applied. Currently, 97% of heavy-duty vehicles, e.g., buses and trucks, are fuelled by diesel and 42% of light-duty vehicles, which means high compatibility with biodiesel [63].

Another potential application of lipids produced by oleaginous yeasts is in the food industry, e.g., as a replacement for palm oil. The primary uses of palm oil are in food and personal care goods [64]. A palm oil similar to that produced by palms' fruit, mostly made up of palmitic, stearic, oleic, and linoleic acids, may be produced by oleaginous yeast species [30], in particular species such as *Rhodotorula glutinis*, *Lipomyces lipofer* or *L. starkeyi* [65,66]. Moreover, one of the key components of chocolate is cocoa butter, which is also utilised in other goods like cosmetics. Cocoa butter contains about 60% saturated fatty acids [67]. Recent studies have determined that, under specific conditions, the yeast *Y. lipolytica* can perfectly mimic the lipid profile to produce cocoa butter equivalent [68]. The price of cocoa butter is relatively high, so using oleaginous yeasts in this field may prove to be a good market opportunity, producing high-quality equivalent cocoa butter. PUFAs, or Poly Unsaturated Fatty Acids, which are usually extracted from deep-water fish oil, are dietary components that are necessary for human health and can also be produced from oleaginous yeasts. Yeasts such as *Yarrowia lipolytica* can accumulate high amounts of internal lipids, which suggests that it has a lot of promise as a host for the industrial synthesis of PUFAs [69]. Notably, new engineering techniques have been used to enhance and enhance *Y. lipolytica*'s capacity to synthesise PUFAs. These techniques include the development and optimisation of PUFA biosynthetic pathways, enhancement of the availability of precursors NADPH and Acetyl-CoA, and regulation of genes involved in lipid metabolism [70].

## 3. Common feedstocks and substrates for oleaginous yeasts producing lipids

Carbohydrates used as substrates can be bio-converted into TAGs or lipids due to adequate intracellular metabolic pathways found in oleaginous yeasts. The yield of such bioconversion can vary depending on several factors, including the cultivation method, the strain used, the extraction strategy or the carbon source used. Considering the possible industrial-scale implementation of TAGs production for subsequent conversion to biodiesel, techno-economic feasibility assessment should be a key parameter in the experimental design. In a techno-economic analysis of microorganisms producing high-value lipids, it was discovered that the utilised carbon source represented 50–80% of the production costs [10,71]. Studying the possible carbon sources to be considered substrates is advisable to reduce production costs and increase lipid accumulation. Table 1 shows some results on lipid production by various oleaginous yeasts using different carbon sources for growth. Fig. 3 shows the processes described below, using different feedstocks for lipid production.

### 3.1. Glucose and other sugars

Oleaginous yeasts can utilise diverse hydrophilic and hydrophobic substrates as carbon sources to accumulate lipids in the form of SCOs. Of all possible sugar carbon sources, glucose has probably been most extensively studied in lab-scale studies, reaching significant fat contents [22]. Other sugars used to grow such yeasts mainly include pure xylose, fructose, sucrose or galactose. This process is illustrated in Fig. 3A.

According to Carsamba and co-workers, three types of lipid accumulations are observed when glucose (or similarly catabolised compounds) are used as a carbon source in batch nitrogen-limited cultures [49]. The first one, known as typical oleaginous metabolism, occurs when large amounts of lipids are accumulated in the cell after nitrogen exhaustion. At the same time, extracellular metabolites production, such as citric acid and polyols, is reduced. The second type (also known as atypical oleaginous metabolism) stores lipids following nitrogen depletion in the medium, and citric acid generation commences and continues unabated at a later stage, accompanied by a decrease in lipid content. The third form of metabolism is atypical metabolism, in which lipids accumulate slowly in yeast cells without being degraded, while citric acid is secreted simultaneously.

Moreover, according to the stoichiometry of glucose (and comparable sugars such as lactose, fructose, and others) metabolism, 100 g of glucose (~ 0.56 mol) catabolised would produce roughly 1.1 mol of acetyl-CoA. As a result, assuming 100% of the acetyl-CoA generated goes towards lipid synthesis, the highest possible yield of SCO produced per gram of glucose ingested would be 0.32 g/g [89]. However, even under optimum SCO production conditions (e.g., well-aerated chemostat cultures), the lipid yield on ingested glucose is seldom more than 0.22 g/g, although, in other instances, this threshold value has been set at 0.20 g/g or even lower [16].

In a study in which the yeast *Cryptococcus* sp. was cultivated on glucose (from 2 to 6%, w/w), when the glucose concentration was increased to 6% (w/w), growth and lipid production were suppressed [74]. Park and co-workers reported similar findings, claiming that the yeast is substrate inhibited when the glucose content in the medium exceeds 40 g/L and, that concentrations, between 20 and 40 g/L of glucose is optimal for yeast development [90].

Moreover, at an initial glucose concentration of 40 g/L, Fontanille and colleagues found that *Y. lipolytica* produced 31 g/L biomass with 40% lipid content [91]. Since wild strains of *Y. lipolytica* cannot assimilate xylose and sucrose as sole carbon sources unless the sugar is hydrolysed first or the strains are modified to increase the range of assimilable carbon sources, they are not particularly efficient sugar utilisation platforms and may even prefer substrates such as glycerol over glucose [92,93]. To solve problems related to the tolerance to xylose and sucrose, metabolic engineering strategies hold great promise for this yeast, together with co-fermentation strategies. Burgstaller and co-workers reached similar conclusions when growing the oleaginous yeasts *Apiotrichum brassicae* and *Pichia kudriavzevii* on different carbon

**Table 1**
Lipids production by oleaginous yeasts on different carbon sources.

| Yeast | Carbon source | C/N | Culture mode | Lipid concentration (g/L) | Lipid content (%, w/w) | Lipid yield ($Y_{L/s}$) | Ref. |
|---|---|---|---|---|---|---|---|
| *C. curvatus* DSM 101032 | Glucose 40 g/L | 40 | Batch culture | 5.23 | 52.6 | 0.15 | [72] |
| *C. curvatus* MUCL 29819 | Glucose 40 g/L | N. D. | Batch culture | 3.5 | 42 | 0.088 | [73] |
| *C. curvatus* MUCL 29819 | Acetic acid | N. D. | Batch culture | 5.01 | 71.7 | >0.139 | [73] |
| *Cryptococcus* sp. SM5S05 | Glucose 4% w/w | N. D. | Batch culture | 6.0 | 63.5 | N.D. | [74] |
| *C. curvatus* ATCC 20509 | Acetic acid 40 g/L | N. D. | Batch culture | 6.32 | 65.3 | 0.17 | [75] |
| *C. curvatus* DSM 70022 | VFAs derived from wastepaper | 40 | Batch culture | 1.78 | 41.2 | 0.11 | [76] |
| *Y. lipolytica* CCMA 0357 | Crude glycerol 100 g/L | N. D. | Batch culture | N.D. | 70 | N.D. | [77] |
| *Y. lipolytica* CICC 31596 | Acetic acid 70 g/L | N. D. | Batch culture | 10.1 | 27.2 | 0.14 | [78] |
| *Y. lipolytica* CICC 31596 | Mix of VFAs 50 g/L (acetic: propionic: butyric acid = 5:2:3) | N. D. | Batch culture | 8.28 | 30.7 | 0.16 | [78] |
| *Y. lipolytica* ACA DC 50109 | Mix of VFAs 15 g/L (acetic:hexanoic = 6:1) | 200 | Batch culture | ~5 | 43.4 | N.D. | [79] |
| *Y. lipolytica* Po1g modified | Glucose (367.4 g/L consumed) | N. D. | Fed-batch bioreactor | 98.9 | 66.8 | 0.269 | [80] |
| *Y. lipolytica* ATCC2046 modified | Glucose 100 g/L initial + 400 g/L feed | N. D. | Bench top bioreactor | 72.7 | 81.4 | 0.252 | [81] |
| *L. starkeyi* NBRC10381 | Lignocellulose 30 g/L | N. D. | Batch culture | N.D. | 68.2 | 0.19 | [82] |
| *L. starkeyi* DSM 70295 | Glucose 64 g/L + Xylose 16 g/L | 72 | Batch culture | 4.9 | 40 | 0.162 | [83] |
| *L. starkeyi* ATCC 58680 | Glucose 20 g/L + Xylose 10 g/L | N. D. | Batch culture | N.D. | 56 | 0.18 | [84] |
| *R. toruloides* AS 2.1389 | Food waste (50 g/L sugars) | 73 | Batch culture | 6.37 | 52.7 | 0.127 | [85] |
| *R. toruloides* AS 2.1389 | Distillery wastewater | 21 | Unsterilised batch | 3.5 | 43.7 | N.D. | [86] |
| *R. toruloides* ATCC 10788 | Crude glycerol 44.5 g/L | 100 | Batch culture | 11.3 | 53.3 | N.D. | [87] |
| *L. lipofer* NRRL Y-1155 | Crude glycerol 61 g/L | N. D. | Batch culture | 5.46 | 57.6 | 0.12 | [66] |
| *R. babjevae* DVBPG 8058 | Lignocellulose hydrolysate (Glucose 56 g/L + Xylose 27.2 g/L + Acetic acid 5 g/L) | N. D. | Batch culture | 18.1 | 64.8 | 0.24 | [88] |

sources, including glucose, sucrose, xylose, and galactose [94]. At an initial concentration of 10 g/L as sugar content, they managed to consume 100% of the glucose and galactose substrates. In contrast, in the case of sucrose or galactose, a consumption of 30–60% of the substrate was reached under otherwise the same conditions [94]. Therefore, this shows a low tolerance to the latter pair of sugars.

The mutant yeast *R. toruloides* R-ZL2 was also used to study the effects of different carbon sources. Research carried out by Ye and co-workers confirmed that sucrose (5% w/w) as a carbon source yields better results in terms of lipid production compared to glucose and xylose, i.e., 2 g/L *vs* 1.5 and 1.25 g/L, respectively [95]. These results are greatly improved by controlling the nitrogen source used and the C/N ratio; ammonium nitrate at a C/N ratio of 200 yields 8.25 g/L lipid production, while 65% lipid content can be achieved (using sucrose at 4% concentration, w/w) [95]. However, according to Manzoor, one of the maximum values achieved in terms of lipid content, with glucose as a carbon source in the culture medium, was 72% (w/w) with the yeast *Rhodotorula glutinis* [96].

### 3.2. Glycerol

Glycerol is a major by-product in the biodiesel manufacturing process. According to production estimates, for every 10 kg of biodiesel produced, roughly 1 kg of crude glycerol is obtained [77]. As a result, in 2021, Europe generated 236 barrels of biodiesel per day, equivalent to more than 1.2 million tonnes per year of crude glycerol [97]. Approximately half of the oleaginous yeasts cultivated on glycerol have been grown on crude glycerol [14].

Glycerol constitutes the structural backbone of TAGs and a recommended substrate for yeast lipids, hence oleaginous yeasts may be suitable for metabolising industrial glycerol. Therefore, a microbial lipid production process may be integrated into a biodiesel producing facility, with surplus biodiesel being transesterified from the microbial lipids [87]. However, the presence of certain impurities or contaminants, like MeOH, may reduce or hinder yeast growth, necessitating purification before fermentation [98]. A typical pre-treatment process, with the subsequent use of the pre-treated substrate to cultivate an oleaginous yeast for lipid production, is depicted in Fig. 3B.

*Y. lipolytica* and *R. toruloides* species have been shown to use glycerol efficiently, making them popular among glycerol researchers [99]. For example, some of the *Rhodotorula* species have demonstrated to generate higher levels of conjugated linoleic acid [100] with lipid yields of roughly 0.27 w/w with pure glycerol [101] and 0.22 w/w with synthetic crude glycerol [102]. These are promising results considering that the theoretical maximum yield in grams of lipid produced per gram of substrate consumed is around ~0.30 g/g [89]. Moreover, Teixeira Souza and co-workers compared the results of growth and lipid production of the yeasts *Y. lipolytica* CCMA 0242, *Y. lipolytica* CCMA 0357, *C. humicola* CCMA 0346 and *W. anomalus* CCMA 0358 grown in culture media containing glucose, glycerol and crude glycerol (from biodiesel industries) [77]. The lipid production of *Y. lipolytica* CCMA 0357 was the highest, with nearly 70% (w/w) lipids produced, using 100 g/L crude glycerol [77]. Other authors, such as Uprety and colleagues, report the capacity of some other microorganisms to assimilate crude glycerol to obtain biodiesel. For example, they also show the capacity of *Trichosporonoides spathulata* JU4-57 to accumulate lipids in the form of SCO from crude glycerol, reaching a lipid content of 56.4% (w/w) when cultivated in fed batch mode [103].

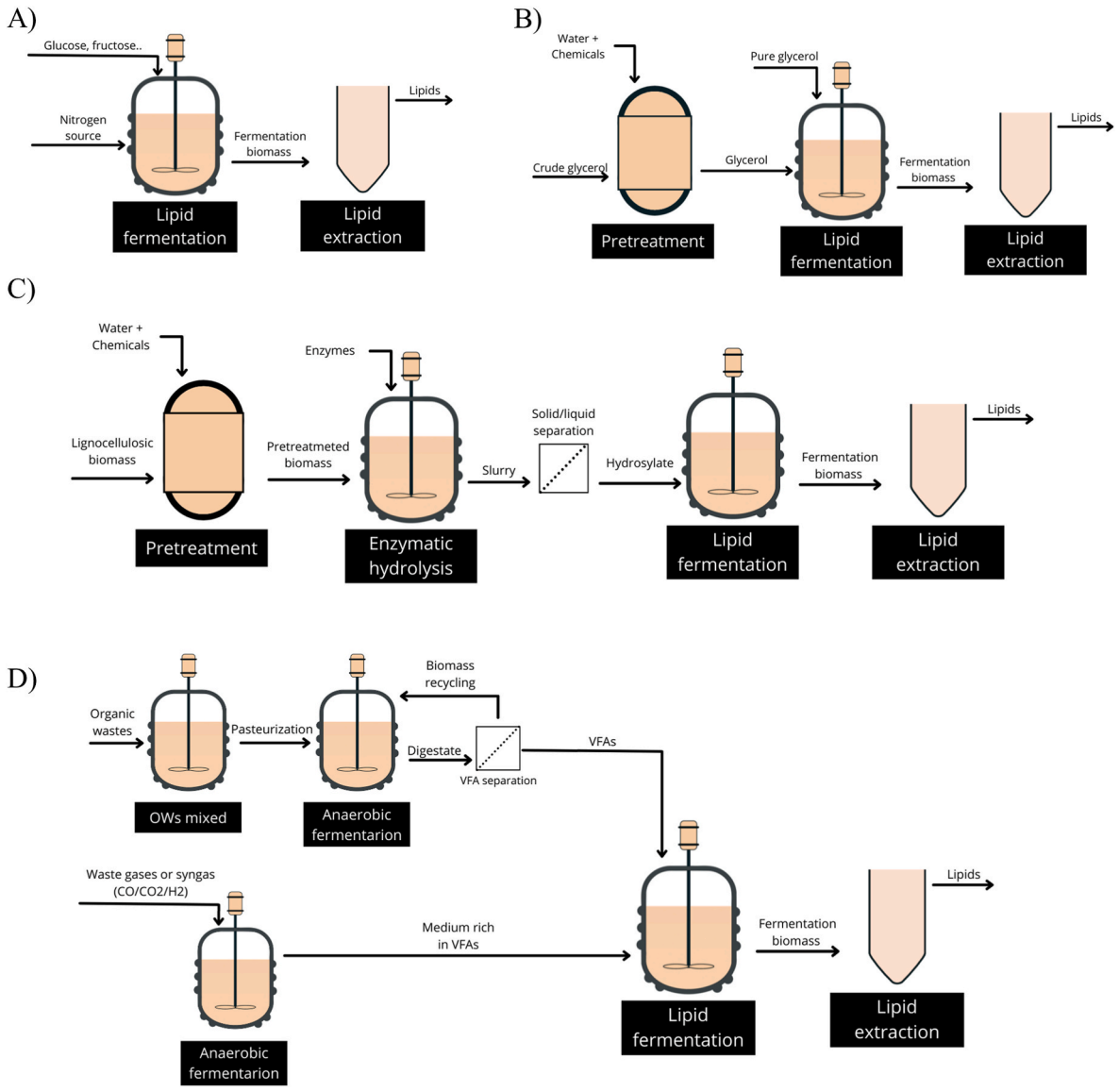


**Fig. 3.** Overview of different lipid production processes according to the different primary carbon sources. A) Production of lipids from glucose. B) Lipid production from crude glycerol. C) Lipid production from lignocellulosic biomass. D) Lipid production from VFAs.

### 3.3. Lignocellulose

The hydrolysis of complex organic feedstocks can be used to provide a suitable culture medium for yeasts. Regarding availability, lignocellulosic biomass is abundant and appealing in the form of hydrolysed feedstock.

In oleaginous yeast research, lignocellulosic materials, primarily maise stover, grass/cane, or other plant wastes, have been employed considerably for hydrolysis [104]. Artificial hydrolysates have also been created to assess a yeast strain's capacity to synthesise lipids, including mixtures of glucose, arabinose, xylose or acetate, as well as cellobiose, from lignocellulosic hydrolysates [105,106]. Regarding xylose, it could be considered that the phosphoketolase pathway is used for its assimilation. Therefore, the theoretical maximum yield of lipid production per gram of xylose consumed is about 0.34 g/g [89]. As the initial step toward microbial lipid synthesis, polysaccharides in lignocellulosic biomass must be transformed into monomeric sugars through enzymatic hydrolysis. High sugar concentrations from enzymatic hydrolysis are desirable because they allow for high-titter lipid synthesis, which is necessary for a commercially viable microbial lipid biorefinery [107]. However, sugar production and concentration are frequently low to moderate due to lignocellulosic biomass's complex structure and refractory nature [108]. A pre-treatment procedure to deconstruct a polysaccharide-lignin complex is required to minimise biomass recalcitrance and maximise biochemical conversion [109]. In addition to sugars, inhibitory chemical compounds (ICCs) are also produced due to the biomass pre-treatment, and they would restrict microbial growth throughout the fermentation process. This process is illustrated in Fig. 3C. Estimating the types of ICCs is challenging since the nature of by-products generated depends on both the kind of biomass treated and the pre-treatment technique used [82].

There are yeast strains which, by their nature, can better metabolise the major components found in the lignocellulosic biomass. Some of these strains are *C. curvatus* [110], *L. starkeyi* [111] and *P. hubeiensis* [105], among others. In particular, several yeasts of the *Lipomyces* genus, such as *L. starkeyi*, *L. doorenjongii* or *L. orientalis*, have proven their ability to consume 100 g/L lignocellulose sugars (50 g/L glucose + 50 g/L xylose), which is relatively high compared to other strains [82]. Of the three strains mentioned above, *L. starkeyi* results are the most promising to date in the use of lignocellulosic biomass, reaching lipid contents of 68.2%, even in media where ICCs were present, such as furfural, vanillin, syringaldehyde or acetic acid [82]. However,

sometimes, the presence of acetic acid or other VFAs can be beneficial for the growth of the strains as long as they are present in relatively small concentrations, as also addressed more in detail in a later section.

In the case of other oleaginous yeasts that show higher inhibition to ICC compounds generated in the pre-treatment, a possible solution that gives positive results is genetic engineering, as in the case of *R. toruloides*. For instance, in a recent study aiming to improve the robustness of *R. toruloides* to inhibitory compounds such as phenols (*p*-hydroxyphenyl, guaiacyl, and syringyl groups), genes degrading phenolic compounds were overexpressed through genetic engineering, resulting in a 25% increase in lipid production and a 30% increase in terms of biomass [112]. This could be a way to improve performance when dealing with inhibitory compounds present in lignocellulose.

### 3.4. Acetic acid and other VFAs

Volatile fatty acids are highly appealing substrates because they may be generated by pure or mixed microbial cultures from feedstocks such as waste or gas emissions [113,114]. To assess their efficiency for growing on lignocellulosic hydrolysate or anaerobic digestion (AD) effluents, oleaginous yeasts have been cultivated on acetate or combined VFAs [115,116]. The most common VFAs generated during AD are acetic, propionic, iso-butyric, butyric, iso-valeric, valeric, and caproic acids [117]. Nevertheless, less is known about the possible use of iso-butyric, valeric, iso-valeric, and caproic acids, which can account for more than 30% of all VFAs in anaerobic fermentation effluents [117]. Many different types of wastes, wastewaters, and other similar feedstocks have been used to produce VFAs. They are suitable for oleaginous yeast growth, e.g., wastepaper [76], tuna waste [113], food waste [118], algae biomass [117], cheese whey [119], sewage sludge [120], brewery wastewater [121], among others. With anaerobic bacteria, the VFAs production profile highly depends on different aspects, such as the nature of the substrate used and the operational conditions in the case of AD processes [118]. This process is illustrated in Fig. 3D.

On the other hand, acid toxicity is one of the critical problems preventing reaching high lipid yields with this type of substrate. Acid concentrations typically employed in batch fermentations can inhibit the yeast's activity, and excessively high VFAs concentrations would thus have adverse effects on oleaginous yeasts [122]. The pH will decrease as the acid concentrations increase in the yeast's culture medium. This is also a key factor affecting their growth, as at pH below 4, most oleaginous yeasts are inhibited [42]. Besides, during VFAs metabolisation, the pH might become basic due to the assimilation of acids, resulting in potential yeast growth suppression. Furthermore, about the use of different volatile fatty acids as substrates, it was found that yeasts assimilate short-chain acids better than long-chain acids. For instance, it was observed that over the first 24 h of fermentation, *Y. lipolytica* could more swiftly metabolise acetic acid than other VFAs (0.035 g/L·h *vs* 0.015 g/L·h) [123].

Despite the problems that can appear during the cultivation on VFAs, either through inhibition and/or because of the type of acid, oleaginous yeasts can effectively convert acids into lipids. For example, under alkaline conditions, Gao and co-workers cultivated the yeast *Y. lipolytica* in batch cultures using synthetic and waste-derived high-content volatile fatty acids [78]. Due to the alkaline conditions, the yeast was able to tolerate acetic acid concentrations of 70 g/L and higher, with a lag phase of no more than 24 h and a lipid concentration and lipid content of 10.1 g/L and 27.2% (w/w), respectively, using an alkaline pH of 8.0 [78]. It was also able to avoid inhibition using a 50 g/L VFAs mixture (acetic:propionic:butyric acids, 25:10:15) as substrate, with results in terms of lipid concentration and lipid content of 8.28 g/L and 30.7% (w/w) respectively, at pH 9.0 [78]. In another study, using a mixture of different VFAs with a total concentration of 30 g/L (acetic:propionic:butyric acids, 15:5:10) to cultivate the yeast *C. curvatus*, maximum concentration and lipid content of 4.93 g/L and 56.8%, respectively, were achieved [42]. So far, one of the highest results obtained in terms of lipid content, using acetic acid as the sole carbon source, was 73.4% (w/w), reached with the yeast *C. curvatus* [115]. Huang and co-workers found that *R. toruloides* was able to accumulate more lipids when grown on 20 g/L acetic acid as the sole carbon source than with 20 g/L glucose, using otherwise the same culture conditions [73].

#### 3.4.1. Acetic acid and other VFAs from carbon capture and $CO_2$ valorisation

Contrary to AD, when gases such as $CO_2$ or syngas are fermented by anaerobic bacteria such as acetogens, acetic acid will essentially be the primary or even single acid produced. Only occasionally have butyric and caproic acids been observed, besides acetic acid, in such $C_1$-gas fermentation processes [124]. Related to this, Robles-Iglesias and co-workers efficiently produced lipids with *R. toruloides* from acetic acid obtained from *Acetobacterium woodii* grown in bioreactors with continuous feed of a mixture of $CO_2$ and $H_2$, aiming at valorising biogenic $CO_2$ [34]. *A. woodii* converts $C_1$ gases through the Wood–Ljungdahl pathway (WLP), with acetic acid as the end metabolite. Suppose longer chain, even fatty acids (i.e., butyric and caproic acids) are produced, besides acetic acid. In that case, such mixture is also efficiently metabolised by oleaginous yeasts such as *Y. lipolytica*, though further optimisation studies are necessary [125]. *C. carboxidivorans* can typically produce such $C_2$, $C_4$, and $C_6$ fatty acids mixtures from $C_1$-gases ($CO_2$, CO) [124].

## 4. Parameters affecting lipid accumulation

Process variables, including the nature of the substrate, C/N ratio, nitrogen source, pH, temperature, or even dissolved oxygen (DO) concentration, can significantly impact on yeast lipid synthesis and their characteristics, including the fatty acid profile. For instance, nitrogen deficiency has generally been identified as the critical factor for lipid accumulation in oleaginous yeasts, as described hereafter [126].

### 4.1. C/N ratio and nitrogen source

According to several studies and as mentioned above, the carbon to nitrogen ratio is one of the main factors which affect the lipid content. An optimal C/N ratio is a crucial factor for the stoichiometric requirement of the carbon flow to generate biomass and leave excess for lipid synthesis [127]. Therefore, an optimal condition for lipid overproduction is a culture medium rich in a carbon source with a limited amount of nitrogen and thus a high C/N value [128]. A study with *R. glutinis* grown on glucose as a carbon source clearly showed that the lipid content increased by 32% when the C/N ratio was raised from 20 to 70, while the lipid concentration was also increased 4.5-fold [129]. This experiment also revealed that at higher C/N ratios (above 100), the yeast's ability to synthesise lipids was inhibited, probably due to inhibition by high glucose concentrations in the culture medium [129], which in the end is more related to substrate inhibition rather than the C/N ratio as such. The maximum C/N ratio that a yeast can tolerate can vary depending on the strain. Indeed, compared to the study above, Award and co-workers, performing experiments with the yeast *C. oleaginous*, determined that the best C/N ratio was 120, reaching 44.3% (w/w) lipid content using glucose and yeast extract as carbon and nitrogen source, respectively [130]. It is also worth highlighting that, besides the C/N ratio, different studies may use different operating conditions (e.g., pH, temperature, macro- and micro-nutrients) which may affect the final results and conclusions. When using acetic acid as the sole carbon source, combined with ammonium chloride as the nitrogen source, to culture the yeast *R. toruloides*, in this case, it was found that a C/N ratio as high as 200 was the optimum, resulting in 48.2% (w/w) lipid content, in batch cultures [116].

On the other hand, increasing the C/N ratio in the growth medium may also have adverse effects on some bioprocess efficiency parameters, increasing lipid yield and productivity while lowering the biomass and growth rate. For example, when growing *R. toruloides* with a C/N ratio =

∞ (no nitrogen source was added), a minimum amount of biomass of 6.8 g/L was obtained when growing the yeast with glucose, while this value increased and basically doubled, to 13 g/L, when the C/N ratio was 20 [95]. Table 2 shows different values obtained in a study by Alexander and co-workers comparing different C/N ratios in three species of the *Rhodotorula* genus [131]. For example, the highest difference in biomass decrease was observed in *R. kratochvilovae*, where increasing the C/N ratio from 2 to 100 resulted in a 4-fold reduction in biomass. Although with a minor difference, this behaviour was similar in the other strains. On the other hand, increasing lipid content was achieved by increasing the C/N ratio in each experiment.

Moreover, in a research carried out by Park and colleagues, the yeast *C. curvatus* was cultured with glucose as the sole carbon source, while $NH_4Cl$ and $NaNO_3$ were used as nitrogen sources [90]. The C/N ratio in both experiments ranged from 5 to 160, at an initial pH of 5.5. As expected in the experiment with $NH_4Cl$, the amount of lipids increased as a function of the C/N ratio, reaching a maximum lipid content of 34% at a C/N ratio of 160. On the other hand, different results were found when $NaNO_3$ was used as the nitrogen source. With $NaNO_3$, the lipid content remained basically constant at all C/N ratios tested, with values close to 50% (w/w) [90]. In another study, Brabender and co-workers assessed the influence of urea as an alternative to ammonium sulfate as nitrogen source in *Y. lipolytica,* and observed an increase in cell growth with glucose as carbon source [132]. Overall, the results in terms of lipid accumulation slightly improved when using urea, reaching the best results (1.160 g/L of lipid concentration) with C/N = 240 and 0.333 g/L urea [132].

Conversely, in some cases, there is evidence that when the C/N ratio is increased, the accumulation of intracellular lipids may decrease. This occurred in a study where acetic acid or other VFAs were supplied as the carbon source at relatively high concentrations. For example, if *C. curvatus* was grown with 30 g/L acetic acid, increasing the C/N ratio from 10 to 200 increased the lipid content, but when cultivating this yeast with 40 g/L acetic acid, increasing the C/N ratio decreased the amount of lipids [75]. This shows that, at high acid concentrations, the source of nitrogen, such as yeast extract, or amino acids, such as arginine and lysine, becomes more necessary for lipid production and arguably prevents acid inhibition [75].

### 4.2. Genetic engineering

The use of metabolic engineering techniques to increase lipid accumulation in oleaginous yeasts has greatly improved and can significantly impact the lipid yield obtained. Nevertheless, most naturally occurring oleaginous yeasts are often untyped strains, making it difficult to use effective genetic modification approaches [133]. Currently, the primary impediment to considerer certain oleaginous yeasts as lipid cell factories is the absence of advanced genetic manipulation techniques. One of the common oleaginous yeasts regarded as a safe organism for biotechnological purposes is *Y. lipolytica*. Since the species' whole genome sequence is publicly accessible in a database, it has often served as a main reference organism in research on genetics, metabolism, and transcriptomics [134]. Although *Y. lipolytica* is probably the most widely reported organism so far in the literature, other species, such as *R. toruloides*, have successfully responded to genetic modifications as well [135]. Table 3 shows the results of different genetic engineering strategies aimed at increasing lipid accumulation.

*Y. lipolytica* and *R. toruloides,* included in Table 3, have shown encouraging results in synthesising lipids and lipid-based chemicals. To create specific strains that may be used on an industrial scale, additional research is required to understand better the metabolic network involved in the push-pull pathways of the target metabolite production. If they are susceptible to genetic modification, several other non-conventional yeasts with intriguing oleaginous traits may be investigated as prospective hosts [9]. Creating such a platform for commercially viable biotransformation might be greatly aided by new recent molecular tools and methods combined with bioprocess optimisation methodologies [9].

**Table 2**
Lipid content and dry biomass in three *Rhodotorula* strains using culture media with C/N values of 2 and 100, adapted from Ref. [131].

| Yeast | C/N | Lipid content (%, w/w) | Dry biomass (g/L) |
|---|---|---|---|
| *R. glutinis* | 2 | 24.5 | 19.01 |
| | 100 | 31.5 | 9.98 |
| *R. mucilaginosa* | 2 | 20.2 | 27.74 |
| | 100 | 23.1 | 13.90 |
| *R. kratochvilovae* | 2 | 21.3 | 41.33 |
| | 100 | 29.7 | 9.80 |

### 4.3. pH

The pH of the medium is also an essential parameter in the assimilation of the carbon source by the yeast and the accumulation of lipids. Besides, the surface features of the cell membrane are influenced by pH, which impacts the carbon absorption process.

Some studies showed that a slightly acidified medium might favour lipid accumulation in some yeast cells (pH 5.0 to 6.5) [141,142]. For instance, a pH range of 5–6 is optimal for lipid synthesis, according to several experiments using *Y. lipolytica* grown on glucose [143]. This tendency is shown in yeasts whose carbon source is glucose since, on the other hand, Gao et al. achieved the best results at an alkaline pH of 8.0 in the yeast *Y. lipolytica* using acidic sources [78]. As explained above, their study suggests that in media-rich in acetic acid and other VFAs, the alkaline pH helps prevent inhibition, thus stimulating further lipid accumulation. Similarly, Liu and co-workers showed that *C. curvatus* grew well in an alkaline medium (pH 8.0–10.0) containing 40 g/L acetic acid [75]. This behaviour was attributable to decreased dissociation of the VFAs at the high pH values.

In another study with the yeast *R. glutinis* grown in potato wastewater and 5% glycerol, the lipid content was affected by inhibition phenomena at pH values below 3. In contrast, the lipid content remained constant at pH 4–7 [144]. For *R. toruloides* grown on food waste hydrolysate, there was a significant increase in lipid content when the initial pH of the medium was set at 11, reaching 46% (w/w), which was 50% higher than at pH 4 [145]. Generally, according to several authors, the most suitable pH for optimal growth of *R. toruloides* ranges between

**Table 3**
Different genetic modifications applied to *Y. lipolytica* and *R. toruloides* that resulted in a remarkable increase in the lipid yield.

| Yeast | Carbon source | Genetic modification | Genes | Lipid yield (g/g substrate) improvement | Ref. |
|---|---|---|---|---|---|
| *Y. lipolytica* | Glucose | Overexpression | ACC1, DGA1, GAPC | 25% | [80] |
| *Y. lipolytica* | Glucose | Overexpression and deletion | DGA1, DGA2, Δtgl3 | 250% | [136] |
| *Y. lipolytica* | Glucose | Overexpression and deletion | DGA1, Δpex10, Δmfe1 | 400% | [137] |
| *Y. lipolytica* | Sucrose | Overexpression and deletion | DGA (x3), Δdga1, Δdga2, Δlro1, Δare1 | 175% | [138] |
| *R. toruloides* | Glucose | Overexpression | ACC1, DGA1, SCD | 42% | [139] |
| *R. toruloides* | Glycerol | Overexpression | DGAT1, SCD1 | 13% | [140] |

4 and 6 [85,146]. In a nutshell, the results of different research studies suggest that the ideal pH value varies from one strain to another and is also dependent on the carbon sources, among others.

### 4.4. Temperature

The best temperature to grow most oleaginous yeasts is around 30 °C, as most of them are mesophilic [13]; however, some oleaginous yeasts have been cultivated at temperatures as low as 3 °C (e.g., *Rhodotorula glacialis*) [147] or also under thermophilic conditions, at 45 °C, as in the case of *Blastobotrys adeninivorans* [148].

Generally, the optimal growth temperature would best be as close to room temperature as possible because this would reduce both heating and/or cooling costs. In a study with a wide range of strains from different families, grown at a C/N ratio of 5 and varying temperature from 20 to 37 °C, it was found that both below and above the optimum temperature, the strains were able to grow but with a lower biomass production, which decreased as the temperature moved away from the optimum temperature [149]. If it is considered that the optimal temperature for yeast cultures, in terms of energy costs, is 20 °C, the yeast *Waltomyces lipofer* showed excellent results, generating an amount of biomass very close to its optimal culture temperature [149].

In a study carried out on the effect of culture temperature using obligate psychrophilic, facultative psychrophilic and mesophilic yeasts, it was concluded that for the latter two types, culturing at 4 °C affected growth rate but not biomass accumulation [150]. In terms of lipid production, the only yeasts that experienced a decrease when cultured at low temperatures were the mesophilic yeasts [150].

Briefly, finding an optimal temperature range where the yeast can produce the maximum amount of biomass will favour lipid accumulation and cost-effectiveness.

### 4.5. Macro-nutrients

Some authors studied how some macro-nutrients of the culture medium influenced the yeast's ability to synthesise lipids. For example, Wierzchowska and co-workers discovered a direct relationship between the $KH_2PO_4$ concentration of the medium and lipid accumulation [151]. The authors reported how under limiting concentrations of $KH_2PO_4$, the amount of lipids accumulated in the yeast *Y. lipolytica* increased. Still, on the other hand, this led to unfavourable conditions for biomass production [151]. Other authors, such as Zhao and co-workers, determined that $FeSO_4$, together with yeast extract and the concentration of the carbon source, are significant components influencing lipid accumulation [152]. Using the yeast *R. toruloides*, the limitation of various nutrients, such as N, P and S, both individually and together, in a medium composed mainly of glucose, was also studied through mathematical modelling [153]. When testing different concentrations of these nutrients, the lipid content varied between 50 and 77% (w/w). N was the compound that most altered the lipid production, with the content increasing as nitrogen was limited. With both P and S, the lipid content was also reduced when limiting their concentration in the culture medium [153].

### 4.6. Physical parameters

Physical parameters such as agitation speed, fermentation time or even the inoculum's age and size can also affect lipid production performance [58]. For example, according to Zhang and co-workers, optimal agitation for the growth of oleaginous yeasts, such as *L. starkeyi*, lays between 150 and 400 rpm [58]. On the other hand, Liu and co-workers studied the effect of inoculum age on lipid production with *L. starkeyi* by growing the inoculum for 36, 40 and 48 h in liquid YPD (Yeast extract-Peptone-Dextrose) and then fermenting it in a sugar mixture for 36 h [154]. The results of this experiment in terms of lipid production were high and very similar, with around 60% w/w lipid content. However, there was a difference in the amount of biomass obtained (g/L), with the inoculum grown for 36 h yielding the lowest amount and the 48-h inoculum the highest. The fermentation time may also affect the lipid content and rate of lipid production, with a significant increase in both variables during the first 24 h of growth and then remaining almost constant [154]. A study on lipid production with *L. starkeyi* dedicated to evaluating the effect of the optical density of the inoculation medium revealed that in the case of inoculation of the culture medium with either a minimum optical density at 600 nm ($OD_{600nm}$) of 0.6 or a maximum $OD_{600nm}$ of 18.0, the final lipid yield was roughly similar. However, the growth rate (g/L·h) varied, even triplicated, when the medium was inoculated with $OD_{600\ nm} = 18.0$ [155]. These results are interesting since inoculating with a higher optical density could shorten growth time, resulting in cost savings at the industrial level.

One of the other factors influencing lipid production by oleaginous yeasts is aeration. For example, increasing the aeration flow rate from zero to 2.0 vvm significantly increased the biomass and lipid yields in *R. glutinis*, while increasing from 2.0 to 3.0 vvm exhibited no significant change [7]. This indicates that if the rate of DO supply is exceeded, virtually no change is observed. On the other hand, it suggests that if a high lipid accumulation performance is desired, the DO should be at least equal to the oxygen demand.

In general, optimising yeast growth to achieve maximum yields of lipid accumulation should be a key factor before industrial scale-up.

## 5. Factors affecting the lipid profile

The experimental conditions are strongly correlated with the lipid profile produced by some yeasts [156]. Typically, lipid profile display variations among different strains and media. Of all the lipids commonly produced by oleaginous yeasts, oleic acid (C18:1) is generally predominant, with a total percentage ranging from 28 to 66%, approximately, of the total lipids produced, followed by palmitic acid (C16:0) with 11–37% [96]. Lipids that are also produced by oleaginous yeasts but which are not as predominant as those mentioned above are linoleic acid (C18:2), stearic acid (C18:0), palmitoleic acid (C16:1) and linolenic acid (C18:3), whose approximate typical abundance as a function of total lipids is, respectively, 3–14%, 1–10%, 1–6% and 1–3% [96]. Their relative ratios are comparable to those of various commercial vegetable oils in certain circumstances, as shown in Table 4.

The ability of oleaginous yeasts to produce different lipid profile, varying, among others, according to both strain and substrate, converts

**Table 4**
Comparison of the major lipid profiles in vegetable oils and different species of oleaginous yeasts.

| Lipid source | Lipid composition (%, w/w) | | | | | | Ref. |
|---|---|---|---|---|---|---|---|
| | C16:0 | C16:1 | C18:0 | C18:1 | C18:2 | C18:3 | |
| Palm | 18.7 | 1.6 | 0.9 | 56.1 | 21.1 | – | [157] |
| Canola | 4.1 | 0.3 | 1.8 | 61 | 21 | 8.8 | [158] |
| Soybean | 10.9 | 0.1 | 5.7 | 27.5 | 51.5 | 3 | [157] |
| Corn | 11.6 | – | 2.5 | 38.7 | 44.7 | 1.4 | [157] |
| Rapeseed | 2.8 | 0.3 | 1.3 | 64.4 | 22.3 | 7.3 | [159] |
| *R. toruloides 32,489* | 22.2 | 2.9 | 5.7 | 21.3 | 39.5 | 6.7 | [98] |
| *R. toruloides CBS 14* | 25.6 | 0.8 | 11.3 | 45.6 | 12 | 2.1 | [36] |
| *Y. lipolytica Po1dL* | 34 | 2.6 | 16.4 | 15.4 | 12.2 | 18.3 | [49] |
| *L. starkeyi DSM 70295* | 43 | 5.2 | 6.5 | 42 | 2.1 | 0.1 | [160] |
| *L. starkeyi DSM 70296* | 29.2 | 4.1 | 4 | 55.5 | 5.7 | – | [161] |
| *R. glutinis T13* | 24.5 | 1.9 | 8.7 | 37.6 | 18.8 | 3.5 | [162] |
| *C. curvatus NRRL Y-151* | 22.9 | <0.1 | 7.6 | 52 | 10.7 | <0.1 | [46] |

them into microorganisms of high industrial interest. For example, in a study by Shaigani and co-workers, the lipid profile of some yeasts was mapped by culturing them with different nitrogen-limited sources containing xylose (Xyl), glucose (Glu), and mannitol, and also phosphate-limited sources containing N-acetylglucosamine [163]. When comparing the N-acetylglucosamine medium to synthetic Glu/Xyl-containing media, the FA profile revealed a higher amount of unsaturated FAs in the N-acetylglucosamine medium. With N-acetylglucosamine, *C. oleaginosus* had a lower C16:0 ratio and generated a double of C18:2. The use of the mannitol medium also led to an increase in unsaturated FAs in *Trichosporon asahii* and *C. oleaginosus* and reduced C18:0 percentages in this medium [163].

Using different concentrations of the same substrate also leads to changes in the lipid profile. For example, with the yeast *R. toruloides* DSM 10134 growing in a medium rich in acetic acid, C18:1 decreased while C16:0 increased as the initial substrate concentration was increased [34]. When different acids are used as carbon sources for the yeast, changes in the lipid profile are also experienced. For example, several studies have shown that raising propionic and butyric acid levels can boost odd chain FAs synthesis in a variety of oleaginous yeasts [164,165].

Both temperature and pH of the medium can also alter the lipid profile produced by yeasts. On the one hand, decreasing the temperature most often increases unsaturated fatty acids, although this is not related to an increase in total lipid content [166]. On the other hand, decreasing the pH of the medium may lead to a rise in C18:1, while the ratio of saturated/unsaturated fatty acids remains almost constant [144]. Finally, many investigations revealed that the lipid profile is comparable to that of vegetable oils. It can be altered by altering the yeast strain, carbon concentration and supply, temperature, and pH, among others.

## 6. SCOs lipids production costs, scale-up, and LCA

As with any process to be brought to an industrial scale, a critical step is to determine whether it can potentially be cost-effective. The feedstock cost is crucial when analysing whether the process will be viable, as it generally represents between 50 and 80% of the total costs [10,71]. Apart from the feedstock, the following two major costs of this process would be the midstream (associated with aspects such as the bioreactor, cultivation, media preparation, sterilisation) and downstream (related to cell harvesting, lipid extraction, recovery, among others) processes, as summarised in Fig. 4. In each of these two processes, the main costs are directly derived from the capital, energy, and wastes. All scientific advances in these three key areas (feedstocks, midstream and downstream) would be essential to make lipid accumulation economically competitive in the form of SCO [167].

Every engineer is aware that scaling requires an organised, stage-gate strategy that outlines the full-scale process from beginning to end and applies rigorous project management approaches throughout. Therefore, a small diagram summarising the steps that should be followed in the industrial scale-up of this fermentation process has been developed (Fig. 5). The scaling process is not a closed process and should continuously be reviewed iteratively as the project progresses [168].

### 6.1. Feedstock costs

Yeasts need an organic carbon source to grow, representing one of the process's high operational costs, as indicated above. Before analysing the costs of the raw material used for yeast cultivation, it is helpful

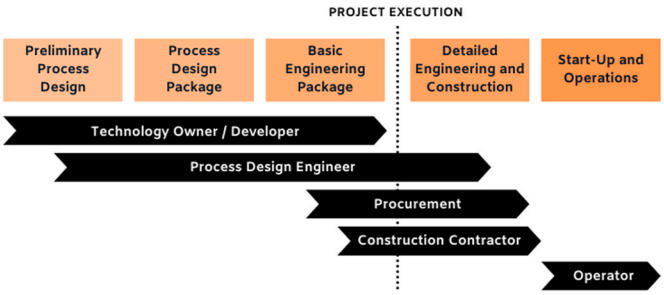


**Fig. 5.** A step-by-step approach to the process of industrial scale-up and construction of a yeast fermentation plant.

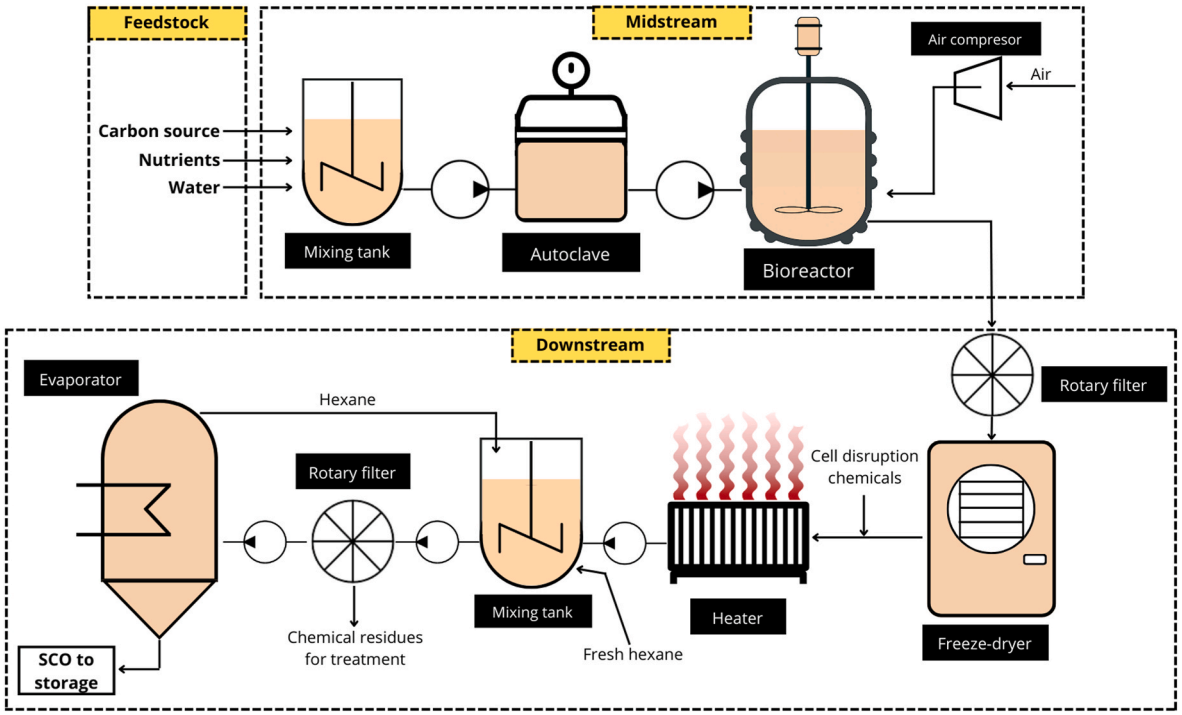


**Fig. 4.** Diagram of the SCO manufacturing process showing the three main cost-critical parts: feedstock, midstream and downstream.

to have a reference of the evolution of feedstock prices of first-generation biodiesel. This information is provided in Fig. 6. Besides, Table 5 shows the prices of different raw materials used as carbon sources by oleaginous yeasts, with some of their main advantages and drawbacks. Looking at the recent rise in the price of first-generation biodiesel, in Fig. 6, and comparing this with the prices of the raw materials used to obtain biodiesel from yeasts (Table 5), it can be deduced that the cost of the raw material is of high relevance. Authors such as Parsons and co-workers conclude in their studies on the techno-economic analysis of microbial oil production that the factor that most influences the process's overall economic performance is the price of the raw material and the lipid yield. However, the valorisation of co-products also has a significant effect [13].

According to the prices appearing in Table 5, volatile fatty acids are among the cheapest raw materials in terms of carbon substrates used by yeasts, with much lower prices compared to other options. Therefore, if a good yield in lipid production can be achieved from VFAs, this may represent the optimal cultivation option.

### 6.2. Midstream costs

The midstream incur costs and can be a problem in terms of industrial scaling. Around 70–90% of all equipment costs within the midstream costs derive from the fermenter [172]. According to Parsons and co-workers, these figures are significantly higher if we compare with anaerobic fermentation, and therefore such an increased capital investment may become somewhat prohibitive today [30]. It is essential to correctly calculate the working volume to be used, to avoid cost overruns. Some authors studied operational alternatives to reduce costs and make scaling up less challenging. For example, costs are considerably reduced if an open fermenter is used instead of a closed fermentation. Braunwald and colleagues estimated that this could result in 27% lower operational costs [173]. Also, a difference was reported in terms of optimal working volume between these two types of fermenters, being 80% in closed fermenters and 85% in open fermenters, with a 5% increase in production in open fermenters. While the main disadvantage of open culture systems is the potential of external contamination and the inability to regulate culture conditions fully, the risk can be reduced by constantly feeding fresh inoculum and operating at a high organic load. It is also worth highlighting that many yeasts often grow well under slightly acidic conditions, which may limit potential bacterial contamination, as the latter generally prefer near-neutral pH conditions. Besides, keeping the nutrient content as low as possible further limits the possibility of contamination. Because lipids accumulate in a nutrient-limited environment, lipid synthesis would not be affected [173].

When trying to determine the best operational conditions for given biofuel production strains, it is important to evaluate from the onset what large-scale production would entail and how large-scale circumstances will vary from those commonly seen in smaller laboratory bioreactors. First, full-scale bioreactors will have vessel mixing periods and gradients in temperature, hydrostatic pressure, nutrient concentrations, and dissolved oxygen concentration that are normally more insignificant at the laboratory scale [174]. In the end, it could be necessary to develop novel process designs to provide enough mixing in the bioreactor vessel such that, with good operation, the size and length of such gradients would stay within what the production strain can tolerate. However, achieving favourable process economics necessitates extreme cost minimisation, which almost certainly entails reducing energy inputs for agitation and air/oxygen compression, typically resulting in larger gradients within the bioreactor. These energy inputs are also necessary for mixing and aerating the culture. Therefore, an optimal selection of strains capable of maintaining high yields while minimising energy use is necessary. Simply said, strains that can maintain productivity in the face of higher concentration and temperature gradients inside the bioreactor throughout the production process will be favoured for large-scale production.

On the other side, if a high process yield is desired, operational parameters such as oxygen demand, good agitation and aeration, optimal growth temperature, and sterilisation, among others, must be optimised. Consequently, costs may also be incurred increase. Humbird and co-workers conducted a study on the costs of aeration in an aerobic reactor. Their analysis suggested that using a bubble-column reactor could reduce 10–20% aeration costs [175]. As a result, a trade-off between productivity, operational, and capital costs may be required within technological feasibility and investment potential.

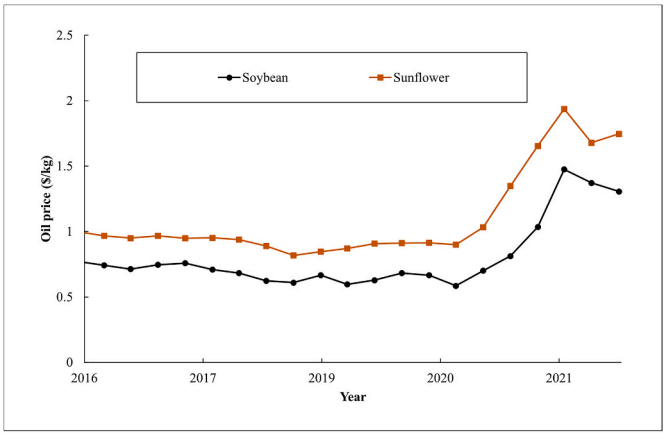


**Fig. 6.** Vegetable oil prices from 2016 to 2022 [169,].

**Table 5**
Recent estimated feedstock costs for yeast cultivation with some advantages and disadvantages.

| Feedstock | Price ($/kg) | Year | Advantages | Disadvantages | Ref. |
|---|---|---|---|---|---|
| Glucose | 0.5 | 2017 | High lipid yields | Price | [77] |
| Glucose | 0.4 | 2020 | | | [170] |
| Glycerol | 0.22 | 2021 | High yield, wide sources | Pre-treatment | [14] |
| Lignocellulose | 0.255 | 2021 | Low raw material costs | Pre-treatment | [14] |
| VFAs | 0.02–0.1 | 2021 | Price, diverse raw material sources, no complicated pre-treatment | Possible toxicity on growth | [171] |

### 6.3. Downstream costs

Lipid extraction and cell harvesting are critical steps in downstream processing after fermentation. In this regard, in a given study, the cost of oil recovery through solvent extraction has been calculated to represent roughly 13% of the entire cost of yeast lipid synthesis [14]. A cell disruption step is necessary to break the cell wall and allow lipid extraction. Commonly, a solvent is used for this purpose. This process could be integrated into a single step on an industrial scale. The solvent would be introduced, the mixture would be homogenised, and then, using a distillation column and a solvent recovery process, the solvent and lipids would be recovered [173]. Ultrasounds, bead grinding, acid or enzymatic hydrolysis and microwave are examples of other disruption techniques. On an industrial scale, hexane-based wet extraction has been modelled [176]. However, enzymatic treatment may be advantageous because it can save on operating costs with low energy consumption; this technique for oleaginous yeasts is still considerably limited [177]. Enzymatic pre-treatment with *L. starkeyi*, for example, is ineffective due to sulphide bonding in its cell wall [178]. Some emerging technologies for lipid extraction, such as supercritical fluid extraction

with $CO_2$, are regarded as effective, moderate, and "green" alternative approaches, but there are not yet enough techno-economic data to draw conclusions on profitability [177].

Based on the diagram shown in Fig. 4, the energy costs of each equipment in the downstream process have been analysed, taking as reference the studies carried out by Karamerou et al. [12] and Bonatsos et al. [170]. The costs, estimated for the production of 10,000 ton SCO/year, are shown in Table 6. An energy price of $0.06 per kWh has been used as a reference.

According to Bonatsos and co-workers, the energy needed in the downstream stage is significantly lower compared to that needed in the midstream stage, by approximately 1 kWh for every 25 kWh in the midstream [170].

As energy-saving methods, apart from the aforementioned solvent recovery, a hot air stream coming out of the autoclave in the sterilisation stage could be used and recirculated to the heater in the downstream stage to save on heating costs. The fermentation's output properties can also ease downstream processing, lowering total production costs. For instance, lipid secretion into the culture broth would aid subsequent lipid recovery by centrifugation and would save on the cost linked to the need to use an extracting solvent [179]. In addition, if not all the substrate has been consumed at the time of lipid extraction, returning the remaining supernatant from the centrifugation to the original medium would be another cost-saving method to be considered.

### 6.4. Life cycle analysis (LCA)

By identifying all energy and material fluxes that occur within the boundaries of a well-defined system, environmental life cycle assessment is a systematic way for assessing the environmental effect of a product, a process, or an activity. Only a few LCA have been published on the generation of oleaginous yeasts because of the dearth of industrial data on the procedure. The sustainability of the feedstock, emphasising waste resources rather than first-generation sugars, is one of the essential needs. Moreover, without a rigorous analysis of its environmental performance, the evaluation of a novel process for the manufacture of a chemical, energy, or fuel cannot be regarded as complete (impacts).

In recent LCA studies, the three steps shown in Fig. 4 have been considered, from the yeast feedstocks' pre-treatment processes to the product's final purification. Emissions resulting from administration, maintenance, and monitoring of their functioning are not included, nor are emissions produced during the building of manufacturing facilities and equipment. Acetic acid generated from poplar biomass utilising a bioconversion process was considered in an LCA research for the synthesis of yeast derived SCOs feedstock (carbon source) [180]. One ton of SCO produced from yeast is used as the functional unit. The LCA data referring to the bioproduction of acetic acid for use as substrate in the process were obtained from Budsberg et al. [180]. On the other side, the data used for the midstream and downstream stages were obtained from Bonatsos et al. [170].

A lipid yield of 0.17 g/g acetic acid, obtained from the study presented in Table 1, has been considered. Therefore, approximately 5.88 tons of acetic acid will be needed to produce 1 ton of SCO. As for yeast extract (nitrogen source) and water, it was calculated that 0.42 and 10 tons are, respectively needed, per ton of SCO [170]. The energy required to bio-produce 5.88 tons of acetic acid will be 24,500 kWh [180], and the process of bio-converting that acetic acid into lipids is 5193 kWh [170]. Therefore, the total energy required will be 29,693 kWh for each ton of SCO. In terms of atmospheric emissions, the balance proposed by Budsberg and colleagues to produce 5.88 tons of acetic acid is negative, at −2175 kg $CO_2$-eq [180], while the process to convert that acetic acid into lipids, 4080 kg $CO_2$-eq [170]. In total, 1905 kg $CO_2$-eq would be emitted for each ton of SCO. As for the hexane required, it was calculated that 2.8 kg were needed for each ton of SCO produced, considering the solvent's recirculation and recovery.

The minimal selling price of SCO was determined from a thorough techno-economic study on six different yearly capacities [170]. If we use the minimum selling price of acetic acid reported in the analysis of Budsberg and co-workers [180] ($0.677 per kg of acetic acid) and considering a yield of 0.17 g SCO/g acetic acid, the minimum selling price of the SCO is estimated to be between $5.5 and $7.3 per kilogram. This value is significantly more than the current prices of vegetable oils, which are around $1.5 per kilogram of vegetable oil (Fig. 6). This scenario changes if we use the VFAs prices presented in Table 5 ($0.02–0.1 per kg of acetic acid). When using these values, the minimum selling price of SCO is estimated to be between $1.2 and $4.4 per kilogram. These values are closer to the current selling price, however, there is still a lot of research to be done in order to lower this threshold and make such a process more profitable. According to the present LCA study, GHG emissions are in the range of 1.9 kg $CO_2$-eq/kg SCO and are not as high as those linked to the manufacturing of popular vegetable oils (3–5 kg $CO_2$-eq/kg veg. oil).

**Table 6**
Summary of electricity and costs estimation for SCO production (10 kt SCO/year).

| Equipment | Electricity (kWh/year) | Cost ($/year) |
|---|---|---|
| Rotary filter | 91,480 | 5,489 |
| Freeze-dryer | 78,400 | 4,704 |
| Heater | – | – |
| Mixing tank | 130,000 | 7,800 |
| Evaporator | – | – |

## 7. Conclusions and future perspectives

SCOs have been proven to have similar lipid profiles to other vegetable oils currently in use, such as palm, canola, or soybean oils. This provides SCOs identical properties as vegetable oils for biodiesel manufacturing. However, despite the academic and technological advances in this area, in recent years, SCOs still represent a relatively new alternative for biodiesel production, currently with some gaps for its industrial implementation. For instance, the total costs of setting up a biofuel plant from microorganisms are still quite high considering the relatively low lipid yields presently reachable. The use of waste-based feedstocks, such as volatile fatty acids, would bring the costs down a considerable amount. Still, the toxicity for growth at high acids concentrations would be a limitation to overcome.

In terms of legislation and accordance with the 2030 agenda, the European Union is committed to third-generation biofuel in regulation 2009/28/EC, which establishes a target of 3.5% of total transport renewable energy use by 2030 (1% by 2025). It plans to phase out the use of other biofuels of plant and food origin to reduce $CO_2$ emissions, among others. This opens the door to possible increases in financial support for both research and implementation of existing technologies in the short term. Together with a clear demonstration of environmental benefits and solid political support, such financial support could boost the roadmap for integrating SCOs in biofuels production processes.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

No data was used for the research described in the article.

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