**OLUFEMI GABRIEL ABIMBOLA**

# SELECTION OF OLEAGINOUS YEASTS ABLE TO ACCUMULATE LIPIDS FROM CULTURE MEDIA CONTAINING GLYCEROL AS THE SOLE CARBON SOURCE

Dissertation submitted to the Agricultural Microbiology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae.*

Adviser: Wendel Batista da Silveira

Co-adviser: Rafaela Zandonade Ventorim

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**OLUFEMI GABRIEL ABIMBOLA**
Data: 05/03/2024 09:48:09-0300
Verifique em https://validar.iti.gov.br

Olufemi Gabriel Abimbola
Author

Documento assinado digitalmente
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**WENDEL BATISTA DA SILVEIRA**
Data: 05/03/2024 10:19:56-0300
Verifique em https://validar.iti.gov.br

Wendel Batista da Silveira
Adviser

*This is dedicated to God, my family, the Microbial Physiology Laboratory, UFV, Brazil, and the Department of Microbiology and Biotechnology, First Technical University, Ibadan Nigeria*

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*Success is not final; failure is not fatal: It is the*
*courage to continue that count." —*
*(Winston S. Churchill)*

*"All our dreams can come true; if we have the courage to pursue them." –*
*(Walt Disney)*

# ABSTRACT

ABIMBOLA, Olufemi Gabriel, M.Sc., Universidade Federal de Viçosa, August, 2023. **Selection of oleaginous yeasts able to accumulate lipids from culture media containing glycerol as the sole carbon source**. Adviser: Wendel Batista da Silveira. Co-adviser: Rafaela Zandonade Ventorim.

The biodiesel is currently produced mainly from edible oils as feedstock, generating glycerol as a by-product. The use of glycerol as a carbon source for oil production by oleaginous yeasts contributes to the development of alternative routes of oil production in the context of the circular economy. Therefore, this study aimed to select oleaginous yeasts capable of producing oil in culture media containing glycerol as a carbon source. A total of 10 yeasts capable of assimilating glycerol as carbon source were evaluated for their ability to produce lipids in a glycerol culture medium to select a promising oleaginous yeast. Herein, the isolate CA8 was selected as the best lipid producer from glycerol considering the achieved values of lipid content (%), lipid titer (g/L), and biomass (g/L). This isolate was taxonomically identified as *Meyerozyma guilliermondii* and designated as *Meyerozyma guilliermondii* UFV-2. Since the C: N ratio notably influences lipid production by oleaginous yeasts, the effect of different C:N ratios on biomass production, lipid content and lipid titer were evaluated. The highest values of biomass formation, lipid content, and lipid titer, recorded in a C: N of 150:1, were 6.77 g/L, 25 % (w/w), and 1.71 g/L, respectively recorded. The lowest biomass formation 4.67 g/L was recorded in a C: N ratio of 75:1. Taken together, these results indicate that *M. guilliermondii* UFV-2 displays the potential to produce lipids from glycerol, opening perspectives for further studies that aim to optimize the production parameters.

**Keywords**: Biodiesel*; Meyerozyma guilliermondii;* Yeast oil; Circular economy; Carbon-nitrogen ratios.

# RESUMO

ABIMBOLA, Olufemi Gabriel, M.Sc., Universidade Federal de Viçosa, agosto de 2023. **Seleção de leveduras oleaginosas capazes de acumular lipídeos a partir de meios de cultura contendo glicerol como única fonte de carbono.** Orientador: Wendel Batista da Silveira. Coorientadora: Rafaela Zandonade Ventorim.

A produção atual de biodiesel utiliza principalmente óleos comestíveis como matéria-prima, gerando o glicerol como subproduto. O aproveitamento de glicerol como fonte de carbono para a produção de lipídios por leveduras oleaginosas contribui para o desenvolvimento de rotas alternativas de geração de óleo alinhado com os princípios de economia circular. Este estudo focou na seleção de leveduras oleaginosas capazes de produzir óleo em meios de cultura contendo glicerol como única fonte de carbono e energia. Dez leveduras capazes de assimilar glicerol como fonte de carbono tiveram a sua capacidade de produção de lipídios avaliada em meio de cultura contendo glicerol objetivando a seleção de uma levedura oleaginosa promissora. O isolado CA8 foi selecionado como o melhor produtor de lipídios em meio de cultivo contendo glicerol com base no teor de lipídios (%), título lipídico (g/L) e biomassa (g/L). Este isolado foi identificado taxonomicamente como *Meyerozyma guilliermondii*, sendo nomeado *Meyerozyma guilliermondii* UFV-2. É bem estabelecido que a razão carbono:nitrogênio (C:N) é um fator que afeta consideravelmente a produção de lipídios por leveduras oleaginosas; portanto, o efeito de diferentes razões C:N sobre a produção de biomassa, teor lipídico e título foi avaliado. A maior produção de biomassa foi observada na razão C:N de 150:1 (6,77 g/L). O maior teor lipídico (25,0% m/m) e título lipídico (1,71 g/L) foram observados na razão C:N 150:1. Os resultados obtidos neste estudo mostram que *M. guilliermondii* UFV-2 apresenta potencial para produzir lipídios em meios de cultivo contendo glicerol e, portanto, abrem perspectivas para estudos futuros visando a otimização dos parâmetros de produção.

**Palavras-chave:** Biodiesel; *Meyerozyma guilliermondii;* Óleo de leveduras; Economia circular; razão carbono-nitrogênio

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# LIST OF ACRONYMS AND ABBREVIATIONS

| | |
|---|---|
| AMP | Adenosine monophosphate |
| ATP | Adenosine triphosphate |
| IMP | Inosine monophosphate |
| TAG | Triacylglycerides |
| DAG | Diacylglycerides |
| FFA | Free fatty Acid |
| PUFAs | Polyunsaturated fatty acids |
| FALs | Fatty alcohols |
| FAMEs | Fatty acid methyl esters |
| FAEEs | Fatty acid ethyl esters |
| DHAP | Dihydroxyacetone phosphate |
| G3P | Glycerol 3-phosphate |
| GK | Glycerol kinase |
| HA | Dihydroxyacetone |
| cG3PDH | cytosolic glycerol 3-phosphate dehydrogenase |
| C: N | Caron-Nitrogen ratio |
| YPD | Yeast extract dextrose |
| YP | Yeast peptone |
| YNB | Yeast nitrogen base |
| DNA | Deoxyribonucleic acid |
| EDTA | Ethylene Diamine Tetra-acetic Acid |

# LIST OF SYMBOLS

| | |
|---|---|
| $Mg^{+2}$ | Magnesium (II) ion |
| $Ca^{+2}$ | Calcium (II) ion |
| $Mn^{+2}$ | Manganese ion |
| $Fe^{+3}$ | Iron (III) ion |
| $Cu^{+2}$ | Copper (II) ion |
| $Zn^{+2}$ | Zinc (II) ion |
| $MnSO_4$ | Manganese tetraoxosulphate (vi) |
| $ZnSO_4$ | Zinc tetraoxosulphate (vi) |
| $MgSO_4$ | Magnesium tetraoxosulphate (vi) |
| $CoCl_2$ | Cobalt chloride |
| $CuSO_4$ | Copper tetraoxosulphate (vi) |
| $FeSO_4$ | Iron tetraoxosulphate (vi) |
| $KH_2PO_4$ | Potassium dihydrogen tetraoxophosphate (vi) |
| $(NH_4)_2SO_4$ | Ammonium tetraoxosulphate (vi) |
| $CaCl_2$ | Calcium chloride |
| NaCl | Sodium chloride |
| % | Percentage |
| g/L | Gram per liter |
| µl | Microliters |
| w/v | Weight per volume |
| rpm | Revolution per minute |
| ml | Milliliters |
| $OD_{600nm}$ | Optical density at 600 nanometers |
| $\mu$ | Specific growth rate |

# SUMMARY

# 1 INTRODUCTION

Biodiesel is a biodegradable, eco-friendly fuel produced by trans esterifying vegetable oils with methanol. Various renewable feedstocks, such as vegetable oils, animal fats, and waste oils, are considered for biodiesel production. However, the use of edible oils competes with their use as food. In order to overcome this limitation, microbial oils are considered an alternative source due to their higher growth, ease of scaling up, and low impact on seasonal, space, and climate change factors. Microbial oils are produced by oleaginous microorganisms such as yeast, microalgae, fungi, and bacteria, which can accumulate lipids or triacyl glycerides over 20% of their dry cell weight.

Oleaginous yeasts are a promising biodiesel producer outperforming other oleaginous microorganisms due to their capacity to achieve high growth rates, as well as easy cultivation. They offer short production cycles, independent of geographical, seasonal, and climate limitations, and can produce lipids from bio-based residues, providing cost-effective carbon sources without land-use change. Oleaginous yeasts can convert low-value substrates into valuable triglycerides (TAGs) similar to plant oil.

Glycerol, a low-cost biodiesel substrate, is being explored for lipid synthesis by oleaginous yeasts in the circular economy. However, only a few yeasts can convert glycerol into lipids. Interest is growing in cultivating oleaginous yeasts in glycerol-based media, and identifying new species for growth and lipid production from natural biodiversity.

Recently, our research team isolated yeasts from soil samples from Caparaó and Serra dos Órgãos national parks. They selected oleaginous yeasts due to their ability to display the oleaginous phenotype in culture media with xylose as the sole carbon source. However, this present study aimed to select an oleaginous yeast from the collection of soil-isolated yeast capable of producing high amounts of lipids from fermentation media with glycerol as the sole carbon source.

The objective of the research was to identify an oleaginous yeast capable of lipid accumulation in glycerol-based culture media. The study examined lipid production, identified oleaginous yeast strains with potential, evaluated the growth parameters of the selected strain, and evaluated lipid production in culture media with varying carbon: nitrogen ratios.

# 2 LITERATURE REVIEW

## 2.1. Biodiesel

Biodiesel is an eco-friendly fuel produced by trans-esterifying oils, mainly edible oils, with alcohol (PRASHANTH et al., 2016). Biodiesel is the most sustainable and renewable substitute for fossil diesel fuel (MAHLIA et al., 2020). It is usually formed by chemical transesterification, in which triacylglycerides (TAG), regardless of their origin, interact with short-chain alcohols (generally ethanol/methanol) to form alkyl esters (methyl/ethyl esters) (PATEL et al., 2017). Biodiesel can be used in the same standard diesel engines, regardless of its origin or the feedstock (microbial oils, vegetable oils, or animal fats) from which it is derived (ELSAYED et al., 2020). The key features that make it environmentally sustainable and eco-friendly are the lowest levels of $CO_2$ emission without sulfur and a significant role in global climate issues, as it reduces greenhouse gasses (SHAFAQUE FIROZ et al., 2017).

Biodiesel has been regarded as a viable and primary substitute for conventional fuel (KARMAKAR et al., 2019). Lower GHG emissions, a molecular structure that is highly biodegradable and has low combustion toxicity, compatibility with already-in-use engines, and ease of fuel distribution infrastructure compatibility are some of its most prized features (CHRISTOPHER et al., 2014). Chemical catalysis is the most commercially utilized route for biodiesel production, but it has limitations concerning catalyst recovery and recycling, as well as the need for high-quality raw materials. Consequently, the biocatalytic process, especially the use of enzyme lipases, offers several advantages, including favorable reaction conditions, reduced waste generation, easy purification, and raw material flexibility, making it a potential way to reduce biodiesel costs. (THOAI et al., 2019).

## 2.2. Generations of biodiesel

Biodiesel generation is classified based on the type of oil source used as feedstock, including edible and non-edible oil crops, waste oils, and oleaginous microorganisms such as yeasts, algae, and bacteria.

### 2.2.1. First-generation biodiesel

First-generation biodiesel is derived from edible feedstock such as soybean, sunflower, palm, rapeseed, coconut, mustard, and olive oil (ELSAYED et al., 2020). Due to high food demand, the use of oils derived from edible resources for large-scale biodiesel production is not a sustainable practice, as it competes with human food and available resources, raising the *food-versus-fuel* debate and leading to soaring food prices. To meet this requirement, a search for novel non-edible resources is required (PATEL et al., 2019).

### 2.2.2. Second-generation biodiesel

Second-generation biodiesel is derived from non-edible feedstock such as oils from non-edible crops, used cooking oil from the food industry, and animal tallow. Second-generation biodiesel is versatile, affordable, easily accessible, and does not affect food availability (ELSAYED et al., 2020). However, oils derived from cooking oil or other waste sources must be refined before being used as a feedstock for the production of biofuels (MANDOLESI et al., 2013). Triacylglycerides (TAG), diacylglycerides (DAG), and monoacylglycerols (MAG) are the primary lipids found in used cooking oil. Additionally, it contains a lot of free fatty acids (FFA), which impair the oils' ability to resist oxidation (LAM et al., 2012).

### 2.2.3. Third-generation biodiesel

Third-generation biodiesel is produced from microbial oils as feedstocks. The oil is produced by oleaginous microorganisms capable of accumulating the highest lipid titers, mainly yeasts and microalgae. Significantly, the similarity between microbial oils and vegetable oils renders microbial oils the most efficient feedstock for biodiesel production (SITEPU et al., 2014). Most oleaginous yeasts produce C18:1 (oleic acid), C16:0 (palmitic acid), and C18:0 (stearic acid) fatty acids, which are also prevalent in plant oils used for biodiesel production, such as canola and sunflower oil (SITEPU et al., 2019). Additionally, they have several benefits such as productivity that is typically higher than that of plants or vegetable oils, easier upstream and downstream processing, simple genetic modification for the desired products, the ability to use a variety of substrates made from industrial and agricultural waste, and ease of growth in a controlled environment independent of climate (SADH et al., 2018).

## 2.3. Microbial lipids

Microbial oils/lipids are intracellular lipid molecules produced and accumulated by certain groups of microbes. The majority of this lipid is in the triacylglycerol form, which has a fatty acid composition similar to that of vegetable oils (BANDHU et al., 2014). Microbial oils have advantages over vegetable oils due to their low land and water requirements, production independent of regional variations, quick process cycles, and scalability (FAKAS et al., 2017).

### 2.3.1. Economic importance of microbial lipids

#### 2.3.1.1. Foods

Lipid-derived compounds such as free fatty acids and fatty acid derivatives are widely used as food flavoring agents or additives. *Yarrowia lipolytica* and *Debaryomyces hansenii* produce free FAs such as pentadecanoic, palmitic, margaric, stearic, oleic, linoleic, and behenic acids, which are essential nutrients for humans (PATRIGNANI et al., 2007). SCOs are typically high in polyunsaturated fatty acids (PUFAs), which are essential nutrients for humans because some of these PUFAs cannot be synthesized by mammals. These are dietary supplements used in the food industry and primarily consist of docosahexaenoic acid (DHA), ARA, and GLA (RATLEDGE, 2005). *Cryptococcus curvatus* (currently named *Cutaneotrichosporon oleaginous*) was genetically engineered to enhance stearate production, resulting in the composition of palmitic, stearic, and oleic acids in the ratio 24:21:30 (%, w/w), similar to that of cocoa-butter (WARD and SINGH, 2005).

#### 2.3.1.2. Health care

Microbial lipids encompass several classes of molecules with high medicinal and nutritional values. PUFAs are precursors of eicosanoid-steroid molecules that play important roles in neural function, the cardiovascular system, and the immune system. PUFAs are essential for maintaining health and are found in thrombocytes, neuronal and muscle cells, and immunocompetent cells. EPA and DHA reduce LDL cholesterol delivery, are involved in inflammatory reactions, and are required for normal functioning (BÉLIGON et al., 2016). Commercial production of PUFA-rich SCO and β-carotenes has been improved by boosting lipid biogenesis in oleaginous yeasts, such as *Yarrowia lipolytica* and *Rhodotorula glutinis* (LV et al., 2019).

#### 2.3.1.3. Oleochemicals

Oleochemical compounds derived from lipids such as fatty alcohols (FALs) fatty acid methyl esters (FAMEs), fatty acid ethyl esters (FAEEs), free fatty acids (FFAs), fatty amines, glycerin, etc. are used in detergents, cosmetics, paints, and medicines/pharmaceuticals (ADRIO, 2017; YAN and PFLEGER, 2020). Fatty alcohol (FALs) production from microbes can be a sustainable alternative to petrochemical and vegetable oil sources (FILLET and

ADRIO, 2016). Fatty alcohol (FALs) is a good biofuel alternative due to their high energy density and compatibility with existing infrastructure. (YAN and PFLEGER, 2020).

#### 2.3.1.4. Biosurfactants

Surfactants are molecules that are used in the commercial production of soaps and detergents. Low molecular weight glycolipids such as rhamnolipids and sophorolipids are used in bioremediation as biosurfactants, detergents, and surface cleaners. Oleaginous yeasts such as *Candida tropicalis* and *Candida bombicola* can produce biosurfactants (JOSHI-NAVARE et al., 2013).

#### 2.3.1.5. Bioemulsifier

Bioemulsifier are structurally complex compounds derived from microorganisms and widely used in food industries, such as mannoprotein and liposan (UZOIGWE et al., 2015). Oleaginous yeasts such as *Yarrowia lipolytica, Candida tropicalis,* and *Rhodotorula spp* produce extracellular emulsifiers under nitrogen-limited conditions which are effective in fixing hydrocarbon emulsions (ALIZADEH-SANI et al., 2018).

## 2.4. Oleaginous microorganisms

Oleaginous microorganisms, including yeasts, microalgae, molds, and bacteria, exhibit the capacity to biosynthesize and amass lipids, whereby the lipid content constitutes above 20% of their whole biomass. The oil obtained from this specific source exhibits promise as a feasible alternative for human consumption and the production of biodiesel, serving as a substitute for traditional oil and fat (PAPANIKOLAOU et al., 2019).

### 2.4.1. Oleaginous bacteria

Oleaginous bacteria are a source of TAGs. They efficiently exploit high-carbon waste, such as sewage sludge and food waste, for lipid production, lowering production costs by up to 45% (KOT et al., 2017). The identification and isolation of oleaginous bacteria from mixed cultures pose significant hurdles due to their relatively poor lipid production compared to other microorganisms, as well as the absence of genetic tools for effectively optimizing their lipid biosynthesis (KUMAR et al., 2020). *Rhodococcus* sp., *Gordonia* sp., *Acinetobacter* sp., *and Arthrobacter* sp among others, have been most extensively studied due to their ability to grow on versatile substrates (WANG et al., 2016).

### 2.4.2. Oleaginous microalgae

Oleaginous microalgae are a promising source of renewable biofuels due to their efficient photosynthesis ability. Microalgae cultivation methods include open cultivation systems like ponds, tanks, and raceway ponds, and controlled closed cultivation systems using bioreactors. Open systems offer minimal capital and operating costs, lower energy requirements, and lower energy requirements for culture mixing (ELRAYIES et al., 2018). The manufacture of biodiesel using microalgae encounters several obstacles, such as the significant expenses associated with cultivation, limited yields per unit area, and adverse environmental consequences, including water use, nutrient contamination, and the release of greenhouse gases (PATNAIK et al., 2021). Among the oleaginous microalgae, *Tetraselmis, Chlorella, Nannochloropsis,* and *Scenedesmus* are the most studied.

### 2.4.3. Oleaginous fungi

Oleaginous fungi are promising microbes for biofuel production because they have unique fatty acid profiles with fatty acids, such as gamma-linolenic acid (GLA), that other oleaginous microorganisms cannot synthesize in large amounts (SUBRAMANIAM et al., 2010; SUBHASH et al., 2011). Oleaginous fungi can be grown on inexpensive feedstocks such as molasses, monosodium glutamate wastewater, sewage sludge, glycerol, and agricultural residues (FAN et al., 2012). However, oleaginous fungi encounter several obstacles in the manufacture of biodiesel, such as the significant expenses and limited effectiveness associated with lipid extraction. Additionally, the use of lignocellulosic substrates as feedstocks introduces complications and variations due to their complex nature. Furthermore, the bioflocculation harvesting technique lacks adequate optimization and scaling-up procedures (AYAD et al., 2023). Among the oleaginous fungi, *Mortierella elongata*, *Aspergillus niger*, and *Aspergillus awamori* were most studied (DU et al., 2018; HOARAU et al., 2018).

### 2.4.4. Oleaginous yeasts

Oleaginous yeasts are a promising oil source for biodiesel production due to their ability to store over 20% of their biomass as lipids, primarily triacylglycerides (TAGs). These lipids can be extracted and converted into biodiesel or other valuable products (SIGNORI et al., 2016; PATEL et al., 2016). Oleaginous yeasts are attractive for biotechnological approaches due to their rapid growth rate, short production cycles, and ability to produce lipids from bio-based residues, providing cost-effective carbon sources without land-use change. The triacylglycerol fraction of oleaginous yeast resembles plant oil, making it an ideal candidate for sustainable biofuels and oleochemical building blocks (SHAIGANI et al., 2021). The main challenge in lipid production by oleaginous yeast is finding suitable and low-cost substrates that can support high biomass and lipid yields. Different strains have different preferences and abilities, making it crucial to screen and select the most suitable strains for each substrate and process condition (VASCONCELOS et al., 2019). Oleaginous yeast strains include *Rhodosporidium toruloides, Rhodotorula glutinis, Trichosporon asahii, Rhodotorula mucilaginosa, Cryptococcus oleaginosus, Yarrowia lipolytica, Cryptococcus curvatus, Lipomyces starkeyi, and Trichosporon cutaneum* (SIGNORI et al., 2016; PATEL et al., 2016; SHAIGANI *et al.,* 2021).

#### 2.4.4.1. *Yarrowia lipolytica*

*Yarrowia lipolytica* (division: *Ascomycota*, class: *Saccharomycetes*, family: *Dipodascaceae*) is a dimorphic yeast belonging to the subphylum *Saccharomycetes*. Since this yeast is non-pathogenic and offers easy genetic manipulation (MADZAK et al., 2021), it is a desirable platform for lipid metabolic engineering and can produce value-added chemicals such as enzymes, organic acids, essential fatty acids, and carotenoids (GAO et al., 2017).

#### 2.4.4.2. *Rhodotorula* sp.

*Rhodotorula* is a yeast belonging to the division: *Basidiomycota,* class: *Microbotryomycetes,* family: *Sporidiobolaceae*. Several species of this genus including *R. toruloides* and *R. glutinis* have been identified to accumulate 40-77% lipids in nitrogen-limited medium (VINATI et al., 2016), and are capable of synthesizing natural value-added compounds like carotenoids, terpenoids, extracellular enzymes (MAZA, 2020). Many other oleaginous yeasts of this genus have been identified, such as *R. fluviale DMKU-RK253, R. minuta, R. kratochvilovae, R. azoricum, R. babjevae, R. diobovatum* with lipid content 48–62% (DONZELLA et al., 2019). *Rhodotorula azoricum* and *R. fluviale* have been engineered for enhanced lipid production (POLBUREE et al., 2018).

#### 2.4.4.3. *Cryptococcus* sp.

This group of *Basidiomycetous* yeasts (division: *Basidiomycota*, class: *Tremellomycetes*, family: *Tremellaceae*) is well-known for its oleaginicity and ability to grow on diverse carbon substrates. *Cryptococcus curvatus* has been extensively explored for its oleaginous characteristics and its ability to grow on diverse carbon substrates (BRACHARZ et al., 2017). It has also demonstrated the potential to withstand toxic compounds in lignocellulosic biomass hydrolysate while accumulating a considerable amount of lipid and biomass (CAPUSONI et al., 2017). *Cryptococcus albidus* has also shown a high lipid accumulating ability, with a maximum lipid content of 56.4% DCW (FU et al., 2018). *Cryptococcus terricola* exhibited 61.96% of DCW as lipid content when cultivated on 5% starch, with high proportions of C16:0 and C18:0 (TANIMURA et al., 2014).

#### 2.4.4.4. *Lipomyces starkeyi*

*Lipomyces starkeyi* belongs to the division: *Ascomycota*, class: *Saccharomycetes*, family: *Dipodascaceae*. *Lipomyces starkeyi* is a unicellular ascomycete that reproduces sexually by producing light amber to brown ascospores (KURTZMAN et al., 2011). It is renowned for its ability of lipid accumulation, which usually takes place in the early stationary phase (CALVEY et al., 2016). Research has focused on increasing lipid titer by optimizing conditions for lipid production, such as pH, cofactor induction, nutrient limitation, mode of culture, and type of substrate (PROBST et al., 2017). It also exhibits high oleaginicity with a maximum lipid content of 85.1% DCW reported (JUANSSILFERO et al., 2018), and is also well-known for its efficient utilization of a wide range of substrates (XUE et al., 2018).

#### 2.4.4.5. *Trichosporon* sp.

*Trichosporon cutaneum* is an oleaginous yeast that has been extensively investigated for lipid production in this genus (division: *Basidiomycota*, class: *Tremellomycetes*, family: *Trichosporonaceae*). It produces high amounts of lipid in lignocellulose hydrolysate and corncob hydrolysate withstanding the effect of inhibitory compounds present (WANG et al., 2016) and has also been reported for essential fatty acid production (KOLOUCHOVA et al., 2016). Other oleaginous yeasts of this genus such as *T. corymbiform, T. fermentans, T. dermatitis*, and *T. loubieri* have been reported to produce high quantities of lipid on lignocellulosic raw materials (CHATTOPADHYAY et al., 2020).

#### 2.4.4.6. *Candida* sp

The genus *Candida* belongs to the division: *Ascomycota* class: *Saccharomycetes*, family: *Debaryomycetaceae*. Lipid production has been studied in many species grown on different carbon sources such as *C. bombicola, C. tropicalis* (SOLAIMAN et al., 2004). *C. tropicalis* is a diploid yeast that can ferment diverse sugars and assimilate them through the pentose phosphate pathway. Lipid accumulation in *C. tropicalis* has been significantly enhanced by genetic engineering (CHATTOPADHYAY et al., 2020) and is the most suitable biotransformation platform for many industrial applications (BLANDIN et al., 2000). It is an ideal platform for dicarboxylic acid (DCA) production due to its ability to utilize hydrophobic substrates, owing to its highly functional cytochrome P450 hydroxylase system (CRAFT et al., 2003).

## 2.5. Regulation of triacylglycerides accumulation by oleaginous yeasts

Nitrogen-deficient conditions often stimulate oleaginous yeast to accumulate TAG. In situations where nitrogen is limited, the TAG synthesis takes place. The nitrogen scavenging enzymes, such as AMP deaminase, which deaminates adenosine monophosphate (AMP) and liberates ammonia, which the cell can use when nitrogen is scarce, are responsible for initiating this change in carbon flux (Figure 2). Isocitrate dehydrogenase, the enzyme accountable for catalyzing the transformation of isocitrate to oxoglutarate in the TCA cycle, is inhibited due to the decreased cellular AMP content (BOTHAM and RATLEDGE, 1979; 1981). This inhibition induces isocitrate to accumulate and restore equilibrium with citrate, which is subsequently transported into the cytoplasm by citrate/malate translocase.

EVANS et al. (1983) found that oleaginous yeasts have a citrate/malate translocase with a higher activity than non-oleaginous species, resulting in a citrate efflux of up to eight times greater. Citrate is converted to oxaloacetate and acetyl-CoA in a reaction catalyzed by ATP citrate lyase (ACL). This reaction is critical for the oleaginous phenotype (BOTHAM and RATLEDGE, 1979; 1981). Importantly, cytosolic acetyl-CoA generated via ACL reaction can be utilized in *de novo* FA synthesis. Employing two distinct pathways, cytosolic oxaloacetate is converted to malate and reintroduced into the TCA cycle: either by translocation across the mitochondrial membrane to aid in citrate shuttling, or by catalysis into pyruvate, NADPH, and $CO_2$ by the malic enzyme.

### 2.5.1. *De novo* lipid synthesis

Oleaginous yeasts synthesize lipids, primarily triacylglycerols (TAGs), by converting carbon sources into lipids using two main pathways: sugar-based substrates such as glucose or xylose, and non-sugar substrates such as fatty acids or alcohols. (PAPANIKOLAOU et al., 2011). The sugar-based pathway involves the formation of acetyl-CoA mainly from reaction catalyzed by ACL. The condensation of acetyl-CoA with bicarbonate to produce malonyl-CoA is catalyzed by acetyl-CoA Carboxylase (ACCase). At this stage, the process of lipid synthesis commences by means of the interaction between malonyl-CoA and acyl carrier protein (ACP), which is a constituent of the fatty acid synthase (FAS) complex. Fatty acids (FAs) are synthesized through a series of cyclic processes involving condensation, reduction, and dehydration (BEOPOULOS et al., 2009). The FAS complexes catalyze the sequential addition of Acetyl-CoA units to form long-chain fatty acids, which are then esterified with glycerol-3-phosphate (G3P) to form TAGs. The TAGs are stored in lipid droplets within the cell (PAPANIKOLAOU et al., 2011). The non-sugar pathway involves the activation of substrates by specific enzymes and their incorporation into TAGs by acyltransferases. The activated substrates can be directly attached to G3P to form TAGs or first elongated and desaturated by elongates and desaturases to form different types of fatty acids, which are then attached to G3P to form TAGs. The TAGs are also stored in lipid droplets (PAPANIKOLAOU et al., 2011)

### 2.5.2. *Ex novo* lipid synthesis

The process of *ex novo* lipid production involves the acquisition of hydrophobic substrates, including esters, triglycerides, fatty acids, sterols, and other similar compounds. The process of actively transporting hydrophobic substrates into the cell is facilitated by secreted lipases. Therefore, only microorganisms that possess the ability to produce lipases are able to incorporate free fatty acids. Upon entering the cell, fatty acids (FAs) undergo activation through the action of acyl-CoA synthetases (ACSs), resulting in the formation of Acyl-CoA. This compound serves as the fundamental precursor for lipid synthesis and metabolism within peroxisomes, mostly through the β-oxidation pathway. Acyl-CoA serves as a precursor for the synthesis of phospholipids, which are essential for the formation of cellular membranes, as well as for the synthesis of triacylglycerols (TAGs), which are involved in lipid storage (Figure 2). The process of synthesizing triacylglycerols (TAGs) consists of a series of three consecutive acylation processes, which are facilitated by three enzymes: glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidic acid acyltransferase (LPAT), and diacylglycerol acyltransferase (DGAT). The TAGs that are produced are sequestered within specialized organelles known as lipid droplets (LDs), which are enclosed by a phospholipid monolayer and a variety of proteins (BEOPOULOS et al., 2009).

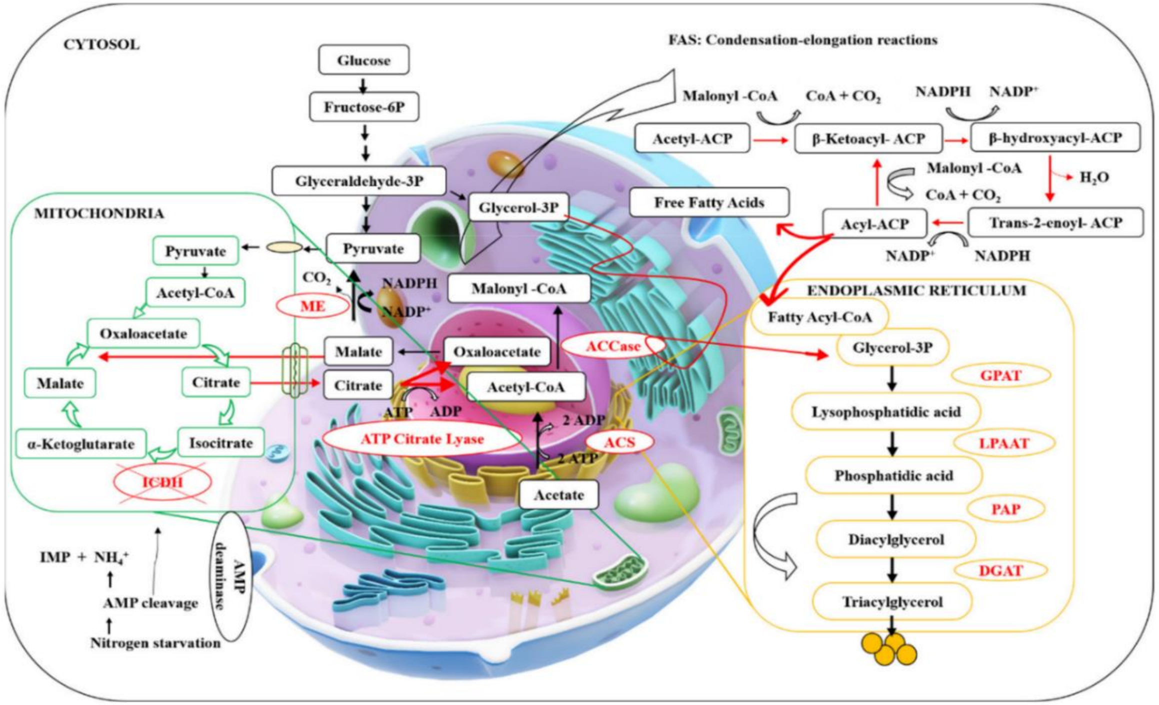
## 2.6. Glycerol uptake by oleaginous yeasts

The process of glycerol uptake in oleaginous yeast is facilitated by a glycerol/H1-symporter, which is encoded by the *STL1* gene. FERREIRA et al. (2005) made a prediction regarding the existence of an active transport system in *S. cerevisiae* based on their observation of the concurrent uptake of glycerol and protons, as well as the accumulation of glycerol within the cell. Moreover, these authors showed that the *STL1* deletion led to the elimination of active glycerol transport and growth in glycerol.

## 2.7. Glycerol assimilation by yeasts

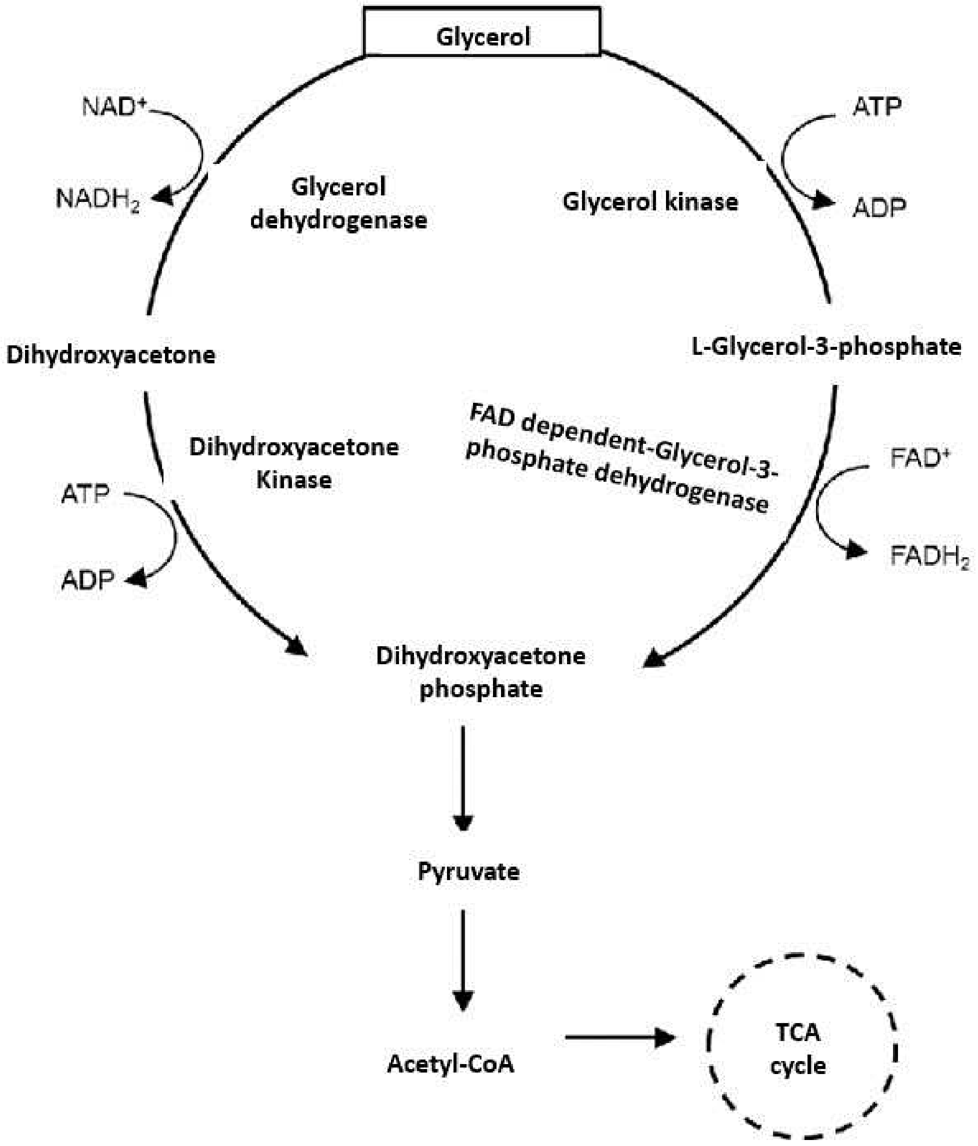
Yeasts employ two distinct metabolic pathways for glycerol assimilation which include the catabolic pathway of Glycerol-3-phosphate (G3P) and the catabolic dihydroxyacetone pathway (DHA) (Figure 2). The phosphorylative glycerol catabolic process yields L-glycerol 3-phosphate (G3P) as an intermediary product. The catabolic pathway of G3P encompasses the participation of two enzymes, namely glycerol kinase (GK) and FAD-dependent glycerol 3-phosphate dehydrogenase (FAD-dependent mG3PDH), which are found on the outer side of the inner mitochondrial membrane. The transfer of electrons from glycerol 3-phosphate dehydrogenase, which is dependent on Flavin adenine dinucleotide (FAD), directly occurs in the respiratory chain through the production of $FADH_2$. The initial step of the catabolic DHA route involves the enzymatic oxidation of glycerol to dihydroxyacetone (DHA) by the action of a glycerol dehydrogenase that is dependent on the coenzyme NAD. Dihydroxyacetone (DHA) is subsequently subjected to phosphorylation by the enzymatic action of DHA kinase (DAK), resulting in the formation of dihydroxyacetone phosphate (DHAP).

**Figure 1** illustrates the metabolic pathways involved in the synthesis of triglycerides (TAG) in oleaginous yeasts. The red circles serve to highlight the key enzymes involved in the process of triglyceride production. The mitochondrial pathway is represented by the colour green, the endoplasmic reticulum pathway is represented by the colour yellow, and the cytosolic pathway is represented by the colour black. The abbreviations used in the illustration are as follows: fatty acid synthase (FAS), isocitrate dehydrogenase (ICDH), malic enzyme (ME), Acetyl-CoA carboxylase (ACCase), Acetyl-CoA synthetase (ACS), glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidic acid acyltransferase (LPAAT), phosphatidate phosphatase (PAP), and diacylglycerol acyltransferase (DGAT).



**Source**: PROBST et al., 2016)

**Figure 2** illustrates the glycerol assimilation by oleaginous yeasts. The catabolic pathway of Glycerol-3-phosphate (G3P) involves two enzymes, glycerol kinase (GK) and FAD-dependent mG3PDH. Glycerol kinase (GK) transfers electrons from glycerol 3-phosphate dehydrogenase, which is dependent on Flavin adenine dinucleotide (FAD), directly into the respiratory chain. The catabolic DHA route involves glycerol oxidation to dihydroxyacetone (DHA), which is phosphorylated by DHA kinase (DAK) to form dihydroxyacetone phosphate (DHAP).



**Source**: RIVALDI et al., 2012

## 2.8. Fermentation conditions for SCOs production

Lipid accumulation in oleaginous yeasts is influenced by nitrogen limitation, carbon source, nitrogen source, C: N ratio, oxygenation, temperature, pH, time of incubation, and mineral salt concentration (CHOPRA et al., 2018, ABELN et al., 2020). Optimization of processes is necessary to improve economic feasibility, influenced by substrate cost, production rate, and lipid yields (KOUTINAS et al., 2014). Different parameters affect SCO production separately.

### 2.8.1. Effect of type and concentrations of carbon source

The type of carbon source significantly impacts the final yield of a yeast's growth process. The main sugars used by yeasts include glucose and xylose, followed by fructose, arabinose, mannose, galactose, and in some cases, glycerol, hydrocarbons, and oils (AWAD et al., 2018). The main biochemical pathway involved in sugar metabolism is glycolysis, where two molecules of acetyl-CoA are generated from one molecule of glucose. The theoretical maximum yield of lipid synthesis is 0.32 g/g and 0.30g/g for glucose and glycerol respectively. The highest theoretical lipid yield derives from the use of acetate as a carbon source, with a of 0.45 g/g (PAPANIKOLAOU et al., 2014). Not all Acetyl-CoA can be channeled towards lipid synthesis, as a portion is used for different metabolic processes. During the lipogenic phase, high quantities of NADPH are required, which greatly reduces the lipid yield. Tight regulation of the metabolic cycle between energy synthesis and lipid production was observed in *Y. lipolytica* (LIU et al., 2016).

A study by AWAD et al. (2018), analyzed the behavior of *C. oleaginosus* on different carbon sources. The highest biomass yield (18.4 $gL^{-1}$) was obtained on lactose, with a lipid content of 49.7%. When sorbitol was the only source of carbon, the lowest biomass and lipid yield were obtained, 4.5 $gL^{-1}$ and 13.4%, respectively. The best conversion of sugars into lipids was obtained with mannose (52% lipid content). *Lipomyces starkeyi* manages to accumulate 85% of intracellular lipids in the presence of a mixture of simple sugars (glucose and xylose) whereas in the presence of only glucose the lipid accumulation was lower (about 79.6%) (JUANSSILFERO et al., 2018). Similarly, SANTEK et al. (2017) found that growing *T. oleaginosus* in the xylose-containing medium increased lipid accumulation while decreasing cell proliferation, whereas glucose-containing media had the reverse effect. Increased sugar concentration in media has opposite effects on lipid and productivity in various oleaginous yeasts such as *Rhodotorula glacialis*, *Trichosporon cutaneum* (HU et al., 2011) *L. starkeyi* (WILD et al., 2010) and *C. curvatus* (DI FIDIO et al., 2019).

In a study using *L. tetrasporus*, an inverse correlation was observed between sugar concentration and lipid synthesis, with decreased lipid synthesis resulting in increased secondary product production (CAPORUSSO et al., 2021). The accumulation of intracellular polysaccharides and polyols observed in *C. curvatus* could be explained by changes in cellular metabolism due to exposure to high osmotic stress (TCHAKOUTEU et al., 2015). Similar phenomena also occur in other yeast species, such as *S. cerevisiae* yeast, which osmotic stress induces a series of molecular, physiological, and morphological events to maintain cellular activity. Yeast cells synthesize and accumulate small molecules, which are supposed to act as osmoprotectants. (BABAZADEH et al., 2017). To investigate the ability to utilize lignocellulosic carbon sources, tolerate inhibitory compounds, and grow in a medium without integrated vitamins. SITEPU et al. (2014) screened 48 oleaginous yeast strains belonging to 45 species, the study found that not all microorganisms were able to grow in low-cost carbon sources, and high variability among different strains of the same species was found.

Glycerol, a by-product of the biodiesel industry, has attracted a lot of interest in recent years, as the flourished production of biodiesel and the huge production of glycerol have stimulated research towards the enhancement of glycerol, used as the carbon source for SCO production (DOBROWOLSKI et al., 2016). *L. starkeyi* DSM 70296 and *L. starkeyi* NRRL Y-11557 have been reported to utilize crude glycerol for lipid production. The lipid yield of *L. starkeyi* DSM 70296 was reported to reach 12.3 g/L after 470 h of fermentation (TCHAKOUTEU et al., 2015; SPIER et al., 2015). Strains of the non-conventional yeasts *Yarrowia lipolytica* and *Rhodosporidium* sp. have been employed as microbial cell factories capable of converting glycerol into a plethora of useful microbial metabolites (TSIRIGKA et al., 2023). *Yarrowia. lipolytica* yeast was employed to produce microbial mass, microbial lipids (single-cell oils; SCOs) (FICKERS et al., 2020).

### 2.8.2. Type and concentrations of nitrogen source and carbon; nitrogen ratio (C: N ratio)

Nitrogen sources can influence lipid accumulation, and its limitation in the medium is crucial. Ammonium sulfate $(NH_4)_2SO_4$, peptone, yeast extract, urea, corn steep liquor, L-arginine, corn gluten, tomato waste hydrolysate, and whey concentrate are the common nitrogen sources used to produce SCO (DONOT et al., 2014). The best organic and inorganic nitrogen sources were yeast extract and ammonium sulfate, respectively. Numerous studies have examined the relationship between nitrogen concentration and source and lipid biosynthesis. According to LOPES (2020), the C: N ratio had no significant effect on the proliferation of *T. oleaginous*, but it did influence the total lipid content. However, increasing the C: N ratio in the growth medium had the opposite effect on the efficacy parameters of the bioprocess, increasing lipid yields and productivity while decreasing biomass and growth rate. Maximum lipid concentration and productivity were 5.4 $gL^1$ and 1.8 $gL^1$, respectively, with a lipid yield of 13%, whereas maximum lipid concentration and productivity were 3.1 $gL^1$ and 1.5 $gL^1$, with a lipid yield of 6%, when the C: N ratio was 80. Similar results were reported for *R. glutinis*, which exhibited an increase in lipid accumulation in the medium with a C: N ratio of 20 to 70 and a decrease when the C: N ratio was increased to 100 (TKÁˇCOVÁ et al., 2017).

According to AWAD et al. (2019), the optimal C: N ratio is 120. Urea and $NO_3$ were determined to be the most effective nitrogen sources for lipid production among those examined. CHOPRA et al. (2018) assessed the impact of various inorganic nitrogen sources, including $NH_4Cl$, $(NH_4+)_2SO_4$, and $NaNO_3$, on *Pichia guilliermondii*. Although it has been demonstrated that organic nitrogen sources such as yeast extract, peptone, and urea are superior for biomass and lipid production, they are not economically viable. The study revealed that $NH_4Cl$ is superior to $NaNO_3$ as a nitrogen source, and the yield of lipids obtained with sodium nitrate was substantially less than with ammonium chloride and $(NH^+)_2SO_4$. These results suggest that $NH_4^+$ is the preferred N source for this microorganism.

### 2.8.3. Hydrogen ion concentration (pH)

The pH of a medium plays a crucial role in the microbial assimilation of carbon sources. The surface properties of the cell membrane influence the carbon assimilation process. Studies have shown that lipid production by *Y. lipolytica* is suitable at pH 6 to 6.5 (TIMOUMI et al., 2017), with a slightly acidic pH (5 to 6.5) being more favorable for lipid accumulation (KUTTIRAJA et al., 2018) The optimal pH value varies among strains and depends on the carbon source (SLININGER et al., 2016). In a study on *Rhodotorula glutinis*, no significant effect was observed on lipid or carotenoid synthesis in the pH range of 4 to 7. However, initial pH of 3.0 led to a reduction in biomass and carotenoid production, and a pH of 2 strongly inhibited microorganism growth (KOT et al., 2017).

### 2.8.4. Temperature

Oleaginous yeasts that can grow at high temperatures are essential in large-scale fermentation processes, as they reduce costs associated with cooling and allow for simultaneous saccharification and fermentation (SSF) mode implementation. Studies have shown that strains with a narrow temperature range and strains with a broad temperature range have different growth behaviors (LAMERS et al., 2016). *Hansenula beijerinckii* and *Saccharomyces cerevisiae* have a narrow optimum of 5°C, while *Pichia anomala, Waltomyces lipofer,* and *Torulaspora delbrueckii,* can vary the growth temperature by 29°C without negatively impacting growth and lipid production (LAMERS et al., 2016). ABELN and CHUCK'S (2020) recent study found that increasing temperature decreases biomass and lipid yield, while arabitol and glycerol yields increase.

### 2.8.5. Effect of oxygenation

Few studies have examined the impact of oxygen on lipid production, with results varying across species. For instance, *C. curvatus* grown on casein whey showed decreased lipid yields with lower oxygenation rates 20% to 15% (CALVEY et al., 2015). However, some studies found contradictory results, with no correlation found between oxygenation and lipid yield in *L. starkeyi* (CHRISTOPHER et al., 2016). *R. glutinis* cells at high dissolved oxygen concentrations had reduced lipid accumulation (CALVEY et al., 2015). *Rhodotorula glutinis* cells in highly aerobic fermenters showed a fast growth rate and high cell mass production, but a 50% reduction in average lipid content (YEN et al., 2014).

### 2.8.6. Effect of mineral salts and other components

Some ions and mineral compounds, such as $Mg^{+2}$, $Ca^{+2}$, $Mn^{+2}$, $Fe^{+3}$, $Cu^{+2}$, and $Zn^{+2}$, can affect biomass and lipid content (BEOPOULOS et al., 2009). ZHAO et al. (2008) showed that $MnSO_4$, $ZnSO_4$, $MgSO_4$, $CoCl_2$, $CuSO_4$, and $FeSO_4$ in appropriate concentrations can increase cell growth and lipid accumulation. Each element has a different impact on growth and lipid production, and their impact is influenced by the presence of other molecules. For example, in *R. toruloides*, biomass production was not affected by $MgSO_4$, while $KH_2PO_4$ positively affected yeast biomass. However, the increase in $MgSO_4$, and yeast extract concentrations affected biomass production until 0.25 $gL^{-1}$, and no further increase was observed. The same effect was observed for glucose, with biomass increased up to 0.35 $gL^{-1}$ of $MgSO_4$ but no effect was observed at concentrations higher than this value (SARAN et al., 2017). KRAISINTU et al. (2010) found the highest lipid production (8.8 $gL^{-1}$) at 0.4 $gL^{-1}$ of $KH_2PO_4$.

# 3 MATERIALS AND METHODS

## 3.1. Collection of the yeast isolates and maintenance

The ten yeast isolates used for this study were obtained from the culture collection of the Microbial Physiology Laboratory, Department of Microbiology, Universidade Federal de Viçosa. They were previously isolated from soil samples from two National parks (Caparaó and Serra dos Órgãos) in the state of Minas Gerais and Rio Janeiro, respectively (VIEIRA et al., 2020). In terms of yeast maintenance, pure cultures of yeast isolates were inoculated in sterile 30 mL Yeast Extract Peptone broth containing glucose (YPD), which consisted of [1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) glucose], in 125 mL Erlenmeyer flasks. They were incubated at 30 °C and 200 rpm for 18 hours in a G25 rotary shaker and centrifuged at 2688 *g* at for 20 minutes. The supernatant was discarded, and the cell pellet was washed twice with 0.9% (w/v) saline solution. The cell pellets were suspended in 5 mL YP medium without glucose. Subsequently, a volume of 800 µL of the suspended culture was transferred into cryovial tubes with a capacity of 1.5 mL, along with an additional 200 µL of sterile glycerol. The stock cultures were cryopreserved using liquid nitrogen and stored in an ultra-low temperature freezer at -80 °C for further use.

## 3.2. Recovery of the cryopreserved yeasts from the culture collection

The yeast isolates were cultivated in a YPD broth, and incubated at 30 °C for a duration of 48 hours. The yeast isolates that demonstrated growth in YPD broth was further cultivated in Yeast Nitrogen Base agar (YNB (0.67% w/v)) (Difco Laboratories, Becton, and Dickinson company) supplemented with 2% glycerol as the carbon source and incubated at 37 °C for 72 hours.

## 3.3. Preparation of culture media and inoculum

The SS2 medium was prepared in agreement with the procedure described by TANIMURA *et al.* (2014), which included the following components: 0.5 g/L yeast extract, 0.133 g/L $(NH_4)_2SO_4$, 0.5 g/L $MgSO_4$, 0.1 g/L $CaCl_2$, 0.1 g/L NaCl, and 20 g/L pure glycerol. The culture medium was used for the purpose of preparing inoculum, cultivating, and screening yeast isolates in order to assess their potential for both biomass and lipid production. Furthermore, the assessment of the impact of various carbon: nitrogen (C: N) ratios on lipid production was conducted using an SS2-glycerol (40 g/L). The ratio of carbon source in glycerol to nitrogen content in yeast extract and ammonium were considered when preparing the SS2 medium, as shown in Table 1.

The inoculum was obtained by transferring a single yeast colony from a freshly cultured YNB plate into 30 mL of SS2 medium supplemented with 2 % (w/v) glucose as a carbon source. The mixture was then incubated at a temperature of 30 °C with a rotational speed of 200 rpm for 18 hours using a G25 rotating shaker. The resulting inoculum was centrifuged at 2688 g for 10 minutes and the supernatant was discarded. The cell pellet was washed twice with 0.9% w/v saline solution and the inoculum was thereafter adjusted to an optical density of 0.1 at a wavelength of 600 nm.

**Table 1:** SS2 culture medium composition for assessing the impact of varying carbon-to-nitrogen ratios on lipid production and growth.

| **Medium component (g/L)** | **C: N 50:1** | **C: N 75:1** | **C: N 100:1** | **C: N 125:1** | **C: N 150:1** |
|---|---|---|---|---|---|
| Yeast extract | 1 | 1 | 1 | 1 | 1 |
| $(NH4)_2SO_4$ | 1.009 | 0.514 | 0.267 | 0.119 | 0.0204 |
| $MgSO_4$ | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| $CaCl_2$ | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| NaCl | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Glycerol | 40 | 40 | 40 | 40 | 40 |

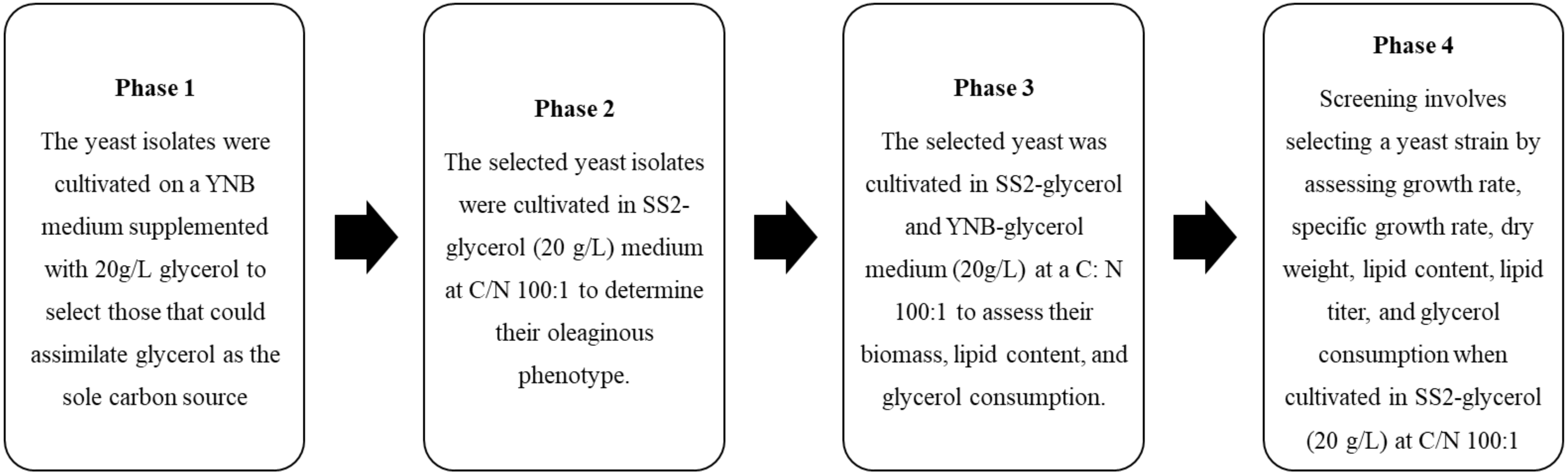
### 3.4. Screening of the yeast isolates

The screening procedure consisted of four sequential phases (Figure 3). Initially, yeast isolates were cultivated on a YNB medium supplemented with glycerol (20 g/L) .to select yeast isolates able to assimilate glycerol as the sole carbon source. Secondly, the selected yeast isolates in the preliminary phase were cultivated in SS2-glycerol (20 g/L) medium at C/N 100:1 to determine their oleaginous phenotype. In the third stage, the yeast isolates that were selected in the previous phase were cultivated in SS2-glycerol medium (20 g/L) and YNB-glycerol (20 g/L) at C/N 100:1, to evaluate their biomass, lipid content, and glycerol consumption. The fourth stage of the screening involves the selection of a yeast strain by determining. its specific growth rate, dry weight, lipid content, lipid titer, and glycerol consumption when cultivated in SS2-glycerol (20 g/L) at C/N 100:1

### 3.4.1. Evaluation of biomass and lipid content of yeast isolates in the screening steps

The yeast isolates were cultivated in 70 mL of SS2 glycerol medium adjusted to a C: N ratio of 100:1 at 30 °C and 200 rpm for 192 hours. Aliquots of one milliliter (1 mL) were withdrawn along the cultivation of isolates and transferred aseptically into 1.5 mL sterile microtubes in order to evaluate the biomass by measuring the $OD_{600}$. After, the aliquots were centrifuged at 19,356 *g* for 10 minutes and the supernatant was collected and stored for evaluation of glycerol consumption. The final culture was recovered after 192 hours, centrifuged at 2688 g for 10 minutes, washed twice with 0.9% (w/v) saline solution, frozen with liquid nitrogen, and lyophilized for 24 hours. The lyophilized biomass was used for lipid quantification.

**Figure 3** shows the screening stage for selecting a promising oleaginous yeast strain, which was conducted in four sequential phases**.** In the first stage, YNB medium-supplemented glycerol was used to select yeast isolates that could assimilate glycerol as the sole carbon source. In the second stage, yeast isolates were cultivated in an SS2-glycerol medium to determine their oleaginous phenotype. In the third stage, yeast isolates were cultivated in both SS2-glycerol and YNB-glycerol mediums to evaluate their biomass, lipid content, and glycerol consumption. The fourth stage involved selecting a yeast strain by determining its specific growth rate, dry weight, lipid content, lipid titer, and glycerol consumption when cultivated in SS2-glycerol medium.



**Phase 1**

The yeast isolates were cultivated on a YNB medium supplemented with 20g/L glycerol to select those that could assimilate glycerol as the sole carbon source

**Phase 2**

The selected yeast isolates were cultivated in SS2-glycerol (20 g/L) medium at C/N 100:1 to determine their oleaginous phenotype.

**Phase 3**

The selected yeast was cultivated in SS2-glycerol and YNB-glycerol medium (20g/L) at a C: N 100:1 to assess their biomass, lipid content, and glycerol consumption.

**Phase 4**

Screening involves selecting a yeast strain by assessing growth rate, specific growth rate, dry weight, lipid content, lipid titer, and glycerol consumption when cultivated in SS2-glycerol (20 g/L) at C/N 100:1

**Source:** The authors

## 3.5. Identification of the selected oleaginous yeasts

The total DNA was extracted according to the rapid isolation of the yeast DNA protocol (SAMBROOK and RUSSEL, 2001). This was done by suspending the cell pellet in 293 µL of 50 mm Ethylene Diamine Tetra-acetic Acid (EDTA) and 7.5 units / µL lyticase. The suspension was homogenized gently, incubated at 37 ° C for 60 minutes, centrifuged at 26,673 *g* for 3 minutes and the supernatant was discarded. The protein precipitation and DNA rehydration were carried out by adding 300 µL nuclei lysis solution, homogenized, incubated for 5 minutes on ice, centrifuged at 26, 673 *g* for 3 minutes and the supernatant was transferred to a new microtube containing 300 µL of isopropanol. The suspension was mixed by inversion, centrifuge at 26, 673 *g* for 2 minutes and the supernatant was discarded. Three hundred microliters (300 µL) of 70% (v/v) ethanol were added and centrifuged at 26, 673 *g* for 2 minutes and allowed to aspirate. The pellet was allowed to be air-dried and 50 µL of DNA rehydration solution and 1.5 µL RNase were added. The suspension was incubated at 37 $^{O}$C for 15 minutes and was allowed to rehydrate at 65 $^{O}$C for 1 hour. This procedure was carried out at the Microbial Physiology Laboratory, Universidade Federal Vicosa, Brazil.

The ribosomal cluster region, which includes the internal transcribed spacers (ITS), and 5.8S rRNA gene, and the D1/D2 domains of the large subunit rRNA gene, was amplified by polymerase chain reaction. (LACHANCE et al., 2003). The amplified region was then subjected to sequencing and sequences underwent analysis using the MEGA11 programme (TAMURA et al., 2013) and were compared with those in GenBank employing BLAST, a Basic Local Alignment Search Tool (BLAST, http://www.ncbi.nlm.nih.gov). This procedure was carried out at the Instituo de Ciências Biológicas, Universidade Federal de Minas Gerais, Brazil.

## 3.6. Effect of the carbon: nitrogen ratio on lipid accumulation by the oleaginous yeast

To obtain different C: N ratios (50:1; 75:1; 100:1; 125:1; 150:1), batch cultures were performed using SS2 glycerol medium (40 g/L) and different concentrations of $(NH_4)_2SO_4$. *Meyerozyma guilliermondii* UFV-2 was cultivated in triplicate in a G25 rotary shaker at 30 °C and 200 rpm. The cells were harvested after 192 hours of growth by centrifugation at 2688 *g* at for 10 minutes, washed twice with 0.9% w/v saline solution, and lyophilized for 24 hours. The lyophilized biomass was used to quantify the lipid production gravimetrically. The $OD_{600nm}$ was used to monitor cell growth, whilst the concentration of glycerol in culture supernatants was determined using high-performance liquid chromatography (HPLC). This experiment was carried out in triplicate

## 3.7. Analytical procedures

### 3.7.1. Determination of specific growth rate and dry weight

The cell growth was monitored using optical density at OD600, and the specific growth rate (μ) was determined using a linear regression between logarithm values of OD600 and cultivation time (h). A calibration curve was constructed to determine the dry weight (DCW) of biomass. The yeast was transferred to 10 mL SS2 glycerol medium and incubated at 30 °C at 200 rpm for 18 h. The culture was then filtered using dried membranes (0.22 m pore and 47 mm diameter), dried in a microwave (low power, -250 W) for 20 minutes), and kept in a desiccator. The dried membrane was weighed to determine the dry weight of the biomass. In parallel, 1 mL of the cell suspensions was diluted six times with 0.9% w/v saline solution, and the OD600 was measured. The DCW was calculated using a linear regression plot of OD600 versus DCW (mg/mL).

### 3.7.2. Evaluation of glycerol consumption by the yeast isolates

The concentration of glycerol was determined using high-performance liquid chromatography (HPLC). The culture supernatant of 20 μL was passed through a 0.13-mm syringe filter (0.45 μm, Merck Millipore Co., Darmstadt, Germany). The Aminex ion exchange column (HPX-87H 300 × 7.8 mm, 9 μm, Bio-Rad, Munich, Germany) was used to separate and quantify the target analytes based on their ionic properties. The eluent of 5 mM $H_2SO_4$ (0.7 mL/min, at 40 °C) was chosen to optimize the separation and ensure good resolution of the peaks detected by a refractive index detector (RID-20A, Shimadzu). The results obtained were plotted with the standard glycerol curve.

### 3.7.3. Quantification of lipids by the yeast isolates

The method described by BLIGH and DYER (1959) as modified by VIEIRA et al. (2020) was used to quantify the lipids accumulated by the yeast isolates. One milliliter (1 ml) of methanol: chloroform solution (2:1, v/v) and 2 tungsten beads were added to 50 mg of each of the lyophilized biomass, and homogenized using Tissuelyser II (Qiagen, Hidden, North Rhine-Westphalia Germany) with a vibrating frequency of 30 shakes per second for 5 minutes, centrifuge at 19,356 *g* for 10 minutes and the supernatant was collected in 15-mL centrifuge glass tubes. This step was repeated two more times. Three milliliters (3 mL) of 100% chloroform and 2 mL of 1% normal saline (w/v) were added and homogenized to obtain a two-phase liquid system. The phases were separated by centrifugation at 4,200 *g* at 4 °C for 10 minutes, and the lower phase (organic phase) was transferred to previously weighed 2 mL microtubes. The samples were then evaporated in a heating blanket at 60 °C for 24 hours, and the lipid content was determined gravimetrically.

### 3.8. Data and statistical analysis

The data were collected in triplicate and reported as the mean value with standard deviation. Subsequently, a one-way analysis of variance (ANOVA) was conducted using the Statistical Package for Social Sciences (Free SPSS version 21.0). Statistically significant differences between means were identified at a confidence level of 95% ($p < 0.05$), and were further assessed using the Duncan multiple range test for comparison. The data gathered was visually represented via the use of Microsoft Excel® 2010, using graphical presentations.

## 4. RESULTS AND DISCUSSION

The utilization of glycerol, a by-product generated during biodiesel manufacture, as a carbon source for oil production by oleaginous is desirable from a circular economy point of view, as this oil can be used as feedstock for the production of this biofuel. This study focused on selecting oleaginous yeasts capable of producing oil in culture media containing glycerol as the sole carbon source. Out of the total of ten isolates that were tested for their ability to assimilate glycerol as the carbon source for lipid accumulation, five (5) isolates displayed the oleaginous phenotype, i.e., lipid contents higher than 20 % (w/w) in SS2 medium containing glycerol as the sole carbon source (Table 2). It is worth mentioning that among the five yeast isolates, isolates CA8 and SO4 exhibited notable performance in terms of achieving the greatest lipid titers and cell densities. Consequently, they were chosen for further characterization.

**Table 2**: Lipid contents (%), Lipid titers (g/L), and Final cell densities ($OD_{600nm}$) of the five yeast isolates selected in the second screening phase. These isolates were cultivated in SS2-glycerol medium (20 g/L) at a C: N ratio of 100: 1 for 192 hours at 30 °C and 200 rpm.

| **Isolates** | **Lipid content (%)** | **Lipid titer (g/L)** | **Final cell density ($OD_{600nm}$)** |
|---|---|---|---|
| CA10 | 20.21 | 0.22 | 5.3360 |
| SO33 | 21.67 | 0.32 | 6.3483 |
| SO4 | 22.03 | 0.43 | 6.6490 |
| SO35 | 20.25 | 0.24 | 5.2350 |
| CA8 | 23.34 | 0.65 | 8.4340 |

To evaluate the parameters of both growth and lipid production, the isolates CA8 and SO4 were cultivated in an SS2-glycerol medium (20 g/L) with C: N 100:1, for 192 hours. The isolate CA8 displayed the highest values of both specific growth rate and final biomass with higher glycerol consumption. However, lipid contents were similar statistically. Otherwise, the lipid titers achieved by the isolate CA8 were higher than the isolate SO4 (Table 3). Taken together, these results underscore the potential of the isolate CA8 to be used in a bioprocess of oil production from fermentation media containing glycerol as the carbon source. As such, it was selected as the best lipid producer and was further characterized.

**Table 3**: Lipid contents [% (w/w)], lipid titers (g/L), biomass concentrations (g/L), specific growth rates (μ), glycerol concentrations (g/L) and percentage of glycerol consumption (%) of both isolates CA8 and SO4. The cultivations were carried out in a shake flask experiment using SS2-glycerol medium (20 g/L) with C: N 100: 1, for 120 hours (*), and for 192 hours, (**), at a temperature of 30°C and 200 rpm. The values were reported as means with the standard deviation when n = 3. Values with distinct superscript letters in each column show a statistically significant difference ($p < 0.05$).

| **Isolates** | **Lipid content [%(w/w)]** | **Lipid titer (g/L)** | **Biomass concentration (g/L)** | **Specific growth rate (μ)** | **Glycerol consumption (g/L)** | **Glycerol consumption (%)** |
|---|---|---|---|---|---|---|
| CA8* | $20.8 \pm 0.64^a$ | $0.56 \pm 0.02^c$ | $2.68 \pm 0.02^c$ | $0.24 \pm 0.00^b$ | $7.67 \pm 0.56^a$ | $38.35 \pm 2.84^a$ |
| SO4* | $20.5 \pm 0.39^a$ | $0.39 \pm 0.01^a$ | $1.90 \pm 0.04^a$ | $0.21 \pm 0.00^a$ | $7.50 \pm 0.05^a$ | $37.50 \pm 0.29^a$ |
| CA8** | $22.7 \pm 0.40^b$ | $0.68 \pm 0.01^d$ | $2.99 \pm 0.06^d$ | $0.24 \pm 0.00^b$ | $10.84 \pm 0.03^c$ | $53.23 \pm 0.01^c$ |
| SO4** | $22.1 \pm 0.38^b$ | $0.45 \pm 0.02^b$ | $2.00 \pm 0.06^b$ | $0.21 \pm 0.00^a$ | $9.95 \pm 0.15^b$ | $49.75 \pm 1.08^b$ |

The isolate CA8 was taxonomically identified as *Meyerozyma guilliermondii* and was designated as *M. guilliermondii* UFV-2 (Table 4). *Meyerozyma guilliermondii* exhibits distinctive biochemical properties and potential for a wide range of biotechnological applications. This is primarily attributed to its remarkable capacity to utilize diverse carbon sources, including hydrophilic and hydrophobic compounds (Wei et al., 2021).

Table 4: Taxonomic identification of the promising yeast isolate *Meyerozyma guilliermondii* UFV-2

| **Collection code** | **Species** | **Strain** | **CBS reference number** | **Access number** | **Identity** |
|---|---|---|---|---|---|
| CA8 | *Meyerozyma guilliermondii* | UFV-2 | CBS 2030 | MH545918.1 | 505/506 (99%) |

Cultivation under nitrogen-limiting conditions has been shown to enhance the accumulation of lipids in oleaginous organisms (BÉLIGON et al., 2016; SHEN et al., 2017). Indeed, the ratio of carbon to nitrogen (C: N ratio) has been identified as a critical parameter that significantly influences the synthesis of lipids. As such, the effect of various C: N ratios on lipid production and biomass formation by *M. guilliermondii* UFV-2 was evaluated (Table 5). The highest biomass production was observed in C: N ratios 150: 1 and C: N 125: 1, while the lowest biomass production was recorded in a C: N 75: 1. The highest lipid content (%) and lipid titer (g/L) were observed at C: N 150: 1; otherwise, the lowest lipid content and lipid titer were recorded in C: N ratios of 50:1 and C: N 75:1, respectively (Table 5).

**Table 5:** Lipid contents [% (w/w)], lipid titers (g/L), biomass concentrations (g/L), specific growth rates (μ), glycerol concentrations (g/L) and percentage of glycerol consumption (%) by *Meyerozyma guilliermondii* UFV-2 with different C: N ratios. The cultivations were carried out in a shake flask experiment using SS2-glycerol medium (40 g/L), for 192 hours at a temperature of 30 °C and 200 rpm.

| **C:N ratios** | **Lipid contents [% (w/w)]** | **Lipid titer (g/L)** | **Biomass concentration (g/L)** | **Specific growth rate (μ)** | **Glycerol consumption (g/L)** | **Percentage of glycerol consumption (%)** |
|---|---|---|---|---|---|---|
| 50:1 | $20.1 \pm 0.01^{a}$ | $1.07 \pm 0.05^{ab}$ | $5.34 \pm 0.25^{c}$ | $0.23 \pm 0.00^{a}$ | $17.91 \pm 0.18^{bc}$ | $44.78 \pm 0.46^{bc}$ |
| 75:1 | $22.1\pm 0.62^{b}$ | $1.03 \pm 0.01^{a}$ | $4.67 \pm 0.13^{a}$ | $0.24 \pm 0.00^{d}$ | $17.44 \pm 0.09^{ab}$ | $43.62 \pm 0.24^{ab}$ |
| 100:1 | $23.0 \pm 0.20^{c}$ | $1.13 \pm 0.06^{b}$ | $4.93 \pm 0.24^{b}$ | $0.24 \pm 0.00^{cd}$ | $17.32 \pm 0.49^{a}$ | $43.31 \pm 1.22^{a}$ |
| 125:1 | $24.1 \pm 0.65^{d}$ | $1.61 \pm 0.03^{d}$ | $6.71 \pm 0.06^{e}$ | $0.23 \pm 0.00^{bc}$ | $18.27 \pm 0.40^{c}$ | $45.69 \pm 1.01^{c}$ |
| 150:1 | $25.1 \pm 0.05^{e}$ | $1.71 \pm 0.02^{e}$ | $6.79\pm 0.05^{e}$ | $0.23 \pm 0.00^{ab}$ | $18.29 \pm 0.14^{c}$ | $45.73 \pm 0.35^{c}$ |

The values were reported as means with the standard deviation, when n = 3. Values with distinct superscript letters in each column show a statistically significant difference ($p < 0.05$).

RAMÍREZ-CASTRILLÓN, et al. (2017), identified *M. guilliermondii B*1281A as a potential oil source for biodiesel production, recording a lipid content of 34.97%. The experiment was conducted under optimized conditions at 26 °C, 150 rpm for 72 hours of cultivation. In addition, HALIMA, et al. (2019), studied the potential of four lipogenic yeasts, *Candida molendinolei* MH267796, *M caribbica* MH267795, *Rhodotorula minuta* MH267794, and *Cryptococcus curvatus* ATCC20509, to produce lipid using glycerol as a carbon substrate. They found that *Meyerozyma caribbica* MH267795 is the best candidate for microbial oil production using crude or pure glycerol. This yeast strain produced 42.12 g/L of dry biomass and 20.28 g/ L of lipids after 220 hours, indicating high lipid accumulation during growth on crude glycerol.

In shake-flask fermentation initial screening, *M. caribbica* MH267795 showed the highest lipid accumulation of up to 40.86% (w/w) dry biomass when cultivated in crude glycerol. This strain outperformed other tested strains from the same species. IIMI, et al. (2021) demonstrated that *Zygosaccharomyces siamensis* AP1 is capable of achieving a lipid content of 30% when carbonated with 75 g/L of glycerol and a C: N ratio of 120. A study on *Candida freyschussii* revealed that an increase in the C: N ratio stimulated lipid production by 33% (RAIMONDI et al., 2014). In addition, LIU, et al. (2017) observed that lipid content in *Lipomyces starkeyi* AS 2.1560 increased with the increase of the C: N ratio, reaching a maximum of 32.3% at a C: N ratio of 110.

This study revealed that *Meyerozyma gulliermondii UFV-2* has limited capacity to completely metabolize all available glycerol in the growth medium, perhaps attributable to variables. Further studies should address the effects of glycerol concentrations, cultivation conditions and C: N ratio on glycerol assimilation. Even though the lipid contents obtained by *M. guilliermondii* UFV-2 were lower compared to some studies described above, the optimal conditions for lipid production by *M. guilliermondii* UFV-2 has not yet been evaluated. This indicates that further optimization strategies could increase lipid contents and titers, suggesting further research is needed to improve growth conditions and glycerol utilization by oleaginous yeast.

# 5. CONCLUSION

*Meyerozyma guilliermondii* UFV-2, selected as the best lipid producer strain in this study, has potential to serve as an oil source for biodiesel production, recording a lipid content of 25% under unoptimized conditions. Therefore, this study opens perspectives of further works of optimization of lipid production by *Meyerozyma guilliermondii* UFV-2 such as varying glycerol concentration, nitrogen sources, optimal initial inoculum, temperature, pH and agitation rate.

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