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REVIEW

Oleaginous fungi and their prospects in bioenergy

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Population expansion and industrialization is leading to increased energy demand all over the globe. In the current scenario, due to the overexploitation of fossil fuels there has been an increasing demand for inexhaustible and sustainable energy sources. Apparently, microbial lipids, especially those obtained from oil-producing fungi have gained significant interest over the years. Oleaginous fungi with more than 20% intracellular lipids of their dry cell weight are feasible substitute to animal and plant-based lipids for the production of biofuel. This review explores the overall biological features of oleaginous fungi, their advantages, lipid composition, extraction methods of fungal lipids, quality parameters related to biodiesel and the various challenges that may arise during the production of biofuel from oleaginous fungi. Moreover, the review deliberates the possibilities of applying agricultural and industrial wastes as the low-cost substrates relevant to fungal growth, contributing to bio-based circular economy. Comparative analysis of the various advantages and limitations of the production of fungal lipid over other microbial and traditional oil sources are also discussed. Prospective of oleaginous fungi in the field of bioenergy, thus demonstrating its suitability as the next-generation biofuel producer has also been discussed.

Keywords : Oleaginous fungi, biodiesel, lipid extraction, transesterification, biodiesel quality

INTRODUCTION

Global energy demand is increasing due to population growth and industrial expansion. Fossil fuels, historically the primary source of energy for activities ranging from household tasks to industrial processes, are facing a crisis due to overexploitation and environmental impact (Nda-Umar *et al.* 2018). The transport sector, heavily reliant on petrol and diesel, contributes significantly to this issue. The increasing global population and energy demand further threaten fossil fuel availability (Raman 2019), while their overuse leads to environmental pollution, including toxic emissions such as carbon monoxide, harming ecosystems (Nda-Umar *et al.* 2018).

In response, bioenergy offers a promising alternative. Derived from biological feedstocks like plant oils, animal waste, and microorganisms, biofuels are renewable and eco-friendly (Nda-Umar *et al*. 2018). However, large-scale biofuel production from plant oils raises concerns due to competition with food production (Christophe *et al*. 2012). Microbial biofuels, including those from microalgae, bacteria, and fungi, are being researched as more sustainable options (Liang *et al*. 2013). Biodiesel production from fungi, though still in early stages, holds potential for future bioenergy systems.The present review describes about the oleaginous fungi and its advantages, lipid composition in oleaginous fungi, lipid production using various carbon sources, efficiency of different extraction methods and recovery, challenges in production and its probable solutions to enhance productivity.

Biofuels as an alternative to fossil fuels

Fossil fuels, including coal, petroleum, and natural gas, take millions of years to form but are consumed at an unsustainable rate, leading to scarcity and rising prices. The demand for fossil fuels is growing rapidly, especially in highly

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populated regions like Asia. For example, India's energy demand is expected to increase significantly, with projections showing it becoming the largest global driver of oil demand by 2030 (International Energy Agency 2021).

Fossil fuel consumption not only contributes to resource depletion but also causes severe environmental pollution, including air, water, and soil contamination, exacerbating global warming and impacting public health (Nda-Umar *et al.* 2018). This has led to calls for alternative energy sources, with biofuels emerging as a key candidate.

Biofuels, which can be solid, liquid, or gaseous, are derived from biomaterials such as food and non-food crops, agricultural residues, animal waste, and microorganisms (Nda-Umar *et al.* 2018). They are a cleaner and renewable energy source compared to fossil fuels. Biofuels also contribute to energy security by reducing dependence on imported oil, stimulating local economies, and creating jobs (Clemente 2015; Francisco and Martin 2021). Biodiesel, for example, can replace petroleum diesel without requiring engine modifications, making it an ideal candidate for global transportation energy needs (Raman 2019).

In India, the National Policy on Biofuels (2018) aims to increase ethanol blending in petrol to 20% by 2025, with a target of 5% biodiesel blending by 2030. The adoption of biofuels is also expected to create significant economic activity, as seen in the US, where the biodiesel industry supports tens of thousands of jobs (Biofuels International 2021).

Biofuels can be classified into conventional (first-generation) and advanced (second- and third-generation) biofuels. First-generation biofuels, made from food crops, face sustainability issues due to competition for land with food production. Second-generation biofuels, made from agricultural residues and non-food crops, are less controversial but still require land. Third-generation biofuels, derived from microorganisms such as fungi, do not face these limitations, making them the most promising candidates for future bioenergy production (Liang *et al.* 2013; Thevenieau *et al.* 2013).

Oleaginous microorganisms

Currently, most biodiesel is produced from plant oils, but large-scale biodiesel production from these feedstocks requires extensive energy and land use. Microbial sources offer several advantages for lipid production over conventional feedstocks (Gujjala *et al.* 2019):

- ❖ No food-versus-fuel controversy.
- ❖ Shorter growth periods compared to plant and animal sources.
- ❖ Lipid production in microbes is independent of climatic, seasonal, and geographic variations.
- ❖ Microbial lipids have similar composition and calorific value to plant and animal oils.
- ❖ Inexpensive agro-industrial and municipal wastes can be converted into microbial oil without compromising lipid quality (Cho and Park, 2018).

Oleaginous microorganisms, capable of accumulating 20% to 90% of their dry weight as lipids, include microalgae, fungi, and some bacteria (Zhu *et al.* 2020). Early research on lipid production from microorganisms dates back to World War I, with strains of *Endomyces* and *Fusarium* used to address cooking oil shortages (Liang *et al.* 2013). Since then, numerous oleaginous microorganisms, including *Rhodotorula glutinis*, *Lipomyces starkeyi*, *Mucor* sp. and *Aspergillus* sp. have been identified (Liang *et al.* 2013).

Microalgae are particularly promising for biodiesel production, with lipid contents ranging from 1% to 70% and reaching up to 90% under specific conditions. Examples include *Chlorella vulgaris*, *Haematococcus pluvialis*, and *Nannochloropsis* sp.(Liang *et al.* 2013; Thevenieau *et al.* 2013). Oleaginous bacteria, such as *Rhodococcus opacus*, *Arthrobacter* sp. and *Acinetobacter calcoaceticus* (Dong *et al.* 2016), can also accumulate lipids, although their oil production is generally lower compared to microalgae.

However, microalgae cultivation requires large areas and long fermentation times, whether autotrophically or heterotrophically, and the use of organic carbon sources limits their potential for biodiesel production (Tsouko *et al.* 2016; Liang *et al.* 2013). Genetic and metabolic engineering are being used to improve these microorganisms for biodiesel production, but oleaginous fungi are emerging as another promising alternative.

Oleaginous fungi

Several species of fungi are reported to accumulate lipids (Table 1), and those accumulate 20% or more intracellular lipids of their dry biomass are called as oleaginous (Bautista *et al.* 2012). Filamentous fungi and yeasts, mainly from the *Mucoromycota* and *Ascomycota* phyla, are known for high-quality oil production (Mhlongo *et al.* 2021). Some fungi, including endophytic species, produce hydrocarbons such as mono-terpenoids and alkanes that are closely related to diesel molecules (Bardi 2018).

Although around 60,000 fungi are identified, only 50 are known to produce intracellular lipids (Meullemiestre *et al.* 2015). Lipid accumulation varies between species and strains, with *Mortierella* sp. exceeding 86% of its dry weight in lipids, while *Trichoderma reesei* and *Mucor circinelloides* accumulate 9.82% and 22.11%, respectively (Zhu *et al.* 2020; Sindhu *et al.* 2019). *Penicillium citrinum* from Assam, India, has been reported to produce60.61±2.08% of lipids (Bardhan *et al.* 2019a). Lipid accumulation depends on factors such as substrate, cultivation method, and the fungus's genetic makeup (Nigam *et al.* 2014).

Oleaginous fungi can utilize various carbon sources, including glucose, lactose, xylose, starch, cellulose, and hydrolysates, making them highly versatile for lipid production (Thevenieau *et al.* 2013). This capability allows for the use of low-cost carbon sources, such as agricultural residues, oil wastes, and municipal organic wastes, reducing production costs and promoting environmental cleanup (Zhu *et al.* 2020). Moreover, the oil produced from fungi has similar characteristics to conventional vegetable oils, making it suitable for biodiesel production (Mhlongo *et al.* 2021).

Other key factors influencing lipid production include pH, temperature, and the carbon-to-nitrogen ratio. The optimal pH for lipid accumulation is between 5.5 and 9, with temperatures ranging from 25°C to 37°C (Zhang *et al.* 2021). Batch and fed-batch cultures are commonly used for cultivating oleaginous fungi (Ayadi *et al.* 2016).Genetic and metabolic engineering techniques, such as RNA interference, electroporation, and CRISPR/Cas9, have been applied to enhance lipid production in fungi (Mhlongo *et al.* 2021). *Mucor circinelloides*, for example, was one of the first fungi genetically engineered for large-scale microbial lipid commercialization (Bautista *et al.* 2012).

Several genes associated with fatty acid and lipid metabolism have been identified in fungi. The gene *ACC1* encodes the enzyme acetyl-CoA carboxylase (ACC), which catalyzes the conversion of acetyl-CoA to malonyl-CoA – a critical, rate-limiting step in fatty acid (FA) biosynthesis (Runguphan and Keasling 2014). *ACC1* has been overexpressed in various fungal strains to significantly enhance lipid accumulation. A study by Yan *et al.* (2020) reported a 56.3% increase in lipid content in *Yarrowia lipolytica* through the overexpression of *ACC1* and deletion of the multifunctional enzyme gene *MFE1*. Diacylglycerol acyltransferase (DGAT) catalyzes triacylglycerol (TAG) synthesis from diacylglycerol (DAG) and fatty acyl-CoA. The genes *DGA1* and *DGA2* are key regulators of TAG biosynthesis and facilitate its transport into lipid droplets (Qiao *et al.* 2015; Gajdos *et al.* 2015; Friedlander *et al.* 2016). Overexpression of *DGA1* in yeast under a constitutive (housekeeping) promoter led to a 1.5-fold increase in lipid content (Runguphan and Keasling 2014). Malic enzyme (ME) genes encode oxidative decarboxylases that convert malate into pyruvate, generating NADPH/NADH in the process (Chang and Tong 2003; Vongsangnak *et al.* 2012). In *Mucor circinelloides*, overexpression of the *ME* gene resulted in a 2.5-fold increase in lipid content (Zhang *et al.* 2007). Fatty acid synthase (FAS) is responsible for de novo fatty acid biosynthesis by incorporating acetyl and malonyl groups for acyl-CoA elongation. In *Saccharomyces cerevisiae*, FAS comprises two non-identical subunits encoded by the *FAS1* and *FAS2* genes. Overexpression of both genes led to a 30% increase in lipid content (Runguphan and Keasling 2014).

Peroxisomal acyl-CoA oxidases, encoded by *POX1–6*, mediate fatty acid degradation via the β-oxidation pathway by targeting fatty acids of varying chain lengths (Soong *et al.* 2023). Deletion of *POX1–6* in *Y. lipolytica* blocked fatty acid degradation, thereby enhancing lipid accumulation (Dulermo and Nicaud 2011). Similarly, deletion of *POX1* in *S. cerevisiae* inhibited â-oxidation and resulted in a fourfold increase in fatty acid content (Valle-Rodríguez *et al*. 2014). The *MFE1* gene encodes a multifunctional enzyme that catalyzes the second step of the β-oxidation pathway (Dulermo and Nicaud, 2011). Deletion of *MFE1* in *Y. lipolytica* has been shown to reduce fatty acid degradation and promote lipid accumulation (Blazeck *et al*. 2014). Deletion of *MFE2*, another key gene in the β-oxidation pathway, also resulted in increased linoleic acid content due to enhanced lipid accumulation (Blazeck *et al*. 2013). *TGL* genes encode triacylglycerol lipases that hydrolyze TAGs into fatty acids and glycerol. Deletion of these genes reduces TAG breakdown, resulting in increased size and number of lipid droplets (LDs). In *S. cerevisiae*, deletion of *TGL3* and *TGL4* led to larger and more abundant lipid droplets (Kohlwein *et al*. 2013).

Advantages of oleaginous fungi over other microbes

Oleaginous fungi offer several advantages over other biological feedstocks for biodiesel production:

- ❖ High lipid yield (up to 90% of dry biomass), e.g.,*Umbelopsis isabellina* producing over 80% lipids (Zhu *et al*. 2020).
- ❖ Unique fatty acid profiles, rich in poly unsaturated fatty acids like ã-linoleic acid, which are not easily synthesized by other microorganisms (Youssef *et al*. 2021).
- ❖ Lipid profiles suitable for biodiesel production, including saponifiable lipids and free fatty acids, as seen in *Mucorcircinelloides* (Zhu *et al*. 2020).
- ❖ Sustainable biodiesel production, as the oils from fungi resemble those of vegetable oils (Mhlongo *et al*. 2021).
- ❖ Easy pellet formation in submerged fermentation, which reduces viscosity and simplifies harvesting (Zhu *et al*. 2020).
- ❖ The ability to utilize low-cost carbon. sources such as agricultural residues, glycerol, and food industry waste (Zhu *et al*. 2020).
- ❖ Oleaginous fungi can metabolize complex, toxic compounds from low-cost carbon sources, reducing the need foradditional enzyme digestion and hydrolysis (Bautista *et al*. 2012).
- ❖ Fungi grow independently of light, seasons, and space availability, with shorter doubling times, making them more suitable for bioreactor cultivation compared to microalgae (Bardhan *et al*. 2019b).
- ❖ Direct transesterification of fungal biomass into biodiesel eliminates the costly oil extraction step (Bardi 2018).

Lipid composition of oleaginous fungi

Lipids are high-energy compounds (39 kJ/g) that function as energy storage molecules in oleaginous fungi, primarily in the form of triacylglycerol (TAG) and sterol esters. These lipids are stored in lipid bodies within the cell and provide fatty acids and sterols for membrane biogenesis and maintenance. The lipid composition and fatty acid profiles of fungi vary depending on the cultivation process and substrates used (Thevenieau *et al*. 2013). Lipids in oleaginous fungi are typically classified into two main categories:

Energy Storage Lipids

These include neutral lipids such as mono-, di-, and triglycerides (TAGs), and free fatty acids, which are primarily esters of fatty acids and glycerol (Bautista *et al*. 2012).

Structural Lipids

Polar lipids, such as phospholipids (e.g., phosphatidic acid, phosphatidylcholine, phosphatidylserine), glycolipids, and sphingolipids, as well as sterols like sitosterol, ergosterol, cholesterol, lanosterol, and campesterol (Meullemiestre *et al*. 2015; Bautista *et al.* 2012).

Fungi have been extensively studied for oil production based on lipids extracted from them, which primarily contain TAGs, the same as in plant oils. When selecting fungal strains for biodiesel production, the degree of branching, unsaturation, and chain length of fatty acids should be considered (Sitepu *et al.* 2014). The fatty acid profile of microbial lipids is species-dependent (Xu *et al.* 2012). Oleaginous fungi typically produce a range of fatty acids, including palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1), linoleic (C18:2), and linolenic (C18:3) acids (Table 2).

For efficient biodiesel production, ideal lipids should include monounsaturated fatty acids (MUFAs), such as palmitoleic (C16:1) and oleic (C18:1) acids, controlled amounts of saturated fatty acids (SFAs) like palmitic (C16:0) and stearic (C18:0), and minimal polyunsaturated fatty acids (PUFAs) such as linoleic (C18:2) and γ-linolenic (C18:3) acids. PUFAs with more than four double bonds are unsuitable for high-quality biodiesel (Yousuf *et al*. 2018).

The fatty acid profile significantly influences biodiesel properties, including cetane number, melting point, heating value, kinematic viscosity, and oxidative stability (Mhlongo *et al*. 2021). Many oleaginous fungi produce the desirable lipids needed for biodiesel, such as *Yarrowia lipolytica*, which contains stearic, oleic, linoleic, and palmitic acids in its single-cell oils (Tsouko *et al*. 2016). Hereditary factors and culture conditions, including agitation, temperature, pH, light, and nutrient availability, play an important role in lipid content (Zhu *et al*. 2020).

Screening methods, such as gas chromatography-mass spectrometry (GC-MS) and gas chromatography-flame ionization detection (GC-FID), are commonly used for evaluating fungal lipid production (Rumin *et al.* 2015). Effective screening for high lipid productivity is essential to optimize the biodiesel production process (Mhlongo *et al*. 2021).

Lipid production from various carbon sources via oleaginous fungi

The economic feasibility of biodiesel production depends significantly on the cost of feedstocks used in the upstream process. Oleaginous fungi can utilize a variety of carbon sources, particularly low-cost substrates (Fig. 1). The most common feedstocks include saccharides, hydrolysates, and glycerol, accounting for around 71% of the feedstocks used. Other substrates include fatty acids, molasses, oils/fats, alcohols, aromatics, aqueous extracts, and waste streams (Abeln *et al*. 2021). The choice of carbon source, particularly whether sugar- or fat-based, significantly impacts lipid synthesis (Arous *et al.* 2017).

For instance, the filamentous fungus *U. isabellina* produced over 60% lipids when cultivated with monosaccharides (xylose, fructose, glucose) and carboxymethyl-cellulose (CMC), compared to 17% and 41% when grown on disaccharides like sucrose and cellobiose (Zhu and Bonito 2020). Starch, a common polysaccharide, serves as a carbon source, with some oleaginous yeasts exhibiting amylolytic activity (Chaturvedi *et al*. 2019). Glycerol, a by-product of biodiesel production, can also serve as a carbon source for oleaginous fungi. For example, the red yeast *Rhodotorula glutinis* TISTR 5159 accumulated lipids and carotenoids when grown on crude glycerol (Babu and Sarma 2019).

Lignocellulosic biomass, including wood, grass, forestry waste, and agricultural residues, is an ideal feedstock for microbial lipid and biodiesel production due to its abundance, renewability, and low cost (Zhu *et al*. 2020). Lignocellulosic polymers can be pretreated and hydrolyzed into fermentable sugars by lignocellulolytic enzymes like cellulases and hemicellulases (Dyk and Pletschke 2012; Kitcha and Cheirsilp 2014). Several oleaginous fungi, such as *Colletotrichum* sp. and *Alternaria* sp., have been shown to effectively utilize lignocellulosic biomass for lipid production (Dey *et al*. 2011).

Oleaginous fungi can also grow on starchy waste substrates without requiring external amylases, as they produce amylase and accumulate lipids simultaneously (Kitcha and Cheirsilp 2014). *Candida viswanathii* Y-E4 has been evaluated for its ability to use various agro-industrial byproducts for oil production (Ayadi *et al*. 2016), showcasing the potential of bioconversion processes to reduce enzyme digestion and hydrolysis costs.

Plant oils like rapeseed, sesame, and linseed oils can enhance biomass and lipid accumulation in oleaginous fungi. For example, *Mortierella rouxii* and *Mortierella* sp. exhibited higher lipid accumulation and biomass when grown on glucose supplemented with sesame oil (Somashekar *et al*. 2003). Additionally, supplem-entation with rapeseed oil resulted in increased levels of polyuns-aturated fatty acids (PUFAs), including n-3 fatty acids (Grygier *et al.* 2020).

Oleaginous fungi have a clear advantage over other microbes by utilizing low-cost substrates like molasses, sugarcane juice, potato wastewater, orange peel extracts, and various hydrolysates. Fungi such as *Yarrowia*, *Cryptococcus*, *Candida*, *Rhodotorula*, *Lipomyces*, and *Trichosporon* have been researched for their feedstock versatility (Nunes *et al*. 2024). For example, sweetwater has been demo-nstrated as a cost-effective feedstock for lipid accumulation and fatty acid uptake in *Rhodotorula toruloides* NRRL Y-6987 (Keita *et al*. 2024).

Other fungi, such as *Cunninghamella echinulata*, can grow on a wide variety of low-cost organic sources like molasses, tomato waste, olive oil mill wastewater, and whey, producing high lipid yields (Mirbagheri *et al*. 2015). *Aspergillus oryzae* has been shown to utilize potato wastewater for lipid production, with yields up to 3.5 g/L/day (Muniraj *et al*. 2013).

Extraction of fungal lipids

Lipid extraction is a crucial step in the downstream processing of fungal lipids, aiming to efficiently separate lipids from other cellular components such as proteins, polysaccharides, and small molecules. Efficient extraction requires knowledge of the cell wall characteristics, lipid content, and the desired location of the lipids within the fungal cell. Additionally, preserving the extracted lipids against oxidation and enzymatic degradation is essential. The choice of extraction methods and solvents should align with the intended application of the lipids (Ochsenreither *et al*. 2016).

Lipid extraction can occur through two main paths: dry and wet routes (Patel *et al.* 2020). The dry route, though favored for its higher lipid yields, requires substantial energy for dewatering, making it more expensive. In contrast, the wet route is more energy-efficient and cost-effective. The choice of solvents depends on lipid polarity. Triacylglycerols (TAGs) are extracted using non-polar solvents like chloroform, while polar lipids, such as phospholipids, require polar solvents like alcohols. Mixtures of polar and non-polar solvents can enhance extraction efficiency by better releasing lipids from protein-lipid complexes (Ochsenreither *et al*. 2016).

Due to the robust fungal cell wall, a pre-treatment or cell disruption step is needed to improve lipid extraction (Fig. 1). This step facilitates extraction from wet biomass and is often combined with conventional extraction methods to enhance efficiency. Pre-treatment methods, including physical, chemical, and biological approaches, should be evaluated based on energy consumption, operational complexity, envir-onmental impact, and cost-effectiveness (Dong *et al.* 2016). Pressurized CO_2 treatment has shown promise in reducing energy consumption while enhancing lipid extraction efficiency (Howlader *et al*. 2018).

The choice of cell disruption method depends on the fungal species, as cell wall structures and lipid compositions vary. Currently, no universal method exists, so extraction strategies must be tailored to each species (Zainuddin *et al.* 2021).

Conventional lipid extraction methods
Soxhlet Extraction Method

Invented in 1879 by Franz Soxhlet, the Soxhlet method is commonly used for lipid extraction from dried biomass. Solvents like hexane, chloroform, or methanol are used to extract lipids through repeated cycles of evaporation, condensation, and filtration. Despite its high lipid yield, this method is energy-intensive and time-consuming (typically 8 hours) and involves substantial solvent usage (Zainuddin *et al*. 2021).

Bligh and Dyer extraction Method

The Bligh and Dyer method is a classical, widely cited approach for lipid extraction from oleaginous fungi. It uses a ternary solvent system (chloroform:methanol:water in a 1:2:0.25 ratio) to create a biphasic mixture. The lower phase contains lipids, while the upper phase contains non-lipid components. Some variations use NaCl to prevent lipid binding to proteins (Zainuddin *et al*. 2021)

Folch extraction Method

Similar to the Bligh and Dyer method, the Folch method uses a 2:1 chloroform:methanol ratio. After extraction, the mixture is washed with salt solution to separate the lipids from the solvents. This method is efficient at the laboratory scale but less sensitive than Bligh and Dyer (Zainuddin *et al*. 2021).

The Bligh and Dyer method is generally preferred for its higher lipid purity and ability to separate proteins from lipids, making it ideal for large-scale extractions (Patel *et al.* 2020). The Folch method, while quicker, has lower sensitivity, and the Soxhlet method, though effective, is energy and time-intensive.Conventional solvents like chloroform and methanol are toxic and pose environmental risks. To address this, less toxic and more eco-friendly solvents like toluene and isopropanol have been explored for lipid extraction (Vasconcelos *et al.* 2018). These alternatives reduce health and environmental risks while maintaining extraction efficiency.

Innovative Lipid Extraction Methods

Several novel methods are emerging to improve lipid extraction efficiency:

1.Supercritical Fluid Extraction (SFE)

Supercritical CO_2 is a green and efficient solvent for lipid extraction. It avoids contamination and thermal degradation, making it an ideal method for fungi like *Candida* (Zainuddin *et al*. 2021).

2. Pressurized Liquid Extraction (PLE)

PLE uses elevated pressure and temperature to extract lipids quickly. It is effective for microbes with high lipid content, such as *Cryptococcus curvatus* (Zainuddin *et al*. 2021).

3.High-Temperature Assisted Pressure Solvent Extraction

This method uses a solvent mixture (hexane and isopropanol) under pressure to reduce extraction time and increase efficiency (Andrich *et al*. 2006).

4.Ultrasound-Assisted Extraction

Ultrasonic waves create cavitation, which disrupts microbial cells and enhances lipid release, reducing both extraction time and solvent volume (Teixeira, 2012).

5.Nanoparticle-Assisted Extraction

Nanoparticles, due to their small size and high surface area, can penetrate cell walls and enhance lipid recovery, although this method is still in its early stages of development (Mhlongo *et al*. 2021).

Trans-esterification

Several techniques are employed for biodiesel production from lipids or oils, including microemulsion, pyrolysis, and trans-esterification. Due to issues like high viscosity, free fatty acid content, and oxidation during storage, direct use of vegetable oils in diesel engines is not recommended (Khot *et al.* 2018).

Transesterification is the most widely adopted method for biodiesel production due to its cost-effectiveness and high conversion efficiency (Lin *et al*. 2011). During this process, triacylglycerols (TAGs) are converted into fatty acid methyl esters (FAME) or fatty acid alkyl esters using methanol or ethanol, with glycerol as a by-product. Catalysts such as acids, alkalis, or enzymes are employed in transesterification (Patel *et al.* 2020). For instance, 93% of the lipids from *Mucor circinelloides* were transesterified into fatty acid ethyl esters (FAEEs) using immobilized lipase from *Candida antarctica* (Carvalho *et al*. 2015).

While enzyme catalysts are efficient and safe, they are costly and require specific conditions. In

Table 1: Lipid content of some oleaginous fungi

Oleaginous fungi	Lipid content (% w/w)	References
Alternaria sp.	55.10	Zhu *et al*. 2020
Apiotrichum porosum	14.1–20.0	Zainuddin *et al*. 2021
Aspergillus caespitosus	37.27	Srinivasan *et al*. 2021
Aspergillus carneus	36.20	Ibrahim *et al*. 2023
Aspergillus niger	41–57	Zhu *et al*. 2020
Aspergillus oryzae	18.15–40	Zhu *et al*. 2020
Aspergillus terreus	41.83	Al-Zaban and El-Aziz 2023
Brevistachys sp.	21.07	Rizki and Ilmi 2020
Candida guilliermondii	30	Nigam *et al*. 2014
Candida intermedia	20	Nigam *et al*. 2014
Candida tropicalis	46.8	Bautista *et al*. 2012
Candida viswanathii	50	Ayedi *et al*. 2016
Cerrena sp.	21.77	Rizki and Ilmi 2020
Clavispora lusitaniae	37	Moran-Mejia *et al*. 2022
Colletotrichum sp.	46.08	Zhu *et al*. 2020
Conidiobolus nanodes	30	Bardi, 2018
Cryptococcus curvatus	70.1–82.7	Zhang *et al*. 2011
Cryptococcus flavescens	41.4	Qvirist *et al*. 2022
Cryptococcus terricolous	55–65	Nigam *et al*. 2014
Cunninghamella echinulata	19.03–57.5	Liang *et al*. 2013
Cutaneotrichosporon curvatum	29.4–33.9	Llamas *et al*. 2020
Cutaneotrichosporon oleaginosus	65	Shaigani *et al*. 2021
Cyberlindnera saturnus	29.2–36.9	Llamas *et al*. 2020
Cystobasidium iriomotense	33	Tanimura *et al*. 2018
Cystobasidium oligophagum	36.46	Bardhan *et al*. 2019b
Fusarium verticillioides	27.27	Wadee *et al*. 2024
Fusarium verticillioides	38	Kamoun *et al*. 2018
Galactomyces geotrichum	32.9	Altun *et al*. 2020
Hannaella higashiohmiensis	24.37	Tanimura *et al*. 2023
Hannaella oleicumulans	36.47–37.72	Tanimura *et al*. 2023
Hansenula anomala	17	Nigam *et al*. 2014
Hansenula ciferii	22	Nigam *et al*. 2014
Hansenula saturnus	20	Nigam *et al*. 2014
Lasiodiplodia exigua	20.2	Paul *et al*. 2020
Lipomyces starkeyi	10–61	Zain *et al*. 2024; Liang *et al*. 2013
Meyerozyma guilliermondii	22.4	Valdes *et al*. 2020
Meyerozyma guilliermondii	34.97	Bardhan *et al*. 2019b
Mortierella alpina	35	Mehni *et al*. 2022
Mortierella isabellina	50–65.5	Liang *et al*. 2013
Mucor circinelloides	22.11	Sindhu *et al*. 2019
Mucor fragilis	46.80	Bardhan *et al*. 2019b
Mucor irregularis	43.46	Haura and Llmi 2024
Mucor racemosus	32.4	Hashem *et al*. 2023
Nigrospora sp.	21.32	Tonato *et al*. 2023

Pellicularia practicola	39	Bardi 2018
Penicillium brevicompactum	57.6	Bardhan *et al*. 2019b
Penicillium brevicompactum	57.6	Ali *et al.* 2017
Penicillium citrinum	60.61	Bardhan *et al*. 2019a
Pestalotiopsis microspora	26.07	Paul *et al.* 2020
Phomopsis sp.	21.1	Paul *et al.* 2020
Pichia guilliermondii	12–40	Zainuddin *et al*. 2021
Pseudozyma hubeiensis	46.8	Qvirist *et al.* 2022
Pythium ultimum	48	Bardi 2018
Rhizopus arrhizus	25.64–57	Wadee *et al*. 2024; Bardi 2018
Rhodosporidiobolus fluvialis	29.2–75	Nakata *et al.* 2020; Bardhan *et al*. 2019b
Rhodosporidium diobovatum	40	Nasirian *et al.* 2018
Rhodosporidium toruloides	29.7–67.5	Liang *et al*. 2013; Naveira-Pazos *et al*. 2024
Rhodosporidium toruloides	43.2	Diamantis *et al*. 2023
Rhodotorula glutinis	35	Qvirist *et al.* 2022
Rhodotorula gracilis	32-67	Nigam *et al*. 2014
Rhodotorula graminis	21.26	Bautista *et al*. 2012
Rhodotorula longissima	20	Bardhan *et al*. 2019b
Rhodotorula minuta	34	Qvirist *et al.* 2022
Rhodotorula mucilaginosa	69.5	Bautista *et al*. 2012
Rhodotorula paludigena	23.9	Gosalawit *et al*. 2021
Saccharomyces cerevisiae	52.96	Watsuntorn *et al.* 2021
Saitozyma podzolica	20.7–43.4	Zainuddin *et al*. 2021
Scheffersomyces coipomensis	24.3	Valdes *et al*. 2020
Sporidiobolus salmonicolor	26.8	Qvirist *et al.* 2022
Syncephalastrum racemosum	25.18	Hashem *et al*. 2020
Thamnidium elegans	71	Tsouko *et al.* 2016
Trichoderma harzianum	20.5	Liang *et al*. 2013
Trichoderma reesei	9.82	Sindhu *et al*. 2019
Trichosporon asahii	31	Shaigani *et al*. 2021
Trichosporon cutaneum	49.1	Liu *et al*. 2022
Trichosporon dermatis	41.50	Zhu *et al*. 2020
Trichosporon fermentans	62.4	Liang *et al*. 2013
Trichosporon mycotoxinivorans	32.61–44.86	Sagia *et al*. 2020
Umbelopsis ramanniana	37.10	Zhu *et al*. 2020
Waltomyces lipofer	64	Bardi 2018
Yarrowia lipolytica	43	Liang *et al*. 2013
Zygorhynchus moelleri	42.40	Zhu *et al*. 2020

contrast, acid and alkali catalysts offer high efficiency but are challenging to remove from crude biodiesel. Recent studies have explored cost-effective alternatives like whole-cell biocatalysts (Almyasheva *et al*. 2018) and biochar composites (Jung *et al*. 2019).

There are two main methods of transesterification:

Indirect Method

This two-stage process involves extracting oil from the fungal cells before transesterifying the oil into FAME (Tsouko et al. 2016).

Direct Method (*In situ* transesterification)

In this method, cell disruption, lipid extraction, and transesterification occur simultaneously, reducing costs and energy consumption. It has been used successfully with fungi like *Lipomyces starkeyi* and *Rhodosporidium toruloides* (Patel *et al.* 2020). Enhancement strategies, including ultrasound and microwave assistance, and supercritical solvents, are being investigated to improve reaction rates (Guldhe *et al.* 2014; Pascoal *et al.* 2020; Son *et al.* 2018).

Energy efficiency of biodiesel

Energy efficiency refers to using less energy to achieve a similar function. The commercialization of biodiesel worldwide was facilitated by the development of biodiesel standards. In the U.S., ASTM D6751 was established for biodiesel in 2002, followed by the EN14214 standard in Europe in 2003. Other countries have developed their own standards based on ASTM and EN14214 (Khot *et al.* 2018). Biodiesel is typically used as a blend with petroleum diesel. B100 refers to pure biodiesel, while blends are denoted by the percentage of biodiesel (NREL 2009).

Key quality parameters for biodiesel include cetane number (CN), density, kinematic viscosity (KV), flash point, acid number, iodine value (IV), ester content, water and sediment, copper corrosion, distillation range, total sulfur, and oxidative stability (NREL 2009). The total lipid content and fatty acid profile are critical factors in determining the suitability of an oleaginous fungus for biodiesel production (Subramaniam *et al.* 2010). Fatty acid composition affects biodiesel properties such as cold flow, ignition quality, oxidative stability, and iodine value. Biodiesel with a higher content of saturated fatty esters typically exhibits higher CN and better oxidative stability, but may have poorer cold flow properties (Knothe, 2005).

Moser and Vaughn (2012) suggested that saturated FAMEs should be below 26% for efficient cold flow properties. Unsaturated fatty acids, especially monounsaturated ones like palmitoleic (C16:1) and oleic (C18:1), are desirable as they improve fuel properties such as cetane number and ignition quality (Ramos *et al.* 2009).

Cetane number (CN) is an important specification for biodiesel. The CN value for B100 is typically 47 (ASTM D6751) or 51 (EN14214). The CN values of lipids produced by various filamentous fungi, including *Aspergillus terreus*, *Cunninghamella elegans*, *Mortierella isabellina*, *Mortierella vinacea*, *Rhizopus oryzae*, and *Thermomyces lanuginosus*, range from 54.8 to 61.6, meeting EN14214 biodiesel standards (Zhang *et al.* 2012).

Engine fuel viscosity is crucial for fuel atomization, mixture formation, and combustion. Higher viscosity can cause engine deposits and fuel injection issues. Biodiesel viscosity is affected by the fatty acid profile; higher chain lengths increase viscosity, while unsaturation decreases it. ASTM D6751 specifies a kinematic viscosity range of 1.9–6.0 mm^2/s, and EN14214 specifies 3.5–5.0 mm^2/s (Knothe 2008). The kinematic viscosities of fungal SCOs from mangrove fungi were found within acceptable ranges, indicating their potential as biodiesel feedstock (Khot *et al.* 2012).

Density, influenced by the fatty acid composition, is another key fuel property. Mismatches between biodiesel and engine parameters due to density differences can affect emissions. Standard density testing methods are outlined in EN-3675 (hydrometer method) and EN-12158 (oscillating U-tube method) (Pratas *et al.* 2011). Biodiesel from *Aspergillus* sp. had a density range of 0.80–0.83 g/cm^3, depending on substrate and transesterification methods (Subhash and Mohan, 2011).

Microbial biodiesel has several advantages over plant-based biodiesel, including similar or superior power output, torque, and energy density. Wahlen *et al.* (2013) compared biodiesel from microalgae, fungi, and bacteria with plant oil biodiesel and found that microbial biodiesels had comparable or slightly lower energy densities. Biodiesel from fungi showed the highest cetane number, followed by soybean and microalgae biodiesel, while bacterial biodiesel had the lowest CN.Microbial biodiesel reduces CO, unburned hydrocarbons, and CO, emissions compared to

Table 2: Lipid profiles of oleaginous fungi (in %) profiles of oleaginous fungi (in %)

Oleaginous fungi	Palmitic acid (C16:0)	Palmitoleic acid (C16:1)	Stearic acid (C18:0)	Oleic acid (C18:1)	Linoleic acid (C18:2)	Linolenic acid (C18:3)	References
Aspergillus caespitosus	0.95	0.32	64.77	14.11	12.81		Srinivasan *et al.* 2021
Aspergillus carneus	16.16	-	43.81	9.53	-	-	Ibrahim*et al.* 2023
Aspergillus sydowii	-	-	-	62.9	-	-	Yousuf *et al.* 2018
Aspergillus terreus	18.69	-	30.17	9.63	12.41	-	Al-Zaban and El-Aziz 2023
Aspergillus tubingensis	21.5	-	9.6	35.8	18.8	-	Zhu *et al.* 2020
Claviceps purpurea	23	-	2	19	8	-	Thevenieau *et al.* 2013
Cryptococcus curvatus	30.1	-	18.5	39.3	8.3	1.2	Zhang *et al.* 2011
Cutaneotrichosporon curvatum	10.9-18.6	3.8-8.4	3.0-6.6	32.6-66.7	3.9-22.0	-	Llamas *et al.* 2020
Cutaneotrichosporon oleaginosus	20.4	-	12.9	53.9	11.3	1.5	Shaigani *et al* 2021
Cyberlindnera saturnus	13.5-25.6	1.2-7.0	1.9-25.2	30.1-68.8	1.9-12.5	1.3-2.2	Llamas *et al.* 2020
Cystobasidium iriomotense	30.24	0.13	8.13	36.33	22.75	0.17	Tanimura *et al.* 2018
Fusarium equiseti	-	-	-	46	-	-	Yousuf *et al.* 2018
Fusarium oxysporum	-	-	-	52.4	-	-	Yousuf *et al.* 2018
Fusarium verticillioides	25.61	-	-	34.65	30.78	0.68	Kamoun *et al.* 2018
Galactomyces geotrichum	6.59	8.22	9.12	22.14	23.67	-	Altun *et al.* 2020
Lasiodiplodia exigua	44.54	-	-	13.88	-	-	Paul *et al.* 2020
Lipomyces starkeyi	27-34	6	6	43-51	3-7	-	Zain *et al.* 2024; Melluemiestre *et al.* 2015
Meyerozyma guilliermondii	18.0	11.3	1.8	64.9	2.7	-	Valdes *et al.* 2020
Microsphaeropsis sp.	27.3	3.5	3.0	46.1	20.0	-	Zhu *et al.* 2020
Mucor circinelloides	22	Trace	5	38	10	15	Melluemiestre *et al.* 2015
Mucor irregularis	-	16.89	-	4.85	45.22	30.79	Haura and Llmi 2024
Pellicularia praticola	8	-	2	11	72	-	Thevenieau *et al.* 2013
Penicillium brevicompactum	0.78	15.72	-	-	61.83	7.97	Ali *et al.* 2017
Penicillium chrysogenum	13	trace	12	19	43	6	Melluemiestre *et al.* 2015
Penicillium citrinum	20.25	-	16.13	30.09	33.14	-	Bardhan *et al.* 2019
Pestalotiopsis microspora	19.39	-	-	20.16	-	-	Paul *et al* 2020
Phomopsis sp.	42.66	-	-	51.53	-	-	Paul *et al.* 2020
Pythium ultimum	15	-	2	20	16	1	Thevenieau *et al.* 2013
Rhizopus arrihizus	18	Trace	6	22	10	12	Melluemiestre *et al.* 20 15
Rhodosporidium diobovatum	30.9-36.5	-	2.1-3.3	39.6-44.7	9.7-13.2	0.1-0.2	Nasirian *et al.* 2018
Rhodosporidium toruloides	20-29	-	12-19	28-47	8-19	6-12	Naveira-Pazos *et al.* 2024
Rhodosporidium toruloides	30.1	-	2.6	54.2	2.0	0.8	Diamantis *et al.* 2023
Rhodotorula glutinis	17	1	2	42	21	9	Melluemiestre *et al.* 2015
Rhodotorula paludigena	23.79	0.98	4.39	53.57	4.72	0.42	Gosalawit *et al.* 2021
Scheffersomyces coipomensis	16.8	11.0	2.4	60.4	9.1	-	Valdes *et al.* 2020
Syncephalastrum racemosum	30.61	0.64	14.43	23.45	2.35	1.75	Hashem *et al.* 2020
Trichosporon asahii	13.9	-	7.2	57.6	17.9	3.4	Shaigani *et al.* 2021
Trichosporon mycotoxinivorans	18.28	7.60	17.64	30.84	2.36	-	Sagia *et al.* 2020
Umbelopsis isabellina	24-35	1-2	3.5-8	49-54	2-11	0.4-2	Zhu *et al.* 2020
Yarrowia lipolytica	13	17	6	55	7	Trace	Melluemiestre *et al.* 2015

petroleum diesel. However, NOx emissions were higher in microbial biodiesel, a common issue with biodiesel in general (Wahlen *et al.* 2013).

In terms of lubricity, biodiesel has better lubricating properties than petroleum diesel, which helps engine efficiency. Biodiesel's higher flash point (150°C) compared to petroleum diesel (77°C) also makes it safer to handle (https://extension.psu.edu/whats-so-different-about-biodiesel-fuel).A drawback of biodiesel is its tendency to thicken and gel in cold temperatures due to its high oxygen content. However, additives can improve cold-weather performance (https://extension.psu.edu/whats-so-different-about-biodiesel-fuel).

Challenges in the production of biofuel from oleaginous fungi

Despite the promise of oleaginous fungi as feedstocks for biofuel, large-scale biodiesel production from these microbes is still limited. Challenges include the high cost of cultivation, fermentation, and lipid extraction processes. Key challenges are:

❖ The species chosen, lipid concentrations, and cell yield directly affect the cost of oil production (Thevenieau *et al.* 2013).

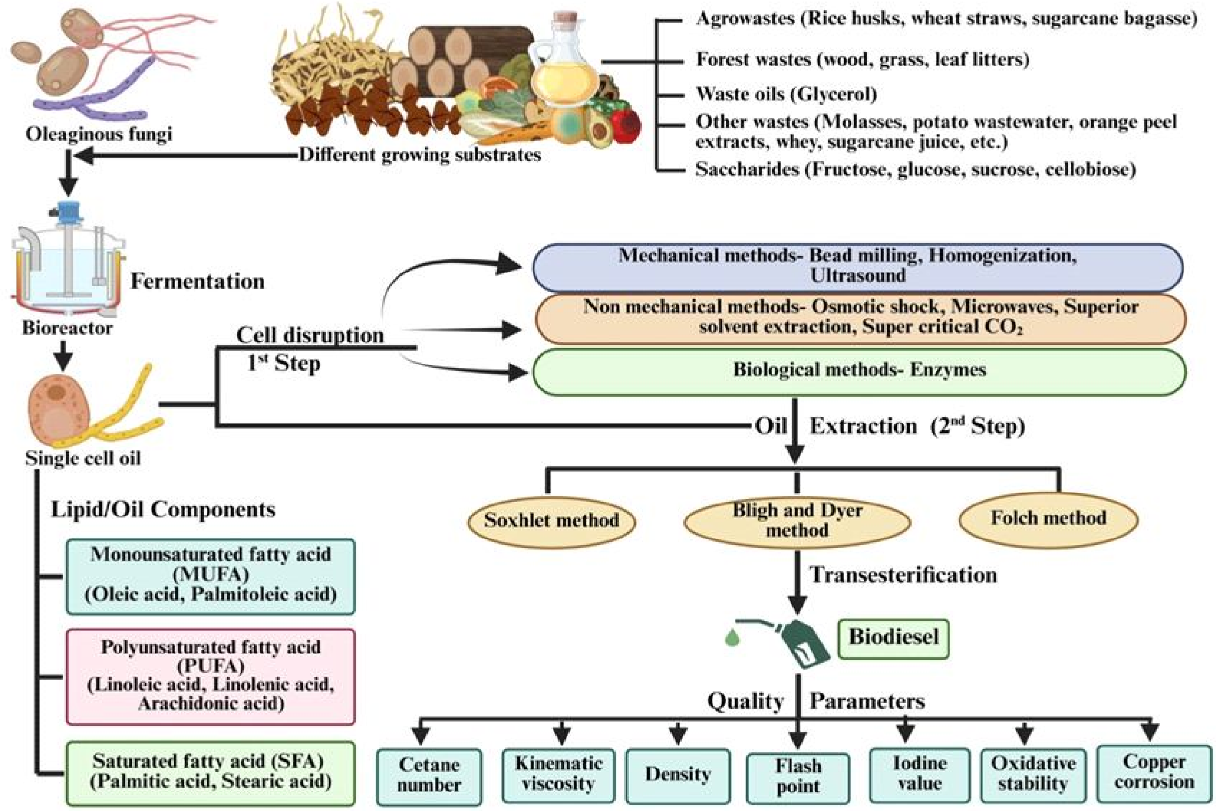

Fig. 1: Overview of fungal biodiesel production

❖ Identifying suitable low-cost carbon sources for microbial oil production is critical, as carbon source costs account for 60–70% of total production costs. Some alternative sources, such as biodiesel-derived waste glycerol, have been explored (Zhu *et al.* 2020).

❖ Lignocellulosic biomass can serve as a low-cost carbon source, but requires energy-intensive pre-treatment and hydrolysis (Zhu *et al*. 2020).

❖ Complex mixtures in cheap carbon sources can inhibit microbial growth (Bautista *et al.* 2012).

❖ Traditional fungal screening methods are labor-intensive and inefficient for large-scale production (Mhlongo *et al*. 2021).

❖ Lipid extraction methods are costly, time-consuming, and produce toxic waste, which hinders commercial scalability (Mhlongo *et al.* 2021; Patel *et al.* 2020).

❖ Rheological issues in submerged cultures complicate fungal cultivation, requiring precise environmental control (Caporusso *et al*. 2021).

❖ Harvesting lipids from filamentous fungi is challenging due to the viscous nature of the mycelial culture (Chattopadhyay and Maiti 2021).

❖ Factors like C/N ratio, pH, temperature, aeration speed, and agitation affect lipid accumulation, leading to variable yields (McNeil and Stuart 2018; Sutanto *et al*. 2018).

❖ High lipid extraction costs, including energy-intensive drying and dewatering, hinder large-scale biodiesel production (Patel *et al*. 2018).

❖ The use of toxic solvents like chloroform and methanol in traditional lipid extraction methods poses environmental and health risks (Patel *et al*. 2018).

❖ Maintaining aseptic conditions during fungal fermentation is costly, complicating industrial-scale production (Gujjala *et al*. 2019).

❖ Limited knowledge of oleaginous microbial feedstocks and the need for genetic engineering research hinder biofuel production efficiency (Nigam *et al*. 2014).

Addressing these challenges, particularly the costs of substrates and lipid extraction, is essential for the commercial viability of fungal biodiesel production.

Potential solutions to overcome challenges in fungal biodiesel production

As aforementioned, the production of biodiesel from oleaginous fungi, come across multiple challenges including insufficient lipid accumulation, high cost of feedstocks and complicated downstream processing. Some of the probable solutions for overcoming these issues include, the screening of lipids on the basis of fatty acid composition such as high monounsaturated fatty acids (MUFA), moderate saturated fatty acids (SFA), and low PUFA (polyunsaturated fatty acids) for the quality of biodiesel, and selecting the efficient fungal strains for large scale lipid production. Optimization of fermentation conditions such as C/N ratio, pH, temperature and aeration according to the nature of the strain which will remarkably boost lipid productivity. Other solutions, such as over expression of genes (*ACC1*, *DGAT*, *DGA1*, *ME*, *FAS1/2*) and knockout or silencing of genes (*POX1-6*, *TGL*, *MFE1*) through genetic engineering will help in efficient lipid accumulation in fungal cells. Mutagenesis or adaptive evolutions are also highly applicable solutions to enhance lipid biosynthesis pathway that will help in the increasing of lipid accumulation and tolerance to stress.

Another major problem is the high cost of substrates such as use of commercial culture media or glucose which limit economic feasibility. The use of abundant and low-cost agricultural waste such as wheat/rice bran, spent grains, fruits/vegetable peels, agricultural residues and leaf litter proved to be most suitable substrates for fungal lipid and biodiesel production. Use of lignocellulosic biomass and its pre-treatment with harsh chemicals or thermal conditions lead to the formation of inhibitory compounds which can result in reduction of lipid production. The inhibitory compounds can be reduced by utilizing fungal assisted degradation and enzymatic hydrolysis. Moreover, the choice of bioreactors such as fed-batch bioreactors (stirred-tank reactor and air-lift bioreactor) and continuous bioreactor systems (continuous stirred tank reactor and membrane bioreactor) will play a critical role in upstream processing.

Extraction of lipid from fungal cells is a very complicated and costly process which hinders the production of biodiesel in a large scale. To maximize the lipid recovery, cell wall disruption techniques such as bead milling, ultrasonication, enzyme lysis or microwave assisted extraction is highly preferred before the extraction process. The use of non-toxic, biodegradable and renewable solvents such as ethanol, isopropanol can be preferred for lipid extraction for easier recovery, purification and cost reduction. To improve the overall efficiency of downstream processing, transesterification combined with extraction process (direct or in-situ transesterification) in a single step and using reusable catalyst will greatly reduce time, cost and waste.

The risk of microbial contamination during large-scale production of fungal biomass and the expense of sterilization or inhibiting the contamination, limits the recovery of lipids or biodiesel production. However, selecting rapid colonizing and antimicrobial metabolite producing fungal strains can efficiently help in solving the problem of contamination. Additionally, optimizing selective conditions such as low pH, high temperature and use of tolerant strains can further help in minimizing contamination.

Conclusions and future prospects

For large-scale biodiesel production from oleaginous fungi, understanding lipid content, properties, and processing is essential. Strategies to reduce production costs and improve lipid quality are key to commercial success. Genetic engineering and gene editing technologies hold promise for enhancing lipid production in fungi. Genomic, transcriptomic, and metabolomic studies will further our understanding of fungal lipid biosynthesis and facilitate the use of low-cost raw materials. Nanoparticle extraction methods and green solvents (e.g., terpenes, isopropanol) should also be explored to replace toxic solvents. Biorefineries must focus on optimizing both upstream and downstream processes for efficient conversion of fungal oils to biodiesel. Economic feasibility and sustainability assessments are crucial for scaling up production.

Oleaginous fungi hold great potential for biofuel production due to their ability to produce high-quality lipids. Although biodiesel production from fungi is still at a small scale, ongoing research and technological advancements could pave the way for fungal biodiesel to become a major player in the bioenergy sector. Raising awareness and fostering scientific exploration of this sustainable energy source will be key to securing the future of biofuels and improving global energy security.

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DECLARATION

Conflict of interest. Authors declare no competing interests.

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