JETIR.ORG ISSN: 2349-5162 | ESTD Year : 2014 | Monthly Issue
JOURNAL OF EMERGING TECHNOLOGIES AND INNOVATIVE RESEARCH (JETIR)
An International Scholarly Open Access, Peer-reviewed, Refereed Journal

Oleaginous Yeast: A Promising Pathway for Biodiesel Production

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Abstract: As the global demand for renewable and sustainable energy sources continues to rise, biodiesel has emerged as a promising alternative to traditional fossil fuels. Oleaginous yeasts have garnered significant attention in recent years as a potential sustainable source for biodiesel production. Their ability to accumulate high levels of lipids, including triacylglycerols (TAGs), makes them an attractive alternative to traditional oil crops and petroleum-based sources.

To ensure the economic viability and environmental sustainability of biodiesel production, optimization of the production process is crucial. Resonance Surface Methodology (RSM) has gained significant attraction as a powerful tool for optimizing biodiesel production processes.

This review explores the characteristics of oleaginous yeast, their lipid accumulation capabilities, and the various strategies employed to enhance lipid production. Additionally, it discusses the potential of oleaginous yeast as a viable alternative to traditional oil sources in biodiesel production. It also provides an in-depth analysis of the applications of RSM in biodiesel production optimization. It explores the principles of RSM, its advantages, and its integration with biodiesel production, highlighting its role in enhancing process efficiency, reducing costs, and minimizing environmental impact. The review also highlights the advantages, challenges, and future prospects of utilizing oleaginous yeast for biodiesel production.

Keywords*:* biodiesel, triglycerols, resonance surface methodology, oleaginous yeast.

I. Introduction

Oleaginous Yeast: Characteristics and Lipid Accumulation

Definition and Classification:

Oleaginous yeasts are microorganisms capable of accumulating a significant proportion of their biomass as lipids. They belong to different taxonomic groups, including the genera R*hodotorula*, *Lipomyces*, and *Yarrowia*. These yeasts possess unique physiological and biochemical characteristics that make them efficient lipid producers. Oleaginous yeasts are often described as superior for possible commercial lipid production over other oleaginous microorganisms, due to their fast growth, high lipid content, and volumetric productivity (Abeln and Chuck, 2021).

Yeast is usually unicellular fungi, however, in some cases, they also form mycelia and are known to have several biotechnological applications such as the production of protein. Since they have a high lipid content, they are considered to be an attractive candidate for the production of various human nutrition products and also biofuel production (Tsai, S., et.al., 2022).

The lipids in yeast are mainly in the form of triacylglycerols, with long-chain fatty acids along with stearic, palmitic, and oleic as the predominant fatty acid. The cultivation of oleaginous yeast for biodiesel production is highly efficient. Oleaginous yeasts can rapidly accumulate lipids, often reaching lipid contents of over 50% of their dry cell weight. Compared to other lipid-producing organisms, such as algae, oleaginous yeast demonstrates faster growth rates and higher lipid yields, thereby increasing the overall productivity of the biodiesel production process.

Moreover, the production of biodiesel from oleaginous yeast offers several environmental benefits. Biodiesel derived from these yeasts exhibits lower greenhouse gas emissions compared to traditional fossil fuels, contributing to the mitigation of climate change. Additionally, the utilization of waste materials as feedstock reduces the environmental impact associated with their disposal, fostering a circular economy approach.

From an economic perspective, the use of oleaginous yeast for biodiesel production holds great potential. The scalability and simplicity of yeast cultivation processes make them suitable for large-scale production. Furthermore, the abundance of diverse feedstock options allows for flexibility in selecting the most economically viable sources, thereby enhancing the commercial viability of biodiesel production.

From several oleaginous yeast species, the one that has attracted much interest due to their lipid content and synthesis of other useful co-products are *Rhodotorula glutinis, Rhodosporidium toruloides, Yarrowia lipolytica, and Cryptococcus curvatus.* The majority of these yeasts are obligate aerobes and can be cultivated to a high cell density using a conventional stirred bioreactor (Shaigani, P., et.al.,2021).

Many natural oleaginous microorganisms are known to accumulate intracellularly lipids under nutrient-limited conditions. The genus *Rhodotorula* is polyphyletic. It contains *Rhodotorula* species that grow as a single cell yeast (monomorphic) and reproduce asexually by budding/ fission (anamorphic).

The yeast phase of *Rhodosporidium* and *Rhodotorula* species are virtually similar. It is known that all *Rhodosporidium* species are isolated as haploid yeasts and show a bipolar mating behavior which means that their strains belong to either one of two complementary mating types, designated as A1 and A2 or A and a. Due to its mating behavior, as an alternative biotechnology platform to *Saccharomyces cerevisiae, Rhodosporidium toruloides* is being developed (Papanikolaou, S., et al., 2017).

Oleaginous Yeast Species:

More than 160 yeast species with lipid levels of 20% (w/w) or higher have been documented in scientific studies. Although some species can accumulate lipids at over 70% (w/w) of their dry cell weight, the average is 42.8% (w/w) ± 15.5% (w/w). Their lipid content, concentration, and composition depend on several factors, including the yeast species, medium composition, and operational conditions.

Y. lipolytica, R. toruloides, C. oleaginous, L. starkeyi, and R. glutinis, are considered to be the most promising oleaginous yeast species. The oleaginous yeast species *M. pulcherrima* has recently gained renewed interest due to its ability to assimilate a wide range of carbon, and it is also capable of showing high inhibition tolerance.

Optimizing the growth conditions of oleaginous yeast is a complex process. It requires experimentation and fine-tuning, and results may vary depending on the specific strain and desired application, whether for the production of biofuels, bioplastics, or other biotechnological applications.

- Nutrient Media Selection: The choice of growth medium is pivotal. It often includes carbon sources like glucose, glycerol, or other carbon-rich substrates. Nitrogen sources can vary, and the type and concentration of these nutrients greatly affect growth and lipid production.
- Carbon to Nitrogen Ratio: The C:N ratio has a significant impact on the results. Higher concentrations of carbon compared to nitrogen promote lipid accumulation. This ratio can be adjusted to maximize lipid production while balancing overall growth.
- pH Control: It is important to maintain the pH in the range of 4 to 6. Fluctuations in pH can stress the yeast and negatively impact growth and lipid production.
- Temperature control: Oleaginous yeast usually grows at temperatures around 25-30°C. To ensure optimal growth, precise temperature control is crucial.
- Aeration and agitation: Proper aeration and agitation are critical to oxygenating the yeast, preventing hypoxia, and maintaining a healthy culture.
- Incubation time: It is important to determine the ideal incubation time. The aim is to track the growth stages of yeast and determine the point at which lipid accumulation reaches its peak.
- Inoculum size: The initial inoculum size should be adjusted based on the culture volume to ensure a homogeneous starting population. Inoculum volume that is too low or too high will affect growth.
- Supplementation: In addition to the primary carbon and nitrogen sources, the culture medium may need to be supplemented with trace elements and vitamins to support the metabolic processes and overall growth of the yeast.
- Genetic Engineering: Genetic modification can be employed to enhance specific metabolic pathways associated with lipid production.
- Fermentation Strategy: The choice of fermentation strategy such as batch, fed-batch, or continuous cultivation depends on the specific goals and equipment available. Fed-batch strategies, for instance, allow for the controlled addition of nutrients during the process.
- Monitoring and Analytics: Regular monitoring of critical parameters such as cell growth, lipid content, and other relevant factors is crucial. Analytical tools, like gas chromatography, can provide detailed insights into the lipid profile.
- Stress Conditions: Introducing mild stress conditions, such as nitrogen limitation or specific nutrient limitations, can sometimes lead to increased lipid production as the yeast responds by accumulating more lipids.

Some of the most promising oleaginous yeast species are as follows:

1. Rhodotorula toruloides:

This oleaginous yeast was discovered in 1944 and is also known as *Rhodosporidium toruloides*, *Rhodotorula toruloides*, and *Rhodotorula gracilis* (Abeln, F., et.al., 2021). It is non-pathogenic,aerobic, oleaginous red yeast that has been in the past isolated from different sources such as dry leaves, soil, wood pulp etc. It is known to accumulate lipids to more than 70% of its dry cell weight. It is classified into the following categories:

- Family: Sporidiobalaceae
- Order: Sporidiobolales
- Class: Microbotryomycetes
- Phylum: Basidiomycota

It can grow well at varying temperatures and pH and use a range of different carbon and energy sources such as hexoses, pentoses, sucrose, maltose , cellobiose, raffinose, ethanol, glycerol, mannitol, sorbitol, acetate, lactate, succinate, citrate and long chain fatty acids. While ammonium, nitrate, cadaverine, amino acids and small peptides are effective as nitrogen sources.

It is well known not only for the production of carotenoids and enzymes but also as a potential lipid producer. Although lipid accumulation is strongly carbon source-dependent, some Rhodotorula strains grow on single monosaccharides and polyols. The importance of *R. toruloides* has significantly increased since the development of various genetic tools. *R. toruloides* is considered to be a gold mine for industrial enzymes due to its ability to produce high titre of esterase enzymes.

2. Cutaneotrichosporon oleaginosus

Cutaneotrichosporon. oleaginosus is considered a promising and versatile biocatalyst for the fermentation of various carbon sources to bio-based oil. It can grow on a variety of carbon and nitrogen sources. Its resistance to hydrolysis by-products makes it a promising biocatalyst for custom tailored microbial oils. Recently, this species has been reclassified as *Cutaneotrichosporon oleaginosus*, although it was previously also known as *Cryptococcus curvatus*. It is used to produce SCP from whey. It is the preferred oleaginous yeast strain because it grows relatively fast and possesses sufficient inhibitor tolerance.

C. oleaginosus is classified as a basidiomycete yeast taxonomically of the TRemellomycetes class and has recently been added to the *Cutaneotrichosporon* genus. Commonly used substrates for *C. oleaginosus* are hydrolysates, whey, or whey permeate because they are capable of growing on lactose, xylose, oligosaccharides, and fatty acids, and have been grown in fed-batch culture up to a cell density of 104.1 g L^{-1}, yet containing a remarkable lipid content of 82.7% (w/w). Recently, the fatty acid profile of this yeast strain was modified by genetic manipulation (Bracharz, F., et.al, 2017).

3. *Yarrowia lipolytica*:

Yarrowia lipolytica is present in a variety of environments and is commonly classified as *Candida lipolytica, Saccharomycopsis lipolytica,* or *Endomycopsis lipolytica*. *Y. lipolytica* can produce a significant amount of SCO from other lipids via the ex-novo conversion pathway (Jach, M.E., et.al, 2022). *Y. lipolytica* is considered a non-conventional yeast due to its distinctive genome structure and its large phylogenetic distance to other yeasts. It accumulates lipids early in its growth cycle, under nitrogen-limiting conditions. Its affinity towards hydrophobic substrates accelerated its industrial relevance to produce single-cell protein (SCP) and citric acid from the late 1950s, as relatively cheap n-alkanes could be used as substrates. It also has natural and engineered traits that makes it suitable for industrial bioproduction of various fuels, chemicals, foods and pharmaceutical products (Park, Y.K. and Ledesma-Amaro, R, 2023).

Y. lipolytica can grow on a variety of substrates it has been in the past isolated from various lipid containing environments. It can utilize various hydrophobic substrates such as glucose, fructose, and glycerol; and organic acids, including acetate, lactate, citrate, and succinate, etc also it can efficiently utilize some preferred low-cost substrates such as glycerol, oil or food waste. In recent years, many efforts have been made to expand the substrate spectrum of this yeast through metabolic engineering (Salimi Khaligh, etla., 2023).

4. *Lipomyces starkeyi*:

It belongs to the family Lipomycetaceae, and was first described by Robert Starkey and is considered to be a strong lipid producer and one of the few prominent types of oleaginous yeasts that have been named since their discovery in 1946. *L. starkeyi* strains are simultaneously capable of fermenting xylose in combination with either acetate or cellobiose. It can proliferate aerobically and is capable of metabolizing a wide range of carbon and nitrogen sources. *L. starkeyi* can tolerate low pH and also metabolize various inhibitors present in cellulosic hydrolysate. Though genetic engineering of *L. starkeyi* has not been very well developed, recombinant strains are constructed that have resulted in the production of long chain polyunsaturated fatty acids (McNeil, B.A. and Stuart, D.T., 2018).

L. starkeyi can induce the synthesis of lipids when carbon is in excess and at least one of the essential nutrients has been depleted. During this particular phase though the cell division slows down but carbon assimilation continues and the production of lipids then shifts to the lipid storage in the form of triglycerides (TAGs) in lipid bodies. TAGs are composed of saturated and mono, polyunsaturated C16 and C18 fatty acyl chains.

5. Rhodotorula glutinis:

It has previously been identified as *Rhodotorula terrea, Torula glutinis, Saccharomyces glutinis,* and *Cryptococcus glutinis. R. glutinis* is considered as a biocontrol agent due to its antagonistic capabilities. It has great industrial importance due to its ability to produce numerous valuable compounds such as lipids, enzymes and carotenoids. It has been isolated from various products such as cheese, milk seawater. They are aerobic and mesophilic though some are known to thrive even under low temperatures and are spherical, ellipsoidal or elongated in shape.

R. glutinis are known to produce several microbial oils such as oleic, linoleic, palmitic and stearic acid. Various factors affect the lipid content such as choice of carbon sources (glucose, molasses, sucrose, glycerol or waste material), nitrogen sources (ammonium sulfate , yeast extract), C/N ratio and cultivation times.

Although lipid yields of up to 0.19 g g^{-1} crude glycerol have been reported and glycerol is frequently used by *R. glutinis* researchers, poor utilization has been demonstrated by some strains, potentially due to passive diffusion, and genetic tools are limited but are under development for the Rhodotorula genera (Ngamsirisomsakul, M, et.al.,2021). *R. glutinis* has also been used as a biocontrol agent to prevent post-harvest microbial disease of fruit.

6. *Metschnikowia pulcherrima*:

This oleaginous yeast is generally isolated from fruits and flowers. They secrete antimicrobial agents and can outcompete other microbes. Its osmophilic and acidophilic abilities further aid in its effectiveness against microbial contamination. In addition to lipid production, it is also capable of producing a wind range of other co-products, such as 2-phenylethanol.

Almost 11% of the 1500 known yeast has been till now identified as oleaginous. With the huge volume of yeast screened for oleaginicity in the past 143 years, it is questionable whether a new species can be discovered with significantly superior characteristics for economic lipid production compared with the previously discussed prominent species (Hicks, R.H., et.al.,2021).

However, in light of favorable economics when producing high-value lipids such as long-chain PUFAs and/or valuable secondary metabolites, diverted yeast species with characteristics, such as *Wickerhamomyces siamensis* producing medium-chain TAGs could be of interest in this field.

Therefore, in the discovery of new species, it is important for researchers to investigate the fatty acid profile and secondary metabolite production, placing the novelty of a potential production process over a simple assessment that the yeast is oleaginous.

Industrial development and key research for oleaginous yeasts:

The production of fats from yeasts has a long history, dating back to 1878. In 1895, a yeast known as *Torula pulcherrima* (now *Metschnikowia pulcherrima*) was discovered to produce an oil droplet. During World War II, microbial lipids gained attention as a potential energy storage mechanism. Researchers observed that when cells were deprived of certain nutrients other than carbon and anaerobic pathways were reduced, lipid synthesis increased. The oil produced by yeast was found to be similar in composition to vegetable oils, predominantly containing triacylglycerols (TAGs) (Maza, D., et al., 2020).

In the later years of World War II and beyond, German factories utilized *Geotrichum candidum* to produce fats with a 20% (w/w) lipid content, using whey and lignocellulosic hydrolysate. In recent times, significant research efforts have been focused on advancing yeast lipid technology as a sustainable source of oil (Sagia, S., et al., 2020).
During the 1980s, *Cutneotrichosporon oleaginous* was considered a promising yeast species capable of producing high-quality lipids. The lipids produced by this yeast were close to commercialization for cocoa butter equivalent (CBE) production, with plans for up to 1000 tons per year production. However, due to low profitability, competition from low-cost CBE derived from palm oil fractionation, market acceptance uncertainties, and a drop in cocoa butter prices, the project was ultimately terminated.

In the 1990s, the rapid development of genome sequencing and genetic engineering regenerated interest in yeast lipid production. This allowed the creation of high-value lipids from yeast, customizable to meet specific demands in the oil market. Commercial production of yeast lipids commenced in 2006, with *Yarrowia lipolytica* being genetically modified to produce C20 fatty acids.

A glance into lipid metabolism:

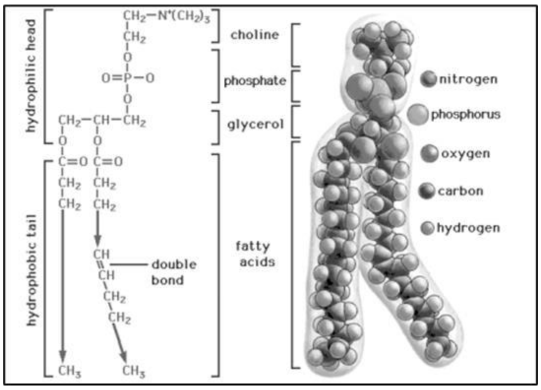

Figure 1: Structure of lipid

Lipids are macromolecules consisting of carbon, oxygen, hydrogen, and also phosphorus in some cases these macromolecules have a non-polar hydrophobic tail and a polar head. Due to the presence of this, the lipids are sparingly soluble or insoluble in water but soluble in selected organic solvents such as benzene, diethyl ether, hexane, chloroform, and also methanol.

Lipids play an important role and are separated into two categories according to their cellular functions:

1. **Accumulated lipids** – these are stored as carbon sources for the cell usually in the form of Triacylglycerol (TAGs) and Free fatty acids.

2. **Structural lipids -** they are part of several cell structures and also play an immense role in acting as barriers between the inner and outer parts of the cell to modulate the transport of amino acids.

Major classifications of lipids are as follows:

[1] Triacylglycerol (TAG):

TAG are molecules of glycerides where three hydroxyl glycerol is esterified by fatty acids. They constitute more than 15% of total structural lipids that are found in the form of glycerophospholipids, sphingolipids, and glycosphingolipids (Patel, A., et.al., 2017).

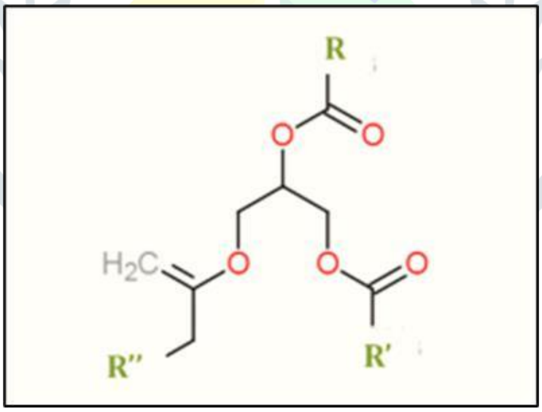

Figure 2: General form of triacylglycerol

[2] Fatty acids:

Fatty acids are considered to be a group of aliphatic chains with hydrophobicity and are normally activated in the form of acyl CoA. Fatty acids in living organisms are found in a paired number of carbons and can be further separated into two main groups:

1. **Unsaturated** – fatty acids with double carbon bonds are classified according to the number of bonds into monounsaturated (one bond) and polyunsaturated (more than one bond).

2. **Saturated -** carbon bonds are single for long and short chains.

3.

Table 1: Fatty acids nomenclature

Common name	Nomenclature	Chemical name
Palmitic acid	16:0	hexadecanoic acid
Stearic acid	18:0	octadecanoic acid
Oleic acid	18:1 (n-6)	9-octadecenoic acid
Linoleic acid	18:2 (n-6)	9,12-octadecadienoic acid
γ -linolenic acid	18:3 (n-6)	6,9,12-octadecatrienoic acid

[3] Phospholipids:

Phospholipids are composed of two fatty acids, and one phosphate group esterified by a glycerol molecule. The fatty acids are hydrophobic (tail) and the phosphate group is hydrophilic (head).

Phospholipids are major constituents of cell membranes. Also, they form a lipid layer when hydrophobic tails line up against one another forming a membrane of hydrophobic heads on both sides. Since the lipid bilayer is semi-permeable it allows the diffusion of molecules through the membrane.

Lipid accumulation in yeasts:

Accumulation of yeast takes place in two ways depending upon the substrate fermentation. The first is known as ***"denovo"*** lipid accumulation and takes place when hydrophilic substrates such as glycerol, commercial sugars, or lignocellulosic hydrolysates are the carbon and the nitrogen source or any other growth essential element is the limiting nutrient. The second mechanisms are the ***"ex novo"*** lipid accumulation and are considered to be a non-growth associated process wherein, hydrophobic substrates such as the oils and fats are acting as the carbon source without any nutrient depletion (Chaturvedi, S, et.al., 2018).

The "Denovo" lipid accumulation is triggered by the depletion of essential components necessary for cell proliferation, specifically nitrogen, in the presence of excess carbon. Lipid synthesis occurs in the cytosol and serves as vital energy storage for various cellular functions. During active cell proliferation, both carbon and nitrogen are available in the medium. However, when nitrogen becomes scarce, excess carbon continues to be taken up by the cell and converted into storage lipids, which are later utilized for the generation of new cells. This process requires a high C/N ratio (Vincent, M., et.al., 2018).

Although triacylglycerol synthesis primarily occurs in the cytosol, its initial steps take place in the mitochondria. The accumulation of lipids results from metabolic cycle disruptions during nitrogen deficiency, as the cell strives to maintain crucial functions.

As nitrogen depletes, cell proliferation ceases, and the synthesis of proteins and nucleic acids halts. Nonetheless, the cells continue to utilize carbon sources, which enter the cytosol and follow the EMP pathway (Glycolysis). Subsequently, pyruvate is formed and transported into the mitochondria, where it undergoes enzymatic conversion to oxaloacetate (OAA) and acetyl CoA via the pyruvate dehydrogenase enzyme. OAA and acetyl CoA further enter the Krebs cycle or TCA cycle, where they are converted to citric acid by the citrate synthase enzyme in an irreversible reaction.

Citrate then gets isomerized to isocitrate and gets transformed to α-ketoglutarate with the help of the enzyme isocitrate dehydrogenase. This is the step where the Krebs cycle is affected by the external nitrogen deficiency and conditions required for triacylglycerol synthesis are created.

Due to a nitrogen deficiency, the cell employs a strategy to acquire nitrogen for protein and nucleic acid synthesis. This involves decomposing adenosine monophosphate (AMP) into inosine monophosphate (IMP) and NH4+. Consequently, the availability of AMP influences the function of isocitrate dehydrogenase, leading to the inhibition of ketoglutaric acid production and an incomplete Krebs cycle. The accumulation of isocitrate slows down its formation. When citrate within the mitochondrion reaches a critical level, it is transported to the cytosol.

In the cytosol, the enzyme ATP-citrate lyase comes into action, breaking down citrate into oxaloacetic acid (OAA) and acetylCoA. OAA is then converted back to pyruvate with the help of malic enzyme, and this conversion also generates NADPH from NADP.

Simultaneously, acetyl-CoA triggers a series of reactions for actual fatty acid biosynthesis, involving the generation of malonyl-CoA. The enzyme responsible for catalyzing these reactions is fatty acid synthetase, which also releases NADPH through the action of malic enzyme. As a result of subsequent esterification with glycerol and processes like desaturation and carbon chain elongation, triacylglycerols (TAGs) are formed.

These TAGs are stored in lipid bodies located within the cytoplasm. The formation of these lipid bodies commences in the endoplasmic reticulum and maintains their individual shape, thanks to proteins and polar lipids surrounding the central TAG core, preventing coalescence (Kankun, I. et al., 2021).

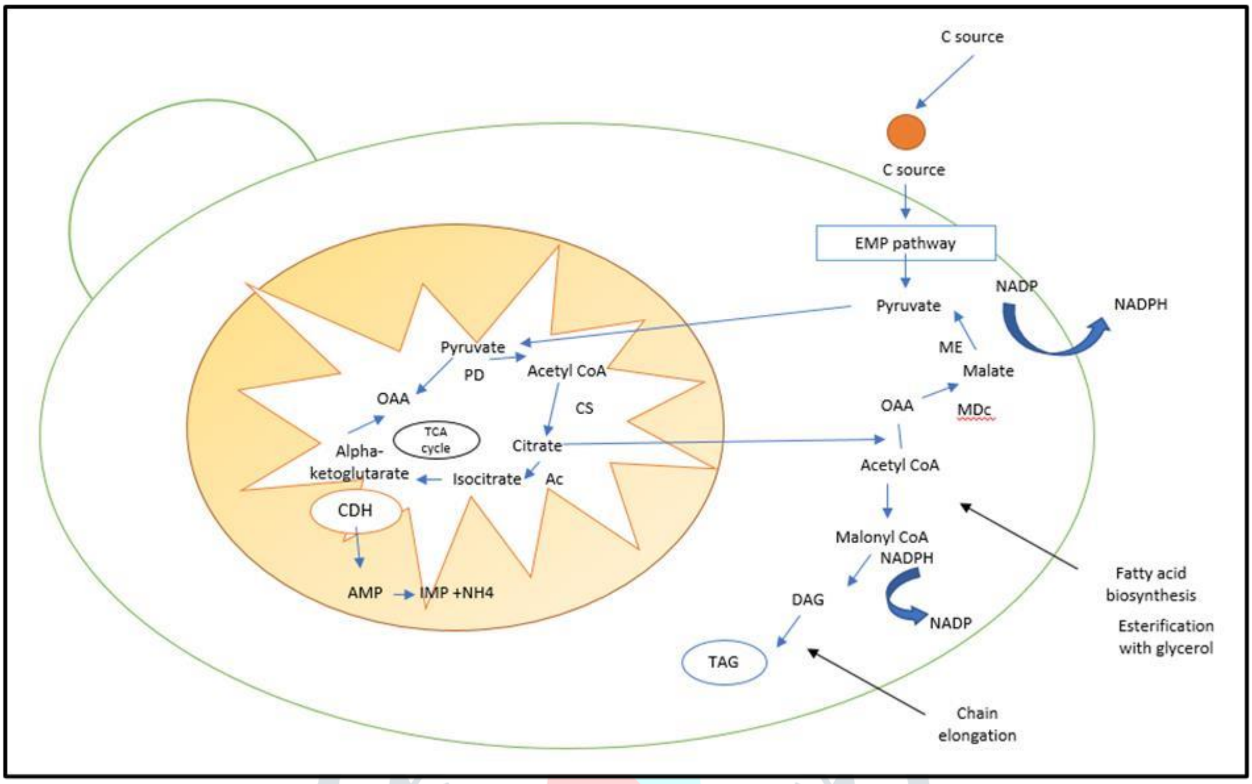

Figure 3: Biosynthesis of lipid accumulation

Factors Influencing Lipid Accumulation:
It has been reported that various cultivation conditions such as carbon source, C/N ratio, nitrogen source, temperature, pH, oxygen can influence oil accumulation.
An important and widely used parameter used for designing fermentation media for the accumulation of lipids is the C/N ratio which indicates the relationship between the carbon and nitrogen amount. A specific ratio can be obtained by using different combinations of carbon and nitrogen. But it is a parameter that indicates how much higher is the carbon than the nitrogen. Usually, for lipid accumulation, a high C/N ratio is required while a low C/N ratio is maintained during the growth phase (Bardhan, P, et.al., 2020).

•Carbon source:
Since yeast is a chemoorganotrophic organism, they obtain carbon and energy from compounds in fixed organic linkages. Most commonly these compounds are sugars of which glucose is most commonly used by yeast.

•Nitrogen source and C/N ratio:
The nitrogen content in yeast cells is around 10% of their dry weight and is capable of utilizing a range of different organic and inorganic sources of nitrogen for incorporation into the structural and functional nitrogenous component of the cell. The imbalance of nutrients in the culture medium is known to trigger the accumulation of lipids by oleaginous microorganisms (Zarea, A. 2021).

To achieve a high- density cell culture for fermentation of microbial lipids, different cultivation modes and substrates are used. Though iron, phosphorus, zinc, nitrogen, magnesium have resulted in the accumulation of lipids, however, nitrogen limitation has been used extensively to create an environment for lipid accumulation.

•Trace elements:
Sulphur is also considered an important element for lipid accumulation. It is required for the construction of proteins such as various amino acids like methionine and cysteine along with other cellular compounds like biotin and coenzyme A. Though sulfur limitation has been reported to induce lipid accumulation it is still not a popular target.

Some researchers have reported that the limitation of phosphorous concentration also affects the accumulation of lipids. Therefore, the phosphorus limitation can also be another strategy to stimulate lipid production.

•Culture conditions:

Temperature is considered to be one of the most important physical parameters which influence the growth of the yeast and lipid accumulation though to a lesser extent than nutrient limitation. Temperature also plays a significant role in regulating the fatty acid composition of membrane lipids of a microorganism. It has been reported that the synthesis of long-chain fatty acids such as C16, C18, C18:1, C18:2 was predominant at 30-32° near the optimal growth temperature, whereas short-chain acids like C7, C8, C9 are predominantly synthesized at 38°C.

For the growth and cultivation of aerobic microorganisms, oxygen is an extremely important substrate. Since the microbial cells are suspended in an aqueous environment that is a culture broth, therefore, oxygen needs to be dissolved in the liquid phase so that it can be utilized by the cells.

The transport of oxygen from the gaseous phase to the liquid phase happens in the following order:

1.Transfer from the gaseous phase to the gas-liquid interphase.
2.Movement within the gas-liquid interphase.
3.Transfer through the liquid film surrounding the gas bubble.
4.Oxygen reaches the bulk liquid area where it is moving through.
5.Before entering the cell, oxygen passes across a stagnant liquid film surrounding the outer part of the cellular wall.
6.It moves through the liquid-cell interphase.
7.It enters the cytosol to reach reaction sites.

Oleaginous yeasts are aerobic microorganisms, and their oil-producing culture systems are supplied with air at a rapid rate, in contrast to processes like alcohol production. High levels of dissolved oxygen are known to enhance the growth rate of these yeasts. Typically, the concentration of dissolved oxygen is influenced by a combination of high airflow and vigorous agitation. The availability of oxygen directly affects the degree of unsaturation in the produced oils. When oxygen becomes limited due to high biomass, the proportion of saturated fatty acids decreases.

Research efforts have been concentrated on expanding the understanding of the diversity of oleaginous yeast species. At present, about 160 yeast species, constituting 8.2% (Abeln and Chuck, 2021), out of a total of 1,958 recognized yeast species (Boekhout et al., 2021), have been identified to harbor oleaginous yeast strains. This count is nearly twice the number reported five years earlier, which accounted for approximately 4.4% of 1,600 known yeast species (Poontawee, R. et al., 2023). The characteristic of lipid accumulation is observed across various strains.

Feedstocks for the growth of oleaginous yeast;

A diverse array of carbon sources has been harnessed for yeast lipid production. Notably, common choices encompass single saccharides, hydrolysates, and glycerol, both in crude and pure forms, constituting approximately 71% of the utilized feedstock. Conversely, less prevalent options encompass fatty acids, wastewaters, oils/fats, and molasses/syrups, while infrequently employed alternatives include alcohols, aromatics, aqueous extracts, and other waste streams. The versatility in substrate utilization constitutes a significant advantage for oleaginous yeast, offering substantial enhancements in process sustainability, in alignment with specific regulatory directives such as the EU Renewable Energy Directive.

The selection of the carbon source notably influences lipid synthesis, particularly determining the pathway—whether sugar-based (de novo) or fat-based (ex novo). Feedstock selection is primarily based on factors like organism compatibility, presence of limiting nutrients, simplicity, and especially on an industrial scale, cost-effectiveness and availability. For instance, while molasses is commonly employed in industrial fermentation, its relatively elevated nutrient content (with a C/N ratio ranging around 20 to 40 g g^{-1}) renders it unsuitable for numerous oleaginous yeast species. Strategies like nutrient removal have been proposed for feedstocks abundant in nutrients. However, the utilization of yeasts like *M. pulcherrima*, *S. terricola*, or *Y. lipolytica* , which do not necessitate nutrient depletion, substantially streamlines the processing.

Lipid Biosynthesis:

Over the years there has been an increase in the development of new methods to obtain lipids (Grubisic, M., 2021). One such method is the production of microbiological lipids called single cell oil (SCO). The lipid bodies in this yeast consist of neutral lipids in the form of triglycerides, and the composition of phospholipids differs from the composition in other cellular organelles.

Lipids in the yeast cells can be accumulated in two ways:

- de Novo (from acetyl- CoA and malonyl-CoA molecules)
- ex Novo

In the de novo method, glycerol and saccharides make up the substrates for lipid production. While in the ex-Novo method hydrophobic compounds are serving as the substrate. In the former method, the overproduction of intracellular lipids takes place only after the depletion of nitrogen compounds from the culture environment, which is related to the AMP deaminase activation. This enzyme can catalyze the decomposition of AMP to IMP and $NH4^+$ ions, which constitutes the additional source of nitrogen. A decrease in the AMP level affects the course of the kKrebs cycle since this compound can activate isocitrate dehydrogenase that catalyzes the transformation of isocitrate to α-ketoglutarate (Awad, et.al., 2019).

In these circumstances, mitochondrion can accumulate isocitrate which remains in balance with citrate due to the activity of aconitase. Citric acid is transported to the cytoplasm from the mitochondrion after attaining a critical concentration where it is split by ATP-citrate lyase to acetyl-CoA and oxaloacetate. During the first stage of fatty acid synthesis carboxylation of acetyl CoA to malonyl CoA takes place after which a sequence of enzymatic reactions occurs that is catalyzed by the complex of fatty acid synthase (Yaakob, M., et.al., 2021).

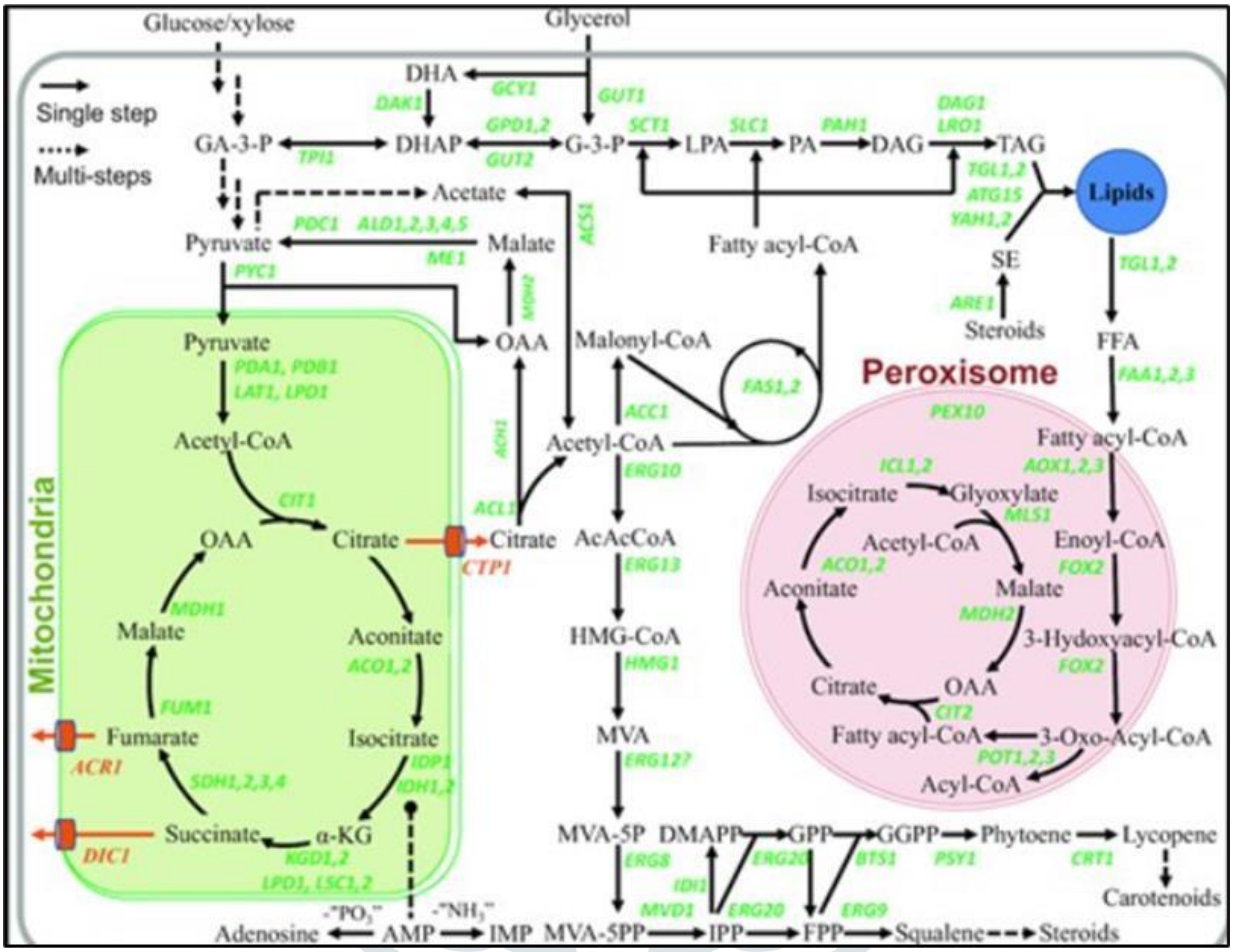

Figure 4: Key genes involved in glycolysis, pentose phosphate pathway, TCA cycle in mitochondria, biosynthesis, and degradation of fatty acids, triacylglycerols and phospholipids, isoprenoid biosynthesis, glyoxylate cycle pathway, and the βoxidation pathways in peroxisomes are highlighted in green.

According to the framework of metabolism, acetyl CoA is regarded as the key precursor for the synthesis of lipids, which is derived mainly from the citric acid cleavage and pyruvate decarboxylation in the cytoplasm. Genes that are important in the conversion of acetyl-CoA into lipids are ACC1 (acetyl -CoA carboxylase), SCD1 (stearic CoA desaturase), and DGAT1 diacylglycerol acyltransferase) these genes are known to catalyze the first, intermediate, and final steps respectively in the triacylglycerol biosynthesis (Shen, H., et.al., 2017).

Low-cost fermentation substrates:

Corn steep liquor as a nitrogen source:

Corn steep liquor (CSL) has been identified as a cheap source of nitrogen in various biochemical industries as an alternative to costly available nitrogen sources. CSL is considered to be a side product of the corn starch industry also it's a cheap source of proteins, amino acids, vitamins, minerals, and trace elements and can be used as a rich and effective nutritional substitute for expensive complex media like beef extract, meat extract, yeast extract, and peptone in fermentation studies (Taiwo, A., et.al., 2018).

Crude glycerol as a carbon source:

Glucose is considered to be one of the most commonly used carbon and energy sources since it is easily assimilated by many microorganisms and the oil and biomass yield is quite considerable. Various energy crops are considered to be low-cost sources of sugars such as sweet sorghum and cassava, lignocellulosic biomass such as straw or bagasse due to a large number of fermentable polysaccharides. However, one of the major drawbacks in the use of lignocellulose biomass is that it is composed of lignin, cellulose, hemicellulose and converts cellulose to assimilable sugars such as xylose, cellobiose, glucose hydrolysis, and pre-treatment is required which add up to the substrate related cost (Boonyarit, J., et.al., 2020).
Wastewaters and effluents are also utilized as substrates for microbial oil production since they are rich in nutrients, contain high COD (Chemical oxygen demand), and high pH suitable for aerobic yeast. However, the cultivation of oleaginous yeast on these materials is more beneficial for growth rather than oil accumulation, and the yields are quite low due to the fast depletion of carbon.

Crude glycerol over recent years had been produced to a surplus, due to immense growth in biodiesel production (Brandenburg, J., et.al.,2021). This crude glycerol obtained from the biodiesel industry is not suitable for direct use in any application and is usually treated as a high-strength pollution waste (Liu, L., et.al., 2016). This has resulted in a decrease in the glycerol price and has negatively affected the viability of biodiesel. Therefore, if the crude glycerol is used as a low-cost carbon source by oleaginous yeast for the production of microbial oil it would be significantly beneficial to the industry.

Properties of crude glycerol:

During the synthesis of biodiesel when glycerol is released it is usually contaminated by salts, methanol, water, or other material due to the transesterification reaction. This mixture is called crude glycerol and is impure and of little economic value. In general, it makes up 65%- 85% of the crude stream.

Crude glycerol is poor quality material and without purification has limited applications the extent of impurities affects the range of industrial applications. The more impure the glycerol is, the more expensive its purification is.

Methanol and free fatty acids(soaps) are the two major impurities present in crude glycerol. Crude glycerol obtained from the synthesis of biodiesel contains alcohol (methanol), free fatty acids, catalysts, salts, mono, di and triacylglycerol, and water which differs according to the type of raw material, catalytic process, stages of biodiesel production, and purification.

The presence of methanol is because the producers of biodiesel use excess methanol to drive the chemical transesterification to completion and do not recover all the methanol. Soaps that are soluble in the glycerol layer originate from a reaction between the free fatty acids present in the initial feedstock and the catalyst (base).

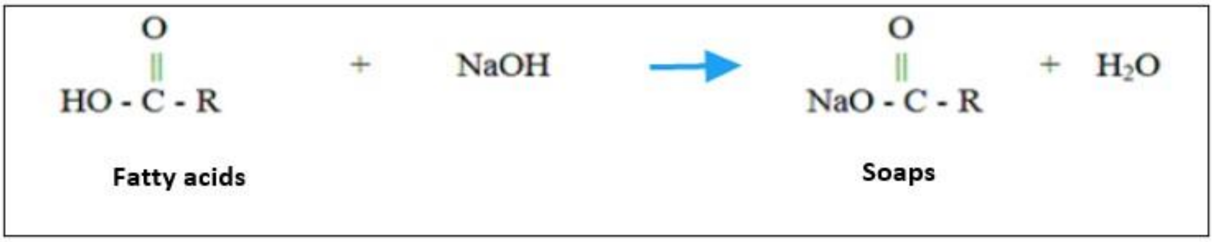

Figure 5: Reaction between the free fatty acids and the catalyst

Crude glycerol can be produced either by oil saponification, hydrolysis, or transesterification. There is an increase in market demand for glycerol to be used in various fields such as food products, cosmetics, medicinal, polymer, and other chemical industries.

The utilization of glycerol obtained from various biodiesel biorefinery is known to enhance the economic production of microbial oil and biodiesel production chains under appropriate cultivation conditions (Chilakamarry, C., et.al., 2021). To trigger microbial oil production, it is important to apply excess carbon over nitrogen limitation which can be provided from high glycerol concentration. It is important to identify and develop species that are capable of assimilating glycerol.

Biosynthesis of single cell oil/lipids:

The production of single cell oil in oleaginous microorganisms depends on the composition of the culture medium. The carbon source has to be present in excess, while the other sources such as nitrogen (N), phosphor (P), magnesium (Mg), zinc (Z), calcium (Ca), or vitamins can be in limited amounts (Ye, Z., et.al., 2021).

In most cases nitrogen is limited, once nitrogen is limited, the cell growth stops, but available carbon will be assimilated into the cell and stored as single cell oil (SCO). The carbon source is converted into pyruvate in the cytosol, pyruvate then is transported into the mitochondrion, decarboxylated to acetyl-CoA (C2) reacts with oxaloacetate (C4) and gets further converted to citrate and subsequently usually to iso-citrate within the citrate cycle (Bettencourt, S., et.al., 2020).

Under nitrogen limitation, the enzyme AMP-deaminase is activated by cleaving adenosine-monophosphate (AMP) into inosinemonophosphate (IMP) and $NH4^+$ to provide cell own nitrogen for cell functions.
AMP is, however, required for the functionality of the enzyme isocitrate-dehydrogenase (ICDH), which converts isocitrate into αketoglutarate within the citrate cycle to produce $NADH^+ + H^+$ for the production of ATP within the respiratory chain.

If AMP is not available then iso-citrate accumulates in the mitochondria. Due to equilibrium reactions, iso-citrate is converted into citrate which then accumulates in the mitochondrion as well and is channeled into the cytosol via malate/citrate transporter. Here, citrate is cleaved under the consumption of ATP into oxaloacetate (C4) and one C2-unit acetyl-CoA which is the chemical precursor for the fatty acid synthesis. This conversion is done by the enzyme ATP-Citrate-Lyase (ACL), which is specific in oleaginous microorganisms.

The extent of fatty acid production depends on the malic enzyme (ME) concentration which converts malate to pyruvate via NADPH release. This chemical conversion is the sole source of NADPH for the enzyme fatty-acid-synthase (FAS), which is required in fatty acid biosynthesis (Bandhu, S., et.al., 2019).

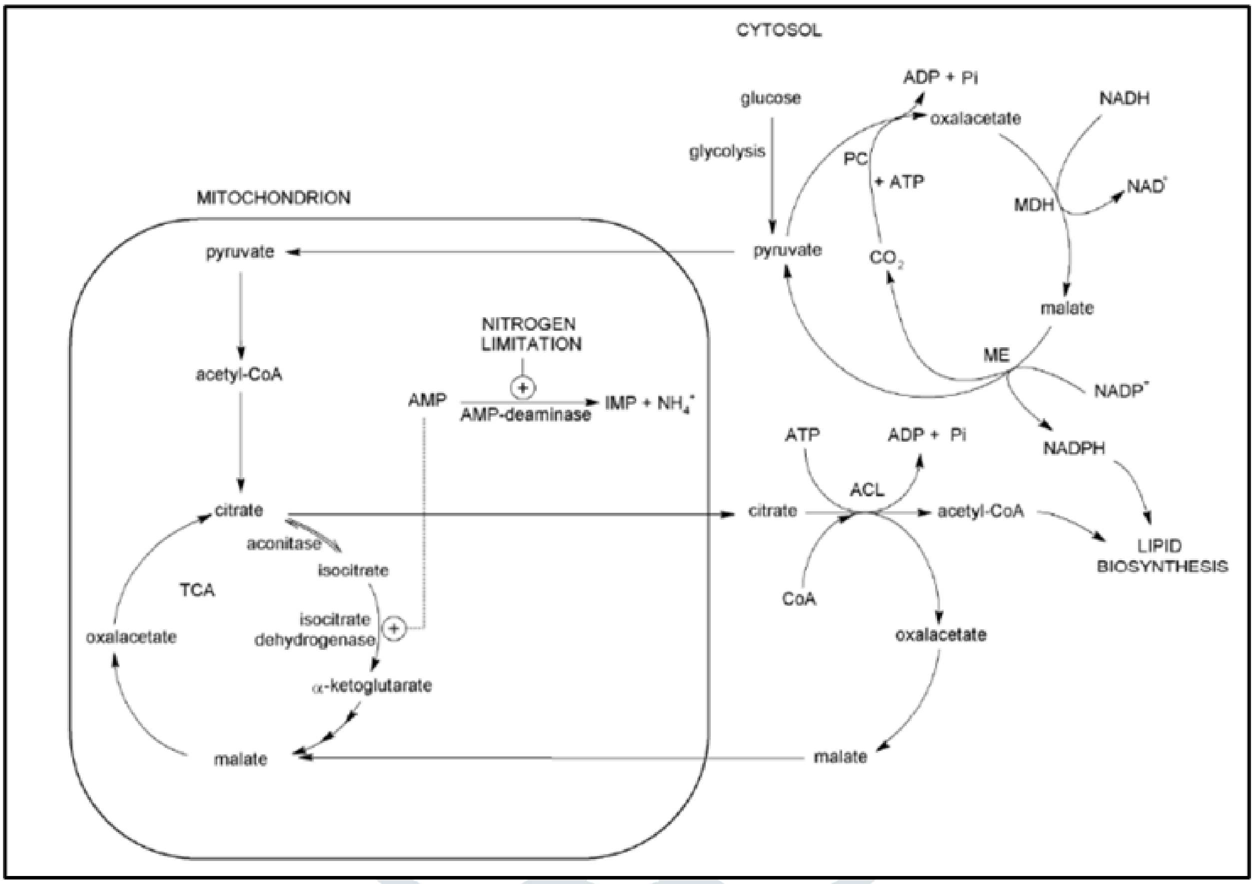

Figure 6: Diagram of the biosynthesis of single-cell oils in oleaginous microorganisms

The research on oleaginous yeast for biodiesel production has faced several shortcomings and hurdles, which have presented challenges in its practical implementation. Some of the key issues include:

Lipid productivity and yield: While oleaginous yeasts have the potential to accumulate high amounts of lipids, achieving consistent and high lipid productivity remains a challenge. Optimizing growth conditions and nutritional requirements to maximize lipid production is an ongoing area of research.

Strain selection: Identifying the most suitable and efficient strains of oleaginous yeast for biodiesel production can be challenging. Some strains may have high lipid content but low growth rates, while others may have faster growth rates but lower lipid yields. Researchers need to find a balance between growth rate and lipid accumulation.

Substrate utilization: Oleaginous yeasts can utilize various feedstocks, including agricultural waste, lignocellulosic materials, and industrial by-products. However, the efficient conversion of these complex substrates into lipids is still a significant challenge, and research is needed to improve substrate utilization.

Genetic manipulation: Although genetic engineering holds promise in enhancing lipid production, it also raises concerns regarding the potential environmental impact and safety. Developing genetically modified strains for commercial biodiesel production requires thorough evaluation and regulatory compliance.

Process scalability: Moving from laboratory-scale research to large-scale industrial production poses challenges in terms of process scalability, cost-effectiveness, and maintaining consistent performance.

Contamination and competition: Oleaginous yeast cultures are susceptible to contamination by other microorganisms, leading to reduced lipid yields. Maintaining pure cultures and preventing contamination is crucial for successful biodiesel production.

Downstream processing: Extracting and refining lipids from oleaginous yeast biomass can be energy-intensive and expensive. Developing cost-effective and efficient downstream processing methods is essential for commercial viability.

Environmental sustainability: While using oleaginous yeast for biodiesel production offers the potential to reduce greenhouse gas emissions, concerns about land use change and competition with food production must be addressed to ensure environmental sustainability.

Despite these challenges, ongoing research and advancements in biotechnology, metabolic engineering, and process optimization are continuously improving the potential of oleaginous yeast for biodiesel production. As the field evolves, addressing these hurdles will be crucial to realizing the full potential of oleaginous yeast as a sustainable and viable source of biodiesel.

Biodiesel global scenario:

The prevailing energy model heavily relies on fossil fuels, supporting various sectors like transportation, industry, and agriculture (Ogunkunle, O., et.al., 2019). However, this model's viability is diminishing due to the depletion of non-renewable energy sources, rising costs, and the associated greenhouse gas emissions, which have been linked to health issues, including cancer. As per the United Nations' Sustainable Development Goals (SDGs) on sustainable energy access (SDG7) and climate change (SDG13), several countries are taking action to reduce carbon emissions by at least 43% by 2030 and limit global temperature increase to 1.5 °C between 2030 and 2050.

To address these challenges, cleaner energy alternatives like biodiesel have emerged. Biodiesel, derived from vegetable and animal fats, can be used in diesel engines without significant modifications, exhibiting physicochemical properties similar to high-performance diesel. This biofuel not only enhances engine performance but also promotes cleaner combustion by reducing CO2 emissions by up to 80% (Maheshwari, P., et.al., 2022).

Over the past decade, biodiesel production has increased by approximately 4–14%, reflecting its growing demand and economic advantage. Europe dominates biodiesel production globally, contributing 34% of the total output in 2021 (Brahma, S., et.al, 2022). Future projections indicate further growth in biodiesel production from 2023 to 2027.

Despite the increasing demand, biodiesel production still struggles to meet requirements in various countries, primarily due to the production cost, which accounts for 60–80% of the lipid raw material expenses. Biodiesel consumption is also highest in Europe (35%), while countries like Canada and Mexico have lower consumption rates (1.4%).

Furthermore, several countries lack legislation promoting biodiesel production and have yet to establish regulations concerning the blending of biodiesel with petroleum diesel. In recent years, biodiesel-producing companies have shifted towards more competitive and accessible feedstocks, such as waste oils, as alternatives to edible seed oils (Malode, S.J., et.al., 2021). Geopolitical conflicts, such as the Ukraine–Russia conflict, have contributed to fluctuations in biofuel prices, prompting some European countries to use cheaper oils or waste cooking oil (WCO) for biodiesel production.

Response Surface Methodology (RSM): An Approach for Optimized Biodiesel Production

RSM is a statistical and mathematical modeling technique used to optimize complex processes. It involves designing experiments, collecting data, and building models to analyze the interactions between multiple variables (Manojkumar, N.,et.al., 2022). RSM employs response surfaces to visualize and navigate the optimization landscape efficiently. By employing statistical methods, RSM guides the process towards optimal conditions and characterizes the relationships between variables.

The utilization of RSM in biodiesel production optimization encompasses various stages of the production process. RSM employs response surfaces, three-dimensional graphical representations, to visualize the relationships between variables and responses (Tan, D.,et.al., 2023). By leveraging statistical techniques, RSM guides the process toward optimal conditions that maximize the desired outcome while minimizing undesirable effects.

RSM facilitates this process by allowing researchers to study the interplay of variables such as feedstock type, reaction temperature, and catalyst concentration (Chumu ang, N, et.al., 2017). By experimenting with various combinations of these factors, RSM helps determine the conditions that yield the highest biodiesel output while minimizing waste and energy consumption.

In the context of biodiesel production, RSM offers a structured methodology to enhance various aspects of the process:

- Feedstock Selection: RSM aids in selecting the most suitable feedstock by analyzing variables such as oil content, fatty acid profile, and impurities. It determines the optimal combination of feedstock attributes to achieve higher biodiesel yields and better fuel properties.
- Reaction Conditions: RSM optimizes reaction parameters like temperature, catalyst concentration, and reaction time. It identifies the ideal conditions that yield the highest conversion rates and product quality.
- Process Parameters: RSM explores the interactions between different process parameters such as mixing intensity, alcohol-to-oil ratio, and agitation speed. By optimizing these variables, RSM enhances the efficiency of the transesterification process.
- Quality Assurance: RSM aids in quality control by analyzing the impact of variables on biodiesel quality parameters such as viscosity, density, and cold-flow properties. This ensures that the final product meets regulatory standards.

Challenges and Future Directions:
While RSM holds immense promise for biodiesel production optimization, challenges such as model complexity, sensitivity to experimental errors, and the need for specialized expertise exist. The integration of RSM with advanced techniques, such as artificial intelligence and machine learning, holds promise in enhancing the accuracy of predictions and extending the method's application to even more intricate processes.

Conclusion:
Oleaginous yeast has demonstrated tremendous potential as a sustainable feedstock for biodiesel production. The utilization of oleaginous yeast for biodiesel production offers a sustainable and efficient alternative to traditional feedstocks. With their ability to accumulate high levels of lipids, adaptability to various carbon sources, and potential for large-scale cultivation, oleaginous yeast provides a promising pathway towards a greener and more economically viable biodiesel industry.

Resonance Surface Methodology stands as a robust approach for optimizing biodiesel production processes. Its systematic exploration of variable interactions empowers researchers and practitioners to identify optimal process conditions. By fine-tuning feedstock selection, reaction parameters, and process variables, RSM plays a pivotal role in achieving economically viable and environmentally sustainable biodiesel production. As the energy landscape evolves, the synergy between RSM and biodiesel production is poised to contribute to a greener and more sustainable future.

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