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BIOENERGY STUDIES

Utilization of Fungal Bioprocess for Biodiesel Production as a Green Energy Source

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Abstract

The trend toward new research studies focused on searching for sustainable sources of clean energy has increased to ensure the sustainability of energy worldwide due to reasons such as the scarcity of fossil-based fuels and the environmental impact of conventional sources. Biofuel is a good candidate to become a world leader in the development and deployment of renewable energy sources among other green energy sources. Biodiesel is an alternative fuel produced from renewable biological resources and can be produced using microbial resources such as algae, bacteria, and fungi. Biodiesel produced from microbial lipids is an important and effective green energy source. Fungi exhibit a high capacity for biodiesel production by accumulating more than 70 percent of intracellular lipids in their biomass during metabolic stress periods. This study aims to examine the studies focused on the use of fungal lipids as a source of biodiesel in recent years. In addition, the conditions in which the highest biodiesel production efficiency was obtained, including preliminary information that will guide further studies on large-scale production of biodiesel from fungi were reviewed.

Introduction

The rapid augment in the world population induces increasing growth in industrialization leading to an increment in energy needs. The greatest challenge facing humankind is the gradual and inevitable depletion of the world's energy from fossil sources. Fossil fuels, which are generously used as fuel in vehicles, are one of the most responsible factors for global warming and environmental pollution. At a time when energy demand is booming, the world is facing dwindling liquid fuel reserves. If consumption continues at the current rate, the fossil fuel supply may have finished before the end of this century. Renewable biofuels are needed to replace limited quantities of petroleum-derived fuels which contribute to global warming. Biodiesel is accepted as the potential renewable fuel receiving the most attention (Papanikolaou et al., 2007). Renewable, carbon-neutral transportation fuels are essential for economical and environmental sustainability. Although it has not been used industrially until now, there has been an increased interest in using microbial systems for biodiesel production.

The ability of microorganisms to grow on an endless variety of food sources can play an important role in bringing society out of the current energy crisis (El Bialy et al., 2011). There has been increased interest in reducing the cost of biodiesel production. Researchers especially focus on studies on biodiesel production using cheap raw materials. For instance, microorganisms are adapted to use a wide variety of carbon sources, such as waste or agricultural by-products, for oil production (Tsigie et al., 2012).

Many molds, yeasts, and algae accumulate more than 70 percent of their biomass for intracellular lipids during periods of metabolic stress (Saygün et al., 2014). Fungi are microorganisms suitable for biodiesel production due to their high oil content (Poli et al., 2014). However, the use of fungal sources for commercial biodiesel production is limited. Therefore, this study aims to examine the studies in the literature on the use of fungal bioprocesses for biodiesel production and to examine the optimization studies carried out for fungal biodiesel production before commercial production.

Fungal Cell and Lipid Profile

In the scientific classification of living organisms, although fungi were initially considered under the kingdom, it belongs to the domain of Eukaryota now (Naranjo-Ortiz & Gabaldón, 2019). Molds (filamentous fungi) and yeasts belong to Fungi. The vegetative thallus of a filamentous fungus (mold) is composed of hyphae forming filaments (Cole, 1996). Yeast is composed of a generally oval-shaped single cell. Fungi are heterotrophic organisms, therefore they need carbon and energy sources (Luiz et al., 2018). Biotrophic fungi obtain their nutritional needs from other living organisms such as plants or animals (Naranjo-Ortiz & Gabaldón, 2019). Saprotrophic fungi obtain their nutrition from dead plants and animals. Necrotrophic fungi infect living hosts to ensure their nutritional demand and mostly killed the host cells (Shao et al., 2021).

Fungal cells have microscopic structures therefore to obtain their nutrition first digest food through their extracellular enzymes and, then absorb formed small molecules after digestion via their cell walls. In the fungal cell structure, lipids are the main component of the membrane systems, as well as the cell wall component and storage material in the lipid droplets. Recently Kothri et al. (2020) showed that lipid droplets present in the fungal and yeast cells were stained with Nile red (Figure 1). In some cases, lipids appear as extracellular products secreted outside of the cell. The diversity of lipid components also contributes to the larger cell size and complexity of fungi. The amount of lipids in fungal cells varies according to the type of fungus, age, developmental stage, nutrition, and environmental conditions (Kothri et al., 2020). The lipid content of fungal species can be manipulated by changing the culture conditions. In this context, the total lipid content is of limited value unless growth medium parameters are disclosed.

Due to the ease of preparation and analysis of total lipid samples in fungi, much information is available about the total lipid composition of fungal cells. The lipid fractions from various filamentous fungi species show a wide range of values for the contents of both polar and neutral lipids (Subramaniam et al., 2010). Triacylglycerols, which represent the main lipid component, are generally considered storage lipids that can be used for energy and carbon skeletons during growth and development. The other main lipid components of oleaginous molds are many sterols, squalene, and other hydrocarbons. Sterols' condensed or liquefied effect on acyl lipids depends on the physical state of the lipid. Sterols can regulate the permeability of membrane lipids by affecting the internal viscosity and molecular movement. Some of the sterols may also act as precursors of steroid hormones in sexual reproduction in some fungal species. The phospholipids are the main polar lipids and also glycolipids are found as other polar lipids in molds. These polar lipids are involved in the active transport of ions across membranes and are also required for the activity of some membrane-bound enzymes (Sancholle et al., 2003; Cohen, 2011).

The potential for use of a microorganism in the production of biodiesel depends on the amount of oil it can produce and accumulate in its cellular structure (Ratledge & Wynn, 2002). In this context, a microbial cell is commercially important in proportion to the amount of oil it can produce. The quality and amount of this produced oil also vary from organism to organism. The relative ratios of all lipid components in the fungal structure can vary with the stage of fungal growth, age, and conditions in which the fungus is cultured.

Previous studies on yeast lipids have divided yeast strains into high-oil and low-oil producers (Sitepu et al., 2013). For example, some yeasts such as *Saccharomyces cerevisiae* or *Candida utilis* never accumulate more than 10% of their cell mass as lipids, while other yeasts such as *Rhodotorula* and *Lipomyces* species can accumulate 70% or even more of their biomass as lipids (Alakraa et al., 2020). In addition, most of the lipid produced by *Rhodotorula* and *Lipomyces* species is in the form of triacylglycerol, which is equivalent to commercial oils and fats in chemical composition (Ratledge & Wynn, 2002). Passoth (2017) reported that molds (filamentous fungal species) can produce longer polyunsaturated fatty acids, but yeasts are able to accumulate more oil in their cell. Although some yeast species are accepted as good oil producers, producing biodiesel commercially using molds is preferable rather than yeasts due to some advantages. One of these advantages is the ability of filamentous fungi to utilize a wider range of waste materials than yeasts, which means a financially more economical way (Hoarau et al., 2018). Another advantage is that molds are capable of producing a wider variety of fatty acids (Akpinar-Bayizit, 2014). Most of the fungal species have major and minor acids (Table 1).

Fungal Lipid Accumulation

Lipid accumulation by fungi has been exhibited under metabolic stress conditions such as limited

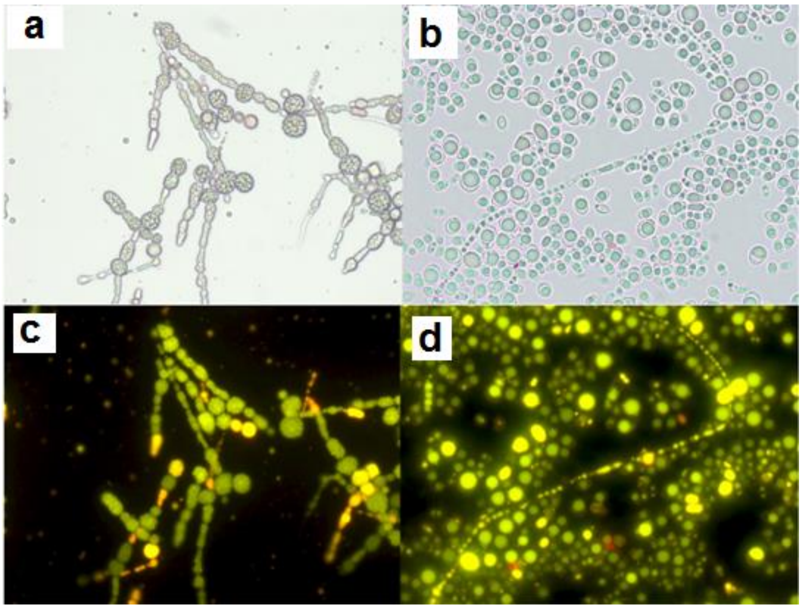

Figure 1. The lipid droplets stained with the Nile red in the filamentous fungal cell: *Umbellopsis isabellina* and yeast cell: *Yarrowia lipolytica* using light (a, b) and fluorescent (c, d) microscopy, respectively.

Table 1. The fatty acids are present in the most fungal species in order of abundance (Akpinar-Bayizit, 2014)

Order	Major Acids	Minor Acids
1	Oleic acid (C18:1)	Stearic acid (C18:0)
2	Palmitic acid (C16:0)	Linolenic acid (C18:3)
3	Linoleic acid (C18:2)	Palmitoleic acid (C16:1)

nutrients and the carbon source in the medium (Shoaib et al., 2018). The limitation of nitrogen is the most effective condition to induce lipogenesis. During the fungal growth phase, nitrogen is required for protein and nucleic acid synthesis and, carbon is used to obtain carbohydrates, lipids, nucleic acid, and protein in energetic and anabolic processes. When the nitrogen in the medium is limited, the fungal growth rate slows down and as a result, the synthesis of proteins and nucleic acids tends to stop. In non-oleaginous fungal species, excess carbon remains unused or is converted to storage polysaccharides, while in oleaginous fungal species the excess carbon is directed to lipid synthesis, leading to the accumulation of TAG in intracellular lipid bodies. Lipid biosynthesis is not very different between eukaryotic organisms, and there is not much difference between non-oleaginous and oleaginous fungal species (Patel et al., 2020).

The accumulation of large amounts of lipids ability is mostly dependent on the regulation of the biosynthetic pathway and the provision of precursors and the cofactor NADPH. Detailed information on fungal lipid metabolism has been obtained from *Saccharomyces cerevisiae*, a non-lipid-accumulating model organism, (Kohlwein, 2010), and *Yarrowia lipolytica*, a model organism for bio-oil production suitable for genetic manipulation (Beopoulos et al., 2009). The utilized fungal species for biodiesel production are given in Table 2.

Fungal Cultivation to Produce Lipids

The two fermentation methods are used in the production of microbial lipids: solid-state fermentation and submerged fermentation. In solid-state fermentation, biomolecules are mostly produced by microorganisms grown on solid support chosen for this purpose. In submerged fermentation, after adding fungi to the sterile environment, when the fermentation process is aerobic, it is constantly mixed and biomolecule production is carried out in a large tank in which the sterile air source is foamed. While continuous culture techniques have advantages for studying lipid accumulation, commercial use of continuous culture is much less common due to high capital costs. Therefore, most lipid accumulation studies are conducted in batch scale-level fermentors. The batch fermentation process has two-stage. In the first stage, fungal cell proliferation and balanced growth occur as a result of the depletion of another nutrient from the environment. The second stage is the phase of lipid accumulation (Anderson & Wynn, 2001).

The lipid accumulation process of fungi is similar to the process that occurred in yeasts. Most of the studies reported that the lipid accumulation process is linked with fungal growth. Although there is a direct correlation between fungal growth and lipid accumulation, the optimal conditions for growth are not necessary to produce the highest yield of lipids. The lipid biosynthesis pathways are carried out by certain key

Table 2. The list of some fungal species used for biodiesel production (Modified from Athenaki et al., 2018)

Oleaginous fungi	Yeast
Aspergillus niger	*Aureobasidium melanogenum*
Aspergillus fumigatus	*Rhodotorula colostri*
Aspergillus flavus	*Sporidiobolus ruineniae*
Penicillium expansum	*Sporobolomyces carnicolor*
Thamnidium elegans	*Yarrowia lipolytic*
Cunninghamella echinulata	*Candida lipolytica*
Mucor circinelloides	*Rhodotorula glutinis*
Mucor circinelloides	*Cryptococcus psychrotolerans*

enzymes present in oleaginous microorganisms (Papanikolaou & Aggelis, 2011). Heterotrophic or autotrophic photosynthetic oleaginous microorganisms use organic carbon sources or CO_2 sequestration as energy sources, respectively (Bellou et al., 2014; Kazemi et al., 2020; Ratledge, 1988; Sharma et al., 2001). Heterotrophic oleaginous microorganisms can use different kinds of organic carbon sources such as hydrophilic (glucose, other hexoses, disaccharides, glycerol, polysaccharides, acetic acid, butyric acid, ethanol, etc.), hydrophobic (free fatty acids (FFA), TGs, n-alkanes, etc.) or their mixtures (FFAs or mixtures of TGs with glucose or glycerol). The percentages of oil content of *Yarrowia lipolytica* using different carbon sources in batch scale level fermentation studies published in the literature are given in Figure 2 (Papanikolaou et al., 2001; Papanikolaou et al., 2007; El Bialy et al., 2011; Tsigie et al., 2012; Saygün et al., 2014; Poli et al., 2014).

Production of Biodiesel from Fungal Biomass

Fungi can act as bioreactors producing biodiesel. To obtain techno-economic results in biodiesel production, it is very important to prefer lower-cost approaches instead of cost-increasing steps such as drying the raw material and oil extraction procedures. In this context, oleaginous microorganisms having high oil content can be used in the production of second-generation biodiesel. Vicente et al. (2009) suggested a new filamentous fungus named *Mucor circinelloides* has a high content of fatty acid to produce biodiesel effectively. Previous studies showed that *Lipomyces starkeyi*, *Rhodosporidium toruloides*, *Rhodotorula glutinis*, and *Yarrowia lipolytica* are oleaginous yeasts and were able to produce and accumulate high amounts of triacylglycerides used to produce biodiesel after transesterification (Ageitos et al., 2011). Zheng et al. (2012) showed that the filamentous fungal species called *Mortierella isabellina* performed the highest lipid production growing on lignocellulosic hydrolysates among eleven tested species and recommended this organism as an effective biodiesel producer using economic substrates and also stated that this fungus provided an easy method to harvest product after fermentation. Sitepu et al. (2013) scanned sixty-nine strains including yeasts belonging to different genera to determine the lipid production and accumulating properties and introduced new oleaginous yeasts such as *Myxozyma melibiosi*, *Kurtzmaniella cleridarum*, *Cryptococcus aff. taibaiensis*, and *Rhodotorula colostri*. Chopra et al., (2016) reduced the processing time by up to 7 hours compared to the ex-situ multi-stage transesterification method, by performing the direct transesterification of the wet biomass of *Pichia guilliermondii*. On the other hand, Naveena et al. (2015) reported that in situ oil extraction from biomass can be performed by using ultrasonic processes. Using this strategy, 92% and 94.3% yields were obtained from the wet biomass of *Cryptococcus curvatus* and *Yarrowia lipolytica* with a 4-minute direct transesterification process, respectively (Yellapu et al., 2017). Biodiesel production was carried out by one-step acid-catalyzed transesterification of oil extracted from *Yarrowia lipolytica* biomass grown in waste cooking oil, and the lipid productivity of the same fungal species was determined as 0.042 g/L/h (Katre et al., 2018). Under optimal conditions (catalyst, 0.2 M H_2SO_4 1.0 mL/g; methanol-to-chloroform-to-biomass ratio, 10:1:4 v/v/w; 50 °C; and 8 h) biodiesel yield was determined as 22 mg in 1 g biomass in the same study. Also, the determining fatty acid methyl ester profile was reported as 32.81% saturated, 36.41% monounsaturated, and 30.59% polyunsaturated methyl esters (Katre et al., 2018). In the newly developed biodiesel production process, the cost is reduced by direct transesterification of wet biomass. Direct transesterification of microorganisms having a high amount of oil significantly reduces both the cost of production (due to shortened processing time) and environmental burdens (such as no requirement for toxic solvents). Recent studies emphasize that fungi are desirable bioengineering resources for the production of biodiesel in a low-cost way (Tabatabaei et al., 2019; Tiwari et al., 2020).

Conclusion

Fungi can produce high levels of oil in their cell structures, and it is possible to use the oils they produce as raw materials in biodiesel production. Figure 3 shows the simple biodiesel production process schema using fungi. In general, fungal systems have advantages such as rapid reproduction ability, high oil production efficiency, low space requirements for development,

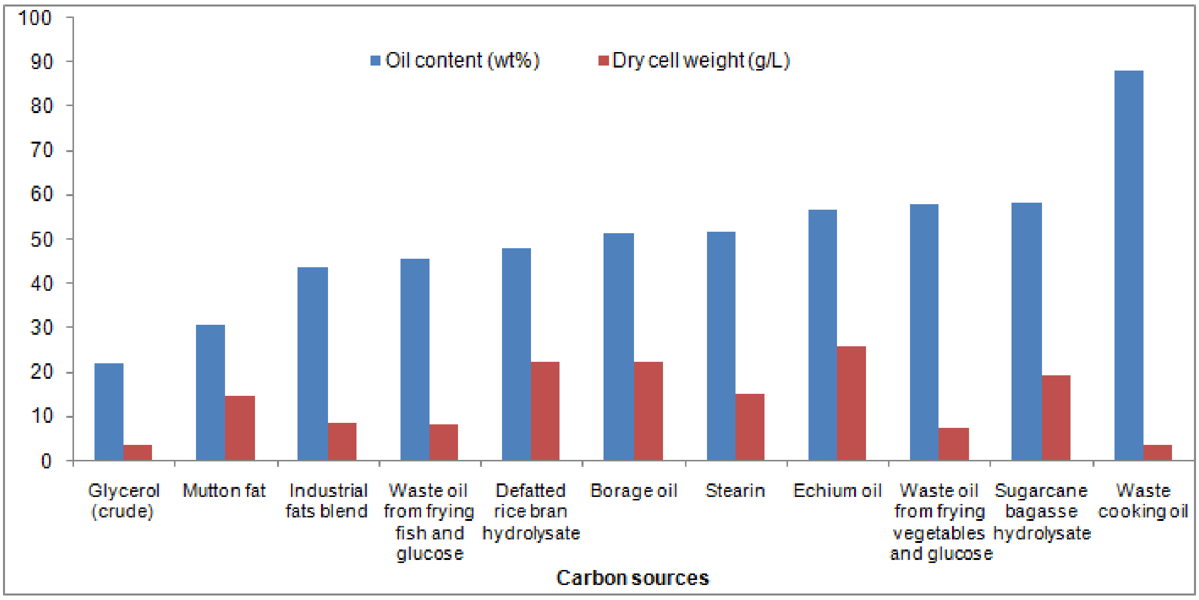

Figure 2. The oil content (wt. %) and dry weight (g/L) of *Y. lipolytica* growing in the presence of different carbon sources (Papanikolaou et al., 2001; Papanikolaou et al., 2007; El Bialy et al., 2011; Tsigie et al., 2012; Saygün et al., 2014; Poli et al., 2014).

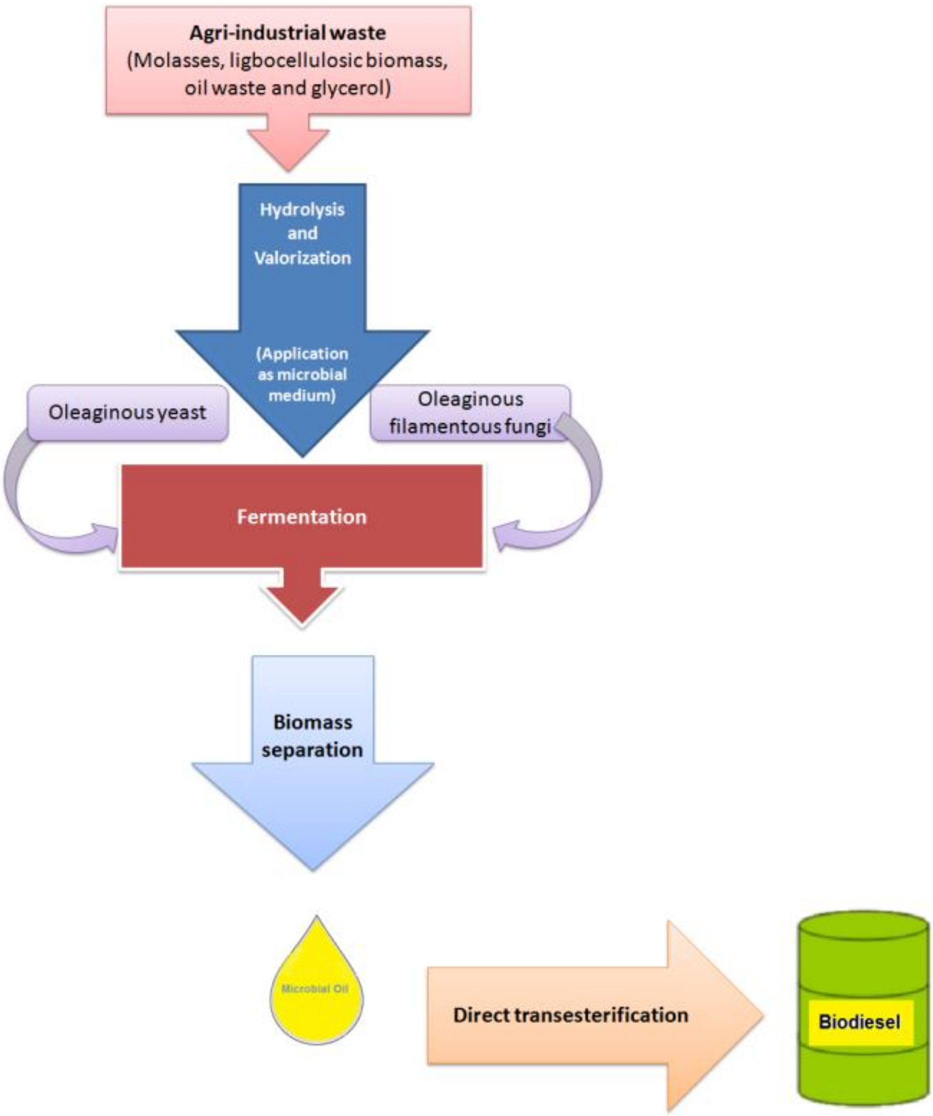

Figure 3. Fungal lipid production and biodiesel conversion scheme.

short oil production periods, and similar fatty acid compositions to vegetable oils (Amara et al., 2016; Athenaki et al., 2018; Cho & Park, 2018; Xu et al., 2015). Therefore, with these properties, fungi can be considered successful bioreactors for biodiesel production. However, economically and environmentally viable biodiesel production technologies should be developed for the direct transesterification of fungal biomass with high wet oil content. In this context, strategies for biodiesel production from fungi with the valorization of agricultural and industrial wastes have gained importance today.

In conclusion, although there are some developments in fungal biodiesel production such as the identification of new oleaginous fungal species, more research is needed on the development of more efficient biodiesel production technologies with low cost. In particular, it is recommended to carry out studies to improve the techno-economic constraints of the fungus-based biodiesel production industry and to evaluate the models that can contribute to the circular economy in this regard.

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