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Production of Biofuel from Oleaginous Microorganisms

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Abstract: ***The increasing demand for sustainable and eco-friendly energy sources has driven significant interest. Production of biodiesel from oleaginous microorganisms is growing along with improvation. These microorganisms, including certain species of yeast, microalgae, fungi, and bacteria, are capable of accumulating of lipids, especially yeast will accumulate high amounts of lipids, primarily in the form of triacylglycerols (TAGs), which can be transesterified into biodiesel. Unlike conventional feedstocks, microbial lipid production does not compete with food crops for arable land, making it a more sustainable alternative. This study shows the isolation, cultivation, and lipid accumulation potential of oleaginous microorganisms under various nutritional and environmental conditions. Factors influencing lipid productivity, such as carbon and nitrogen sources, C/N ratio, temperature, and pH, are critically analysed. Additionally, downstream processing methods, including cell harvesting, lipid extraction, and transesterification, are discussed. The results shows the feasibility of utilising microbial lipids for biodiesel production and suggest that oleaginous microorganisms represent a promising avenue for the development of next-generation biofuels. Biodiesel can be mixed with normal diesel.***

Keywords: Biodiesel production, Oleaginous microorganisms, Lipid accumulation, Brewery wastewater, Sustainable energy, Microbial lipids, Transesterification, Fatty acid methyl esters (FAMEs)

I. INTRODUCTION

A. Global Energy Crisis And Environmental Concerns

The rapid increase in global energy consumption over recent decades has led to a heavy dependence on fossil fuels such as petroleum, coal, and natural gas. This has resulted in the accumulation of greenhouse gases (GHGs), especially carbon dioxide (CO_2), which leads to global warming and climate change. In 2018, approximately 89% of global CO_2 emissions originated from fossil fuel combustion and various industrial activities. In response to these dangerous trends, international agreements such as the Glasgow Climate Pact have been established to mitigate GHG emissions and promote the use of renewable energy. Among renewable alternatives, biodiesel has gained attention due to its lower pollutant emissions compared to conventional diesel. However, traditional biodiesel feedstocks like palm and soybean oils compete with food production and contribute to escalating production costs. Hence, there is a growing interest in oleaginous microorganisms, particularly yeasts and microalgae, as alternative lipid sources. These microbes can accumulate 20–80% of their dry cell weight as lipids, grow faster than oilseed crops, and require less land and water. (Liang et al., 2013)

B. Biofuels As Sustainable Alternatives

Among the renewable biofuels, biodiesel is produced by the transesterification of natural triglycerides typically from plant oils or animal fats with an alcohol - mostly methanol - into fatty acid alkyl (methyl) esters. The biodiesel from this process has similar characteristics to that of mineral diesel.

Biomass lipid-based liquid fuels like biodiesel and renewable diesel arewidely hated as clean and renewable fuels. These biofuels are conventionally produced from vegetable oils like every other mode of transport, aviation has to respond to the challenges raised by climate change. The combustion of fuels contributes to the greenhouse effect due to carbon emission. In the face of petroleum resource depletion and concern about the environment, the development of renewable, sustainable and efficient energy sources is being strongly encouraged.(Breil et al., 2016)

C. Oleaginous Microorganisms As Biofuel Producers

This review describes recent advances in the four most promising oleaginous yeast:
Yarrowia lipolytica, *Lipomyces starkeyi*, *Rhodosporidium toruloides*, and *Cutaneotrichosporon oleaginosus*.

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These yeast are compared to each other based on critical factors that influence host selection:
1) the substrates they can natively metabolise, 2) the availability of genetic engineering tools, and 3) the scope of biofuel- relevant products that have been reported. While the nascent nature of these studies precludes any definitive winners, we will provide our thoughts on which host is currently most promising. Of course, the benefits and detractors of any host can and likely will change as more is learned about the native metabolism of these organisms and the availability of genetic engineering tools increases.
Fuels are produced from biomass feedstocks have the potential to reduce the CO_2 generation rate from industrial and transportation combustion processes. Microbial systems are capable of efficiently utilising biomass feedstocks of varying quality and composition. The most promising microbes for biofuel production are oleaginous yeast, characterised by their significant accumulation of lipids.which are useful precursors for conversion to biodiesel, green diesel, and jet fuel. The naturally high flux pathways for precursors used in fatty acid biosynthesis can also motivate metabolic engineering of oleaginous yeasts to produce non-native molecules that are potentially useful for biofuels and could have better fuel properties. Other benefits of oleaginous yeast include a broader metabolism of different feedstocks and a wider range of tolerance to operational conditions including pH, inhibitors, and ionic strength. Despite significant efforts to engineer model conventional yeast *Saccharomyces cerevisiae* to produce large quantities of lipids that have recently been reported, oleaginous yeast continues to outperform engineered non-oleaginous yeast. The major disadvantages production costs is factor to utilise microbial oils for biodiesel production, since the feedstocks to cultivate microorganisms account for 60% to 80% of the overall. (Spagnuolo et al., 2019)

D. Feedstocks For Om Cultivation

The majority of bioprocesses use glucose as a feedstock; however, alternative sugars, such as xylose and arabinose, are economically beneficial options over glucose. This encourages interest robust metabolism of a variety of different cellulosic and hemicellulosic hydrolysates. Xylose has received the most attention as it is the major constituent of hemicellulose. The oleaginous yeast have greater metabolic flexibility than *S. cerevisiae,* with *L. starkeyi and R. toruloides* demonstrating more metabolic flexibility than *Y. lipolytica;* however, C. oleaginosus clearly has the most diverse sugar metabolisms. In particular, the ability of C. oleaginous to metabolise xylose without catabolite repression and at rates similar to glucose give it an advantage over other oleaginous yeast.Of the four oleaginous yeast described herein, only *Y. lipolytica* does not readily metabolise xylose as a sole carbon source,due it the presence of cryptic xylose metabolism. (Spagnuolo et al., 2019)

E. Brewery Wastewater (BWW) As A Growth Medium

The brewery industry is a major consumer of water, using between 3 to 10 litres of water for each litre of beer produced. This results in the generation of large volumes of brewery wastewater (BWW), which is rich in organic matter (e.g., sugars, ethanol, volatile fatty acids), nitrogen, and phosphorus. The chemical oxygen demand (COD) in BWW can range from 2000 to 6000 mg/L, with variable pH and temperature. Despite being largely biodegradable, untreated BWW will be having many environmental hazards and requires costly treatment.Oleaginous yeasts, such as *Rhodotorula glutinis* and *Rhodosporidium toruloides,* have been reported for their ability to grow on BWW and synthesise lipids and pigments. Studies show that with appropriate nutrient supplementation (e.g., sugarcane molasses and urea), these yeasts can produce moderate biomass and lipid yields. However, yeast growth in untreated BWW is more.Brewery industries, despite being a important role of the producing country's economy, consume large volumes of water during the production processes, and later release about 70% of it as wastewater. (Amenorfenyo et al., 2019)
Wastewater byproducts such as yeast surplus spent grains, produced from two main beer production stages (brewing and packaging) are the main contributors to environmental pollution when mixed with effluent Furthermore, flushing of human excreta, cleaning of floors, bottles, tanks and machines also contribute to the contamination of water bodies This effluent contains chemical oxygen demand (COD), nitrogen, phosphorous and other high organic loads that makes it unsuitable for any beneficial use Brewery wastewater may be discharged either directly into: municipal sewers, water bodies, or the brewery's wastewater treatment plant and water bodies/municipal sewer system after pretreatment.Discharge of untreated/partially treated brewery wastewater into water bodies raises environmental concerns. The major environmental concerns raised by the operation of breweries include water consumption, wastewater, solid waste and by-product generation, energy use and emissions to air. This phenomenon leads to environmental problems such as water scarcity, excessive growth of undesirable microbes that cause loss of aquatic lifeforms and health-related problems in communities around the discharge areas.There is therefore a need for brewery industries to adequately treat and manage their wastewater’s before their final discharge into the environment.The worldwide energy consumption has been amplified in the past decades, mainly due to factors of population growth and economic development attended with an increasing level of mechanisation

.To cover this demand mainly non-renewable fossil fuels such as petroleum, coal and natural gas are exploited Especially in the transport sector almost the entire energy consumption (~95%) is covered by petroleum based fuels . How-ever, the finiteness along with the uneven distribution of this, resource. mostly in favour to politically unstable regions. has led to issues related with the security of energy supply as well as a strong volatility of the oil price. So, the scientific and political interest into biofuels as renewable alternatives to fossil fuels increased and was accelerated by events as for example the first oil crisis of the1970s. More recently the aspect of the climate change mitigation. (Patel et al., 2018)

The use of fossil fuels is causing serious global problems, including climate change and an energy crisis, due to the emission of greenhouse gases and limited resources. Carbon dioxide (CO_2) one of the major greenhouse gases, is considered to be a significant cause of climate change. It is estimated that 75% of total anthropogenic emissions are from the use of fossil fuels. The use of conventional fossil fuels is challenged by fluctuating prices and depleting resources. In recent years, public awareness of the need to reduce CO_2 emissions and find alternative resources has been heightened. (Dias et al., 2015)

Oleaginous microorganisms (OMs) are capable of utilising inexpensive feedstocks [agro-residues, lignocellulosic substrates (LCSs)] and waste substrates for higher lipid accumulation (Kumar et al., 2020d). Besides, employing OMs could realise the potential of circular economy and development of cost-effective processes. For example, crude glycerol, a by-product of the biodiesel industry, can be supplemented as a carbon source for lipid accumulation (Kumar et al., 2020e). Thereafter, these lipids can be converted to fatty acid methyl esters (FAMEs) and glycerol that again can be recycled. Envisaging this process could realise the viability of the process and hence cost-effective biodiesel production using agro-residues, glycerol, and other waste substrates (Chandel et al., 2020).

Production of OMs is easy to scale up, does not require land and acreage, and results in higher lipid accumulation within shorter incubation times with desirable lipid composition. In case of oleaginous microalgae, they can assimilate atmospheric carbon dioxide into lipid synthesis that ultimately helps in carbon sequestration (Kumar et al., 2017a). Tapping the potential of OMs and lignocellulosic biomass (LCB), this paper is mainly aimed to disseminate the usage of LCS for biodiesel production using OMs. (Chintagunta et al., 2021)

Biodiesel arewidely hated as clean and renewable fuels. These biofuels are conventionally produced from vegetable oils. However, there are a number of challenges associated with the use of traditional vegetable oils as biofuel feedstock, including the high cost of feedstock; low productivity; slow growth rate, low photosynthetic efficiency; competition of feedstock plants for land, moisture and nutrients needed by food crops; rise in prices of food commodities; land use and land cover changes; and other factors. Biodiesel produced by transesterification of triacyglycerols (TAGs) is miscible and can be blended with petroleum diesel in various proportions. Biodiesel is a renewable, non-toxic and relatively clean burning fuel for combustion in compression ignition engines. Renewable diesel, also known as green diesel or hydrotreated vegetable oil, is similar to mineral diesel in composition, consisting of long chain alkanes. Renewable diesel can be produced by hydrotreat-ment of lipids and biodiesel. Renewable diesel is a relatively clean fuel and has better cold-flow properties than biodiesel. Biofuels derived from vegetable oils cannot significantly replace their fossil fuel-based counterparts as the oil content is very low. Therefore, development and utilisation of alternative feedstocks that can overcome these limitations is important. Single cell oil (SCO) from oleaginous microorganisms (including some yeast, fungi, algae, and bacteria) have gained significant attention recently as these organisms can accumulate significant quantities of lipids, mostly in the form of TAGs, and have fatty acid (FA) profiles similar to those of vegetable oils conventionally used as feedstock for biodiesel. Elimination of the food vs. fuel dilemma, insignificant changes in land use and land cover, short growth period, and high accumulation of lipids are among the major advantages offered by oleaginous organisms over traditional vegetable oils However, economic feasibility of biofuel production using SCO from oleaginous microorganisms is hampered by the high cost of sugar-based growth. (Kumar et al., 2021)

In order to achieve sustainable and economical production of lipids by oleaginous microorganisms, a cheap and abundantly available growth substrate is required.The earliest research on producing lipids from microorganisms could be traced back to the First World War, when Germany had prepared with some strains of Endomyces and Fusarium sp. to produce lipids to solve the cooking oil shortage problem. In recent years, high energy prices, energy and environment security, concerns about petroleum supplies are drawn great attention and drive us to find a renewable biofuel. One of the most promising renewable biofuels is biodiesel, a mixture of fatty acid methyl esters, and generally speaking, it is produced from vegetable oils, animal fats or wasting oils by transesterification of triacylglycerols (TAGs).. (Liang et al., 2013)

Fuels produced from biomass feedstocks have the potential to reduce the net generation rate from industrial and transportation combustion processes. Microbial systems are capable of efficiently utilising biomass feedstocks of varying quality and composition.

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The most promising microbes for biofuel production are oleaginous yeast, characterised by their significant accumulation of fatty acid in the form of triglycerides, which are useful precursors for conversion to biodiesel, green diesel, and jet fuel.Other advantages of oleaginous yeast include a broader metabolism of different feedstocks and a wider range of tolerance to operational conditions including pH, inhibitors, and ionic strength. Despite significant efforts to engineer model conventional yeast *Saccharomyces cerevisiae* to produce large quantities of fatty acids that have recently been reported, oleaginous yeast continues to outperform engineered non-oleaginous yeast. (Spagnuolo et al., 2019)

F. Integrated Waste Management And Circular Bioeconomy

Furthermore, some strains also produce carotenoids—valuable antioxidant pigments with commercial significance.Despite their potential, microbial biodiesel production remainseconomically unviable due to high cultivation costs. Therefore, integrating waste valorisation strategies using industrial effluents as nutrient-rich media has emerged as a promising approach to reduce production costs and enhance sustainability.Traditional BWW treatment methods, such as anaerobic digestion and activated sludge processes, are often resource-intensive, generating sludge and using chemicals that raise environmental concerns. Hence, coupling wastewater treatment with biomass valorisation —specifically microbial lipid and carotenoid production—offers a circular and economically viable solution.Unintacunta er alLignocellulosic Biofuels Using Oleaginous Microbes of waste substrates could be a viable option to reduce the cost of the process. In addition, encouraging circular economy process is gaining significant interest to reduce the wastage of resources. (Kumar et al., 2019)

Besides, an integration biofuel production such as bioethanol and biodiesel can also be produced through circular economy process.Concerns about growing energy demand, energy independence. limited reserves, and environmental degradation associated with consumption of fossil fuels have spurred worldwide interest in the development and utilisation of sustainable, biomass-based liquid fuels.((Kumar et al., 2021)

G. Research And Industrial Trends

The global markets for biodiesel are entering a period of rapid and transitional growth. In the year 2007, there were only 20 nations producing biodiesel for the needs of over 200 nations; by the year 2010, more than 200 nations became biodiesel producing nations and suppliers. Global Biodiesel production massively Increased to 18.2 billion litres per year from 2000 to 2010. However, the plant oil materials requires large energy and acreage for sufficient production of oilseed crops.Over the last decades, the production of biofuels from renewable sources has gained more attention due to critical environmental issues, such as greenhouse gas emission, rapid depletion of fossil fuel supplies, and high energy cost. (Liang et al., 2013)

Microorganisms predominate Earth's biota. They encode the majority of genetic diversity on the planet and underpin nearly all biogeochemical processes. Advances in molecular methods are now allowing us to uncover the extensive gene pool contained with microbial ecosystems, and this resource will ultimately drive and sustain our societal and environmental needs. Among the renewable commodities of immediate interest which can be produced by microbes in significant quantities are biofuels. (Muller et al., 2014)

Serial No.	Microorganis m Species	Substrate Source	Carbon and Nitrogen Profile	Biomass Production	References
1	*Scenedesmus sp.*	Brewery wastewater	C: CO_2, organic matterN: NH_4^+, NO_3^-	Lipid accumulation increased under stress	(Kukwa & Chetty, 2022)
2	*Scenedesmus dimorphus*	Brewery wastewater	C: Brewery wastewaterN: TN 61.8–247 mg/L	44.26% lipid content	(Lutzu et al., 2015)
3	*Scenedesmus obliquus*	Brewery effluent	C & N: Not specified	Not specified	(Ferreira et al., 2019)

4	Anaerobic bacteria (*Methanosaeta, Methanobacterium*)	Brewery wastewater	Balanced C:N through co-digestion	Not applicable	(Sganzerla et al., 2023)
5	*Trichosporonoides sapthula, R. mucilaginosa, Y. lipolytica*	Hydrolysates of BSG and SYC	C: BSG (47), SYC (8.84)N: Protein-rich SYC	62.9 mg/g (BSG), 39.9 mg/g (SYC)	Saithip Sae-ngae et al.
6	*R. toruloides, Chlorella pyrenoidosa*	Distillery & domestic wastewater	C:N High (promoting lipid accumulation)	Enhanced in mixed cultures	Ling et al., 2014
7	Consortium of 10 oleaginous microbes	Primary effluent wastewater	C:N: 1:1 (suboptimal, 40:1 better)	465.52 mg/L (positive control)	Hall et al., 2011
8	*Cryptococcus curvatus* (MUCL 29819)	Glucose, Acetate	C: Glucose, acetateN:	Up to 50% DCW lipid	Christophe et al., 2011
			Yeast extract, peptone		
9	*Candida parapsilosis* Y19	Orange peel	C: GlucoseN: $(NH_4)_2SO_4$, yeast extract	39.1% lipid (DCW)	Matouk et al., 2025
10	*R. toruloides* Y4	Glucose	C: GlucoseN: Varies	37.09% lipid (DCW)	Zhou et al., 2011
11	Various engineered oleaginous organisms	Glucose, glycerol, hydrolysates	C: CO_2, glycerolN: Nitrogen starvation used	Not specified	Levering et al., 2015
12	*L. starkeyi, R. toruloides*, etc.	Multiple (lignocellulosic)	C:N: HighN starvation enhances lipids	Predominantly C16–C18 fatty acids	Sitepu et al., 2013
13	Various microalgae incl. *S. dimorphus, N. cincta*	Varied	Nitrogen starvation boosts lipids	Variable (species-dependent)	Ganesan et al., 2020
14	Yeasts, fungi, algae	Various microbial lipids	Not specified	Not specified	Franz & Yothers, 2019
15	Microalgae, yeasts, fungi, bacteria	Various low-cost substrates	Limited N enhances lipids	Up to 70% lipid (DCW)	Patel et al., 2019
16	*T. cutaneum, R. glutinis*, etc.	Lignocellulosic hydrolysates	High C:N promotes lipid production	40% lipid (DCW), 28.6 g/L biomass	Chintagunta et al., 2021

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This is the research studies focused on microbial lipid production for biodiesel and bioproduct applications. The reviewed works explore a variety of microbial strains—including microalgae, yeasts, and bacterial consortia—utilized on diverse low-cost substrates like brewery waste, lignocellulosic biomass, and domestic wastewater. The strategic manipulation of carbon and nitrogen availability, particularly high C:N ratios and nitrogen limitation, to enhance lipid accumulation is the recurring theme. The data extracted helps identify the most promising organisms and cultivation strategies for sustainable and economically viable biofuel production, forming a critical basis for selecting microbial candidates and process conditions for future experimental work.

II. NEED FOR SUSTAINABLE ALTERNATIVES

Fossil fuel combustion started during the Industrial Revolution and has since been crucial in supplying global energy demands. The exponential increase in industrialisation, population and urbanisation over recent years has resulted in a global energy crisis and concern regarding the dependence on non-renewable energy sources. Fossil fuels, including petrol, diesel, coal and natural gas, supplied 84% of the global, primary energy consumption in 2019 [1], making them the dominant source of energy worldwide but at current consumption rates it is predicted that gas and oil reserves will run out in ~50 years [2].

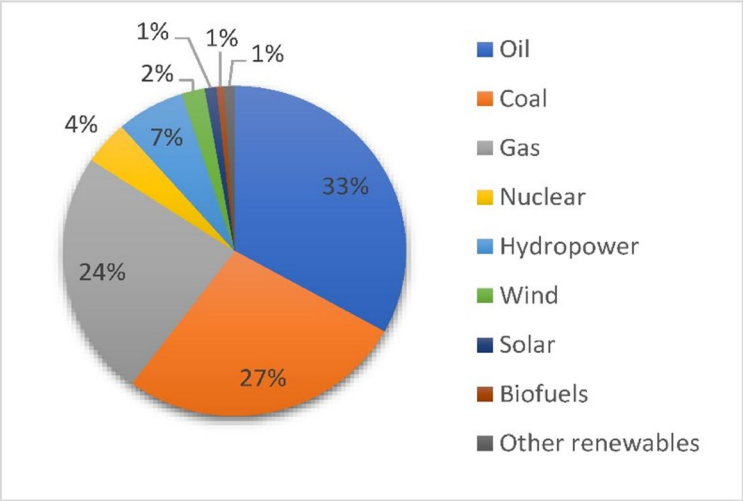

Fig 2.1: Renewable energy consumed across the globe

An estimated 58% of fossil fuels are consumed by the transportation of people and goods via road, rail, air and marine travel [3]. In 2016, the transport sector alone was responsible for 16% of the total, global GHG emissions, highlighting the pressing need for green alternatives to petrol and diesel [2].

A. Biofuel as A Potential Renewable Energy Source

Considering the different renewable energies, biofuels are arguably a potential renewable energy source in the transportation industry. Almost all other renewable energies, particularly solar, wind, hydro and nuclear power sources, only generate electricity and hence cannot equally compete with oil [4]. There are multiple difficulties associated with electricity, which make these energy sources less appealing, such as transmission over long distances and conversion to different types of energy sources. In addition, biofuels can be used within current infrastructures and require less technological advances compared with other energy sources. For this reason, both developed and developing countries have focused on expanding their bioenergy market and set up intergovernmental strategies for the use of biofuels. The introduction of such policies, particularly in Europe, the US and Brazil, has caused the biofuel industry to grow in the last decade with biofuels now representing around 3% of transport fuels in use globally [5,6].

Biofuels are combustible fuels produced from organic matter such as plant material and animal waste. They can exist in solid, liquid, and gaseous forms; however, considerable research focuses on liquid biofuels as they have the greatest potential to help decarbonise the transport sector due to easier integration with existing technology [7]. Ethanol is currently the most widely used biofuel globally, accounting for approximately 80% of all liquid biofuel production. The use of global ethanol as a biofuel (so-called “bioethanol”) production [8,9] has increased significantly in recent years, with the global production predicted to be over 135 billion L by 2024 with the largest contributions from the USA (42%) and Brazil (31%) biofuel industries [10].

B. Types Of Biofuel

Four categories are used to group biofuels based on the type of feedstock used to produce them, their limitations as a renewable source, and their technological progress. First generation biofuels are produced from edible feedstocks, e.g., bioethanol from corn and sugar cane and biodiesel from oil seed crops (soybean, oil palm, rapeseed ad sunflower) using well understood, economically viable technologies and processes, such as fermentation, distillation and transesterification [11,12]. First generation biofuels only provide minimum benefit over fossil fuels in terms of greenhouse gas emissions as they require a large amount of energy (from fossil fuels) to grow, collect and process.

Second generation biofuels are produced from agricultural by-products or cellulosic materials such as wood, leaves and grass and can be grown on marginal land [13,14,15,16]. They are produced by converting cellulose into sugar units, which can then be converted to ultimately produce alcohol. Cellulosic sources that grow alongside food crops could be used for biomass, but this process takes away so many nutrients from the soil and would need to be restored nutrients by applying fertilizer. This process is both costly (chemically and economically) and time-consuming, requiring sophisticated equipment and larger-scale facilities.

Third-generation biofuels are made from aquatic cultivated feedstock, i.e., algae [17,18]. Algae have been shown to have great potential as biofuel feedstocks, due to their capabilities of producing much higher yields with reduced resource inputs [19,20,21]. The use of algae also has other environmental advantages, as a result of their ability to fix CO_2, which has been proposed as a method for removing CO_2 from flue gases from power plants, thus reducing GHG emissions [22,23,24]. However, there has been little research on the economic and environmental feasibility of using algae as a biofuel feedstock, with concerns regarding its commercial-scale production. The growth of seaweed is highly seasonal, meaning that preservation methods need to be developed to allow year-round storage of the feedstock for fuel manufacturing processes [25]. The drying stage is the key part of the energy extraction method. The high water content of algae compared with terrestrial crops [26], means that this process is highly energy intensive [27]. Sun-drying has been used as a low-energy alternative method [28]. However, this has its own limitations, being highly weather dependent. These factors highlight the growing need for research into algae as biofuel feedstocks, with its future applicability being highly dependent on the development of biomass-to-fuel conversion technology which can work with wet feedstocks, or drying processes with much reduced energy requirements [29]. The fourth-generation biofuels are found from the bioengineered microorganisms, e.g., bioengineered algae, yeast, fungi and cyanobacteria [30,31]. Second, third and fourth generation biofuels are commonly referred to as ‘advanced biofuels’ and thought to hold many advantages over first generation fuels, but they are still in the research and development phase and have not reached their full commercial potential.

C. Types of Oleaginous Microorganisms

1) Oleaginous Microalgae

Oleaginous microalgae are a promising source for the production of renewable biofuels owing to their efficient photosynthesis capabilities, the reduced needs for growth area compared to terrestrial plants, and their ability to channel the majority of their energy into cell division, which enhances biomass productivity . Microalgae can use both inorganic and organic carbon sources through four different modes of cultivation, namely, autotrophic, mixotrophic, heterotrophic, and photoheterotrophic. Synthesis of TAGs in microalgae takes place mainly in the sub-cellular compartments such as chloroplast and endoplasmic reticulum as a result of multiple enzymatic reactions. Fatty acid synthesis in the chloroplast, assembly of glycerolipids in endoplasmic reticulum, and accumulation of TAGs into the oil bodies are the three major steps involved in the accumulation of lipids in microalgae. It has been proven that different stress conditions such as physical, chemical, or environmental, individually or in combination, facilitate the synthesis of high amounts of lipids [31]. Under different stress conditions, microalgae can switch their metabolism towards the formation and accumulation of neutral lipids in the form of TAGs, which serves as a form of carbon and energy storage [32,33]. Microalgae synthesize lipids via the de novo pathway, which starts in the chloroplast by CO_2 fixation into sugars, which are further metabolized to form acetyl-CoA, which is a precursor of fatty acid synthesis. Photosynthetic reactions occurring in autotrophic cultivation provide not only a carbon source but also assist in generating reducing power (NADH and NADPH) that is finally used for lipid synthesis [34]. However, low biomass and lipid productivity and the requirement of appropriate photobioreactors are major drawbacks of industrial-scale applications of autotrophic cultivation [35,36]. In the past decades, researchers have been focusing more on the heterotrophic cultivation of algae as it has many advantages over the photoautotrophic cultivation, such as cost-effectiveness and being relatively easy to cultivate with quite low daily maintenance [37]. Furthermore, heterotrophic cultivation can be carried out in any fermenter that is utilized for yeast and bacteria without illumination, and as such, the use of photobioreactor is not required, which in turn reduces the overall production cost. Glucose is a commonly used carbon source for the heterotrophic mode of cultivation; however, it must be obtained from renewable sources to avoid the high cost

associated with feedstocks [38]. Various inexpensive raw materials obtained from inedible lignocellulosic biomass from forests such birch, spruce, and beech, or agricultural residues such as rice and wheat straw, sugarcane bagasse, corn stover, waste molasses, soy whey, and industrial wastewater, have been successfully applied to support heterotrophic cultivation [31].

2) Oleaginous Yeast and Filamentous Fungi

Typically, oleaginous yeasts are chosen when it comes to the production of lipids. Oleaginous yeasts are well-studied microorganisms and include species of the genera *Candida, Rhodosporidium, Yarrowia, Cryptococcus*, *Rhodotorula*, *Lipomyces*, and *Trichosporon*, some of which can accumulate lipids up to 80% *w/w* of their dry cell weight [39]. Additionally, the lipid metabolism of these oleaginous yeasts is well- known [40]. Other potential strains for lipid production are continuously sought for and selected, with several strains engineered for increased lipid production [41,42]. There are certain criteria that these strains should meet, such as the ability to grow to high cell densities along with high lipid content on various carbon sources and robust process conditions. To improve economic feasibility, oleaginous yeast strains have thus been cultivated on various non-food competing carbon sources, such as lignocellulosic materials [43,44]. The non-oleaginous yeast *Saccharomyces cerevisiae* is used in many industrial applications since it is easy to cultivate and its genetic tools are well-established. Consequently, *S. cerevisiae* has also been exploited and subjected to metabolic engineering approaches for lipid production [45,46].

Oleaginous filamentous fungi are promising microbes for biofuel production and have certain advantages such as unique fatty acid profiles with fatty acids such as γ-linolenic acid (GLA) that cannot be synthesized in high amounts by other oleaginous microorganisms [47]. Fungi can be cultivated on inexpensive feedstocks such as waste molasses, monosodium glutamate wastewater, sewage sludge, glycerol, and agricultural residues [48]. An oleaginous fungus, *Cunninghamella echinulate*, cultivated on orange peel and glucose, synthesized 46.6% of total lipids, including 14.1% of γ-linolenic acid (GLA). Similarly, the fungus *Mortierella alpina* LPM 301, cultivated on glucose with potassium nitrate, synthesized high amounts of lipids (31.1%) that contained 60.4% of arachidonic acid (ARA) [49]. Oleaginous fungi were used to grow along with microalgae for enhanced lipid productivity, e.g., a marine microalgae *Nannochloropsis oceanica* and an oleaginous fungus *Mortierella elongata* were co-cultivated to initiate bio-flocculation that yielded high amounts of TAGs and PUFAs (polyunsaturated fatty acids), along with total lipids. *Aspergillus niger* cultivated on sugarcane distillery wastewater or vinasse as low- cost feedstock was utilized for the production of biodiesel. In a study, *A. niger* cultivated on pure vinasse showed highest cell dry weight of 24.05 g/L, while the highest lipid produced (2.27 g/L) was by *Aspergillus awamori* among 28 different strains tested [50].

3) Oleaginous Bacteria

Oleaginous bacteria are also a good source of TAGs; however, their utilization for biodiesel production is limited compared to microalgae and yeast. Some important genera of oleaginous bacteria are *Rhodococcus* sp., *Gordonia* sp., *Acinetobacter* sp., and *Arthrobacter* species. Among them, *Rhodococcus* sp. has been the most extensively studied as a result of their ability to grow on versatile substrates [51]. Within the biorefinery concept for the production of biofuels, lignin is often left underutilized. Only certain fungi (mainly white-rot fungi) and prokaryotes have lignin-depolymerizing enzymes. Recently, *Rhodococcus* sp. was studied for its potential to degrade lignin and finally assimilate lignin monomeric compounds into the lipid accumulation pathway [52]. In a study, *Rhodococcus opacus* attained a lipid content of 26.8% *w/w* when cultivated on aromatics obtained from organosolv pretreatment of loblolly pine along with lignocellulosic pretreatment effluents containing various sugars. This species was also applied to convert oxygen-pretreated Kraft lignin into valuable lipids [53].

D. LIPID Accumulation Mechanism

Oleaginous microorganisms, including yeasts, fungi, and microalgae, accumulate lipids as intracellular storage compounds, primarily in the form of triacylglycerols (TAGs), under conditions of nutrient stress. The Kennedy pathway is the central metabolic route for TAG synthesis, where glycerol-3-phosphate is sequentially acylated to form TAGs [54]. This process is tightly regulated by key enzymes such as acetyl- CoA carboxylase (ACC), which catalyzes the conversion of acetyl-CoA to malonyl-CoA, and ATP-citrate lyase (ACL), which generates cytosolic acetyl-CoA from citrate [55]. Recent studies have shown that nitrogen limitation (C:N ratio >40:1) is a critical trigger for lipid accumulation, as it shifts cellular metabolism from growth to storage lipid production. For example, Rhodotorula toruloides has been reported to accumulate lipids up to 70% of its dry weight under nitrogen-starved conditions [56]. Advances in genetic engineering have further enhanced our understanding of lipid accumulation. CRISPR- Cas9-mediated gene editing has been employed to overexpress key enzymes like diacylglycerol acyltransferase (DGAT), which catalyzes the final step of TAG synthesis.

In Yarrowia lipolytica, overexpression of DGAT2 resulted in a 30% increase in lipid yield [57]. Additionally, blocking competing pathways, such as β-oxidation in Chlorella vulgaris, has been shown to improve lipid retention by 50% [58]. Another emerging area of research is phospholipid remodeling, where microorganisms convert membrane phospholipids like phosphatidylcholine into storage TAGs under stress conditions, thereby increasing lipid droplet size and stability [59]. The role of lipid droplets (LDs) as dynamic organelles has also been elucidated. LDs are coated with proteins such as perilipins, which protect stored lipids from degradation by lipases [60]. Recent work has demonstrated that modulating the expression of these proteins can significantly impact lipid accumulation. For instance, silencing the gene encoding the LD-associated protein Plin2 in Y. lipolytica led to a 20% reduction in lipid degradation, thereby increasing net lipid yields [61]. These findings highlight the potential of targeted genetic modifications to optimize lipid production in oleaginous microorganisms.

E. Substrates Used For Growth

The economic viability of microbial lipid production heavily depends on the choice of substrate. While synthetic media, such as glucose-based YPD (yeast extract-peptone-dextrose), offer reproducibility, they are cost-prohibitive for large-scale applications, accounting for ~60% of total production costs [62]. Consequently, there has been a significant shift toward low-cost, renewable substrates, including industrial and agricultural wastes. Brewery wastewater is a promising substrate due to its high organic carbon content (chemical oxygen demand, COD: 2,000–6,000 mg/L) and abundance of fermentable sugars like maltose.

Studies have shown that Cryptococcus curvatus cultivated in brewery wastewater can achieve lipid yields of 5.8 g/L, with lipid content exceeding 50% of dry cell weight [63]. Similarly, food waste hydrolysates, rich in glucose and free fatty acids, have been successfully used to cultivate Aspergillus oryzae, yielding lipid productivities of 0.18 g/L/h [64].

Lignocellulosic biomass, such as corn stover and sugarcane bagasse, represents another attractive substrate due to its low cost and widespread availability.

However, the complex structure of lignocellulose necessitates pretreatment (e.g., acid/alkali hydrolysis or enzymatic digestion) to release fermentable sugars. For example, Lipomyces starkeyi grown on NaOH-pretreated rice straw achieved 55% lipid content, demonstrating the potential of lignocellulosic feedstocks for microbial lipid production [65]. Despite these advantages, challenges remain, including the presence of inhibitory compounds (e.g., furfurals and phenolics) generated during pretreatment, which can suppress microbial growth. Detoxification strategies, such as activated charcoal adsorption or biological detoxification using Coniochaeta ligniaria, have been explored to mitigate these effects [66].

The use of mixed microbial consortia has also gained attention for its ability to utilize complex substrates more efficiently. For instance, co-cultures of R. toruloides and Bacillus subtilis demonstrated synergistic lipid production when grown on lignocellulosic hydrolysates, with lipid yields 25% higher than monocultures [67]. These findings underscore the importance of substrate selection and pretreatment in optimizing lipid production from oleaginous microorganisms.

F. Fermentation And Cultivation Techniques

The choice of fermentation strategy significantly impacts lipid productivity and scalability. Batch fermentation, while simple to operate, often suffers from substrate inhibition and low yields. In contrast, fed-batch fermentation has emerged as the preferred method for high-density microbial cultures. For example, Yarrowia lipolytica cultivated in fed-batch mode achieved 85 g/L biomass with 60% lipid content, a significant improvement over batch systems [68].

Continuous fermentation, though complex, offers advantages such as steady-state operation and higher volumetric productivity. A recent study demonstrated that Rhodotorula glutinis in a continuous bioreactor with cell recycling achieved a lipid productivity of 0.22 g/L/h, nearly 50% higher than batch cultivation [69].

Solid-state fermentation (SSF) is another promising approach, particularly for agro-industrial wastes. SSF mimics the natural habitat of many oleaginous fungi, leading to higher lipid yields. For instance, Aspergillus oryzae grown on sugarcane bagasse under SSF conditions produced 30% more lipids than submerged fermentation [70].

Critical parameters influencing lipid production include:

pH: Optimal ranges vary by strain; C. curvatus performs best at pH 5.0–6.0 [18] Temperature: Mesophilic strains (e.g., Y. lipolytica) typically require 25–30°C [19]

Aeration and agitation: Enhanced oxygen transfer improves lipid yields; R. toruloides showed 20% higher lipid content at 150 rpm compared to static conditions [71].

G. Lipid Extraction Methods

Cell disruption is a critical step in lipid recovery. Mechanical methods, such as bead milling and high- pressure homogenization, are effective but energy-intensive. Chemical methods (e.g., acid/alkali treatment) are cost-effective but may degrade lipids [72].

Solvent extraction remains the gold standard, with the Bligh & Dyer method (chloroform-methanol-water) achieving >95% lipid recovery [73]. However, green solvents like supercritical CO_2 are gaining traction due to their non-toxicity and selectivity. A 2023 study reported 90% lipid extraction efficiency from Nannochloropsis using supercritical CO_2 at 40°C and 300 bar [74].

Enzymatic lysis (e.g., using lysozyme or cellulase) is another eco-friendly alternative, though enzyme costs remain a barrier [75].

H. Biofuel Conversion Process

The conversion of microbial lipids into biodiesel primarily occurs through transesterification, where triglycerides react with short-chain alcohols (typically methanol) to produce fatty acid methyl esters (FAMEs) and glycerol [76]. This process is catalyzed by acid/base catalysts or lipases, with base-catalyzed (NaOH/KOH) transesterification being the most industrially adopted method due to its high efficiency (>90% yield) and rapid reaction kinetics (1–2 hours at 60°C) [26]. Recent advances (2020–2025) highlight the use of heterogeneous catalysts (e.g., CaO, MgO) to simplify product separation and reduce wastewater generation [77]. For instance, *Yarrowia lipolytica* lipids converted with CaO nanoparticles achieved 95% FAME yield while enabling catalyst reuse for 5 cycles [78].

Enzymatic transesterification using immobilized lipases (e.g., *Candida antarctica* Lipase B) offers milder reaction conditions (30–40°C) and higher specificity, avoiding soap formation—a common issue with base catalysts [79]. A 2023 study demonstrated that lipase-mediated conversion of *Rhodotorula glutinis* lipids reduced energy consumption by 40% compared to alkaline catalysis [80]. However, enzyme costs remain prohibitive for large-scale applications ($300–500/kg) [81].

Fuel Properties of Microbial Biodiesel:

- Cetane number (CN): Microbial FAMEs exhibit CN values of 50–65, exceeding ASTM D6751 standards (min. 47) due to high saturation levels [82].
- Oxidative stability: Lower polyunsaturated FAME content (<5%) in Cryptococcus curvatus- derived biodiesel increases shelf-life to 6 months vs. 3 months for plant-based biodiesel [83].
- Cold-flow properties: Branched-chain FAMEs from *Isochrysis galbana* improve cold-filter plugging points (-10°C) [84].

Emerging Techniques (2020–2025):

- In situ transesterification: Combines lipid extraction and conversion in one step, reducing processing time by 50% [85].
- Microwave-assisted reactions: Enhance reaction rates 3-fold while lowering methanol requirements [86]

III. MATERIALS AND METHODOLOGY

A. Sudan B Black Test

This test is done to determine the presence of lipids in the sample Principle for Sudan B Black Staining:

Sudan stain uses frozen tissue sections that are fixed using formalin solution or paraffinized sections. Sudan

Black B dye is the most used dye from the Sudan dye groups. Sudan Black B is a slightly basic dye that combines with the acidic groups in the lipid compounds, hence staining the phospholipids, lipoproteins, and triglycerides found in the staining specimen.

1) Sample Collection And Initial Culturing

Fig 3.1: Cultured samples of oleaginous organisms

The primary microbial source utilized in this study was brewery wastewater, which is known to contain a diverse population of microorganisms, including oleaginous strains capable of lipid accumulation. A 1 mL aliquot of brewery wastewater was aseptically inoculated onto a nutrient-rich agar plate within a Laminar Air Flow (LAF) cabinet to maintain a sterile environment and prevent external contamination. The agar plate was incubated at 37°C for 24 hours, promoting the growth of a mixed microbial culture. This step was essential to isolate active microorganisms potentially capable of intracellular lipid production.

2) Sudan Black B Staining For Lipid Detection

To identify the presence of oleaginous microorganisms, a Sudan Black B staining procedure was employed. This staining technique is used to determine the presence of lipids hence confirming the presence of oleaginous microorganisms.

Fig 3.2: Prepared Sudan Black B stain

3) Smear Preparation

A loopful of cultured biomass was spread onto a clean glass microscope slide. The smear was spread evenly, air-dried, and subjected to heat fixation by passing the slide gently through a Bunsen flame or alternatively by treatment with 70% ethanol for 2 minutes.

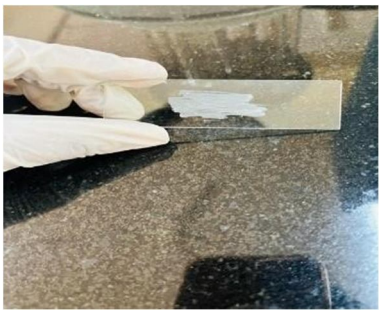

Fig 3.3: Microorganisms smeared on slide

4) Staining Procedure

The fixed smear was flooded with 0.3% Sudan Black B solution, a lysochrome (fat-soluble dye), and allowed to react for 10–15 minutes. After staining, the slide was rinsed thoroughly with distilled water to remove excess dye and then examined under a compound light microscope.

Fig 3.4: Staining of smeared slides

B. Negative Control (Blank Test)

This sample serves as a baseline to evaluate the effects of dilution and dissolved oxygen variation on biomass and lipid production. This section details the observations and results from the control setup, where no dilution or modification was applied to the brewery wastewater.

Materials are not much needed as there will be no alteration for Blank test

1) Incubation Conditions

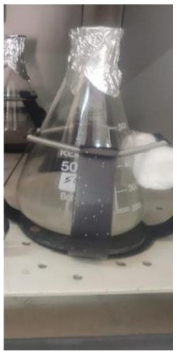

Fig 3.5: Sample kept for harvesting

A volume of 200 ml of the untreated brewery wastewater was placed in a conical flask and incubated for three days on a rotary shaker at 130 rpm. This setup maintained aerobic conditions and mixing to simulate natural fermentation without any external enhancement.

2) Biomass Harvesting And Drying

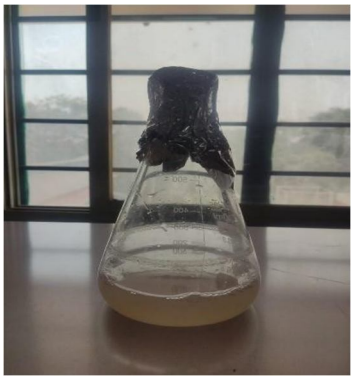

Fig 3.6: After harvesting for three days

Fig 3.7: Harvest kept for drying

After the incubation period, the wastewater was centrifuged, and the pellet was collected and was labelled, and it was kept for drying, the drying period varied with the amount of the pellet collected.

3) Extraction

After the incubation period, the wastewater was centrifuged, and the pellet was collected and waslabelled, and it was kept for drying, the drying period varied with the amount of the pellet collected.

4) Lipid Extraction

Lipid extraction from the dried biomass was performed using the Folch method (chloroform : methanol, 2:1).
Screening of Chloroform and Methanol Efficiency
Chloroform dissolves non-polar lipids
Methanol disrupts cell membranes and solubilizes polar lipids and proteins. The combination helps in comprehensive lipid extraction.

Selective
Ratio affects lipid class extraction: more chloroform = more non-polar lipids; more methanol = better protein precipitation and polar lipid extraction.
Screening different ratios can optimize recovery of specific lipid classes (e.g., phospholipids, glycolipids).

Safety and environmental concerns
Chloroform is toxic and carcinogenic, raising disposal and safety issues.
Methanol is also toxic but less environmentally persistent.
Screening may aim to replace or reduce chloroform use (e.g., with less hazardous solvents like methyl tert-butyl ether [MTBE]).
After addition of solvents, the mixtures were centrifuged, resulting in three distinct layers: Top layer – Lipids
Middle layer – Biomass residue
Bottom layer – Methanol and chloroform
The top lipid layer was carefully pipetted and quantified.

5) Transesterification Process For Biodiesel Production

The extracted lipids were converted into Fatty Acid Methyl Esters (FAME) via the transesterification process, using methanol as the alcohol and sodium hydroxide (NaOH) as the base catalyst.

6) Process Parameters

Methanol to lipid molar ratio: 6:1 Catalyst concentration: 5% (w/w of lipid)
The methoxide solution was prepared by mixing methanol and NaOH, then reacted with the lipid extract. The reaction mixture was allowed to settle, forming two distinct layers:
Top layer – Biodiesel (FAME) Bottom layer – Glycerol
The biodiesel produced.

C. Addition Of Carbon Source

1) Chemicals And Reagents

Brewery wastewater.
Carbon sources – Glucose, sucrose, lactose, and maltose (1g each). Urea – Nitrogen source (0.04 g per flask).
Sudan Black B (0.3%) – For lipid staining.
Methanol and chloroform (1:2 ratio) – For lipid extraction. Sodium hydroxide (NaOH) – Catalyst for transesterification. Ethanol (70%) – For fixation and cleaning.
Distilled water – For all preparations.

2) Glassware And Equipment

Conical flasks, test tubes, petri dishes, beakers, pipettes, and slides. Laminar Air Flow cabinet – For aseptic work.
Incubator (30–37°C) – For microbial growth. Shake flask incubator – For aerated culturing. Hot air oven – For biomass drying (55°C).
Centrifuge – For separation steps. Microscope – For staining observation.
Magnetic stirrer, pH meter, weighing balance – For media and measurement.

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3) Safety Materials

Gloves, masks, cotton swabs, labels, and autoclave bags.

To evaluate the lipid accumulation efficiency of microbes, different carbon sources were tested.

Fig 3.8: Different carbon sources used

4) Preparation Of Carbon Source Solutions

1g each of glucose, sucrose, maltose, and lactose was dissolved in 100 mL of distilled water. These solutions were mixed thoroughly using a magnetic stirrer to ensure complete dissolution. All carbon source solutions, except for the lactose solution, were autoclaved to achieve sterilization.

5) Substrate Formulation

Each carbon source solution was mixed with freshly collected brewery wastewater, acting as the microbial substrate. The pH of the mixture was adjusted to the optimal range of 4.0 to 6.0, as lipid accumulation efficiency is pH sensitive. The recorded pH values were:

Subsequently, 0.04 g of urea was added to each flask as a nitrogen source to stimulate microbial metabolism without suppressing lipid biosynthesis.

6) Incubation And Biomass Development

The prepared flasks were placed in a shake flask incubator set at 30°C and 150 rpm for a duration of 72 hours (3 days). This ensured optimal aeration and homogeneous mixing of nutrients. At the end of the incubation period, it was observed that:

Fig 3.9: a) In the incubator Fig 3.9: b) After the harvesting was done

Flasks with glucose and sucrose showed a volume reduction to 50mL. Flasks with maltose and lactose showed a reduction to 20–30mL.

This suggested higher metabolic activity and substrate utilization in the glucose and sucrose samples, as these sugars are more readily metabolizable compared to lactose and maltose, which require additional enzymatic hydrolysis.

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7) Extraction

After the incubation period, the wastewater was centrifuged, and the pellet was collected and was labelled, and it was kept for drying, the drying period varied with the amount of the pellet collected.

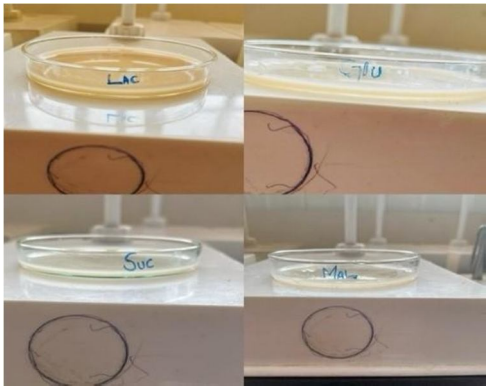

Fig 3.10: Pellets containing biomassFig 3.11: Dried biomass

8) Lipid Extraction

Lipid extraction from the dried biomass was performed using the Folch method (chloroform : methanol, 2:1).
Screening of Chloroform and Methanol Efficiency
Chloroform dissolves non-polar lipids
Methanol disrupts cell membranes and solubilizes polar lipids and proteins. The combination helps in comprehensive lipid extraction.

Selective
Ratio affects lipid class extraction: more chloroform = more non-polar lipids; more methanol = better protein precipitation and polar lipid extraction.
Screening different ratios can optimize recovery of specific lipid classes (e.g., phospholipids, glycolipids).

Safety and environmental concerns
Chloroform is toxic and carcinogenic, raising disposal and safety issues.
Methanol is also toxic but less environmentally persistent.
Screening may aim to replace or reduce chloroform use (e.g., with less hazardous solvents like methyl tert-butyl ether [MTBE]).
After addition of solvents, the mixtures were centrifuged, resulting in three distinct layers: Top layer – Lipids
Middle layer – Biomass residue
Bottom layer – Methanol and chloroform

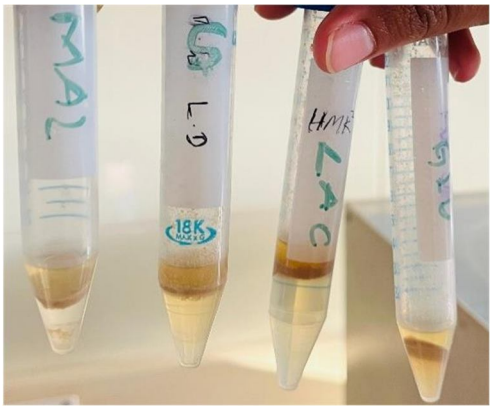

Fig 3.12: Different layers containing lipid and Floch's reagent

The top lipid layer was carefully pipetted and quantified.

9) Transesterification Process For Biodiesel Production

The extracted lipids were converted into Fatty Acid Methyl Esters (FAME) via the transesterification process, using methanol as the alcohol and sodium hydroxide (NaOH) as the base catalyst.

10) Process Parameters

Methanol to lipid molar ratio: 6:1 Catalyst concentration: 5% (w/w of lipid)

The methoxide solution was prepared by mixing methanol and NaOH, then reacted with the lipid extract. The reaction mixture was allowed to settle, forming two distinct layers:

Top layer – Biodiesel (FAME) Bottom layer – Glycerol

The biodiesel produced.

D. Changing Of Dissolved Oxygen

Concentrated brewery wastewater was collected in the purpose of this experiment and the COD was 2550

And 100ml of brewery wastewater was taken in conical flask and dilution of 25ml, 50ml, 100ml of distilled water was added to each test tube making the COD

25ml diluted with 100 ml conc. Brewery wastewater - 1800 50ml diluted with 100 ml conc. Brewery wastewater – 1500 100ml diluted with 100 ml conc. Brewery wastewater – 1125

To calculate Chemical Oxygen Demand (COD) after dilution, we can use the dilution equation: C1.V1=C2.V2

Where,

C1 = Initial COD (2550mg/L)

V2 = Initial volume of brewery wastewater C2 = Final COD after dilution

V*diluent* = 25ml, 50ml, 100ml of distilled water

Table 3.1: Change in COD after addition of distilled water

Sample	Distilled Water Added (ml)	Total Volume (ml)	COD (ppm)
A	25	125	1800
B	50	150	1500
C	100	200	1125

1) Harvesting

Added 25ml, 50ml, 100ml distilled water to 100ml of brewery wastewater

Fig 3.13: Flasks after harvesting for three days

The prepared flasks were placed in a shake flask incubator set at 30°C and 150 rpm for a duration of 72 hours (3 days).

2) Extraction

After the incubation period, the wastewater was centrifuged, and the pellet was collected and was labelled, and it was kept for drying, the drying period varied with the amount of the pellet collected.

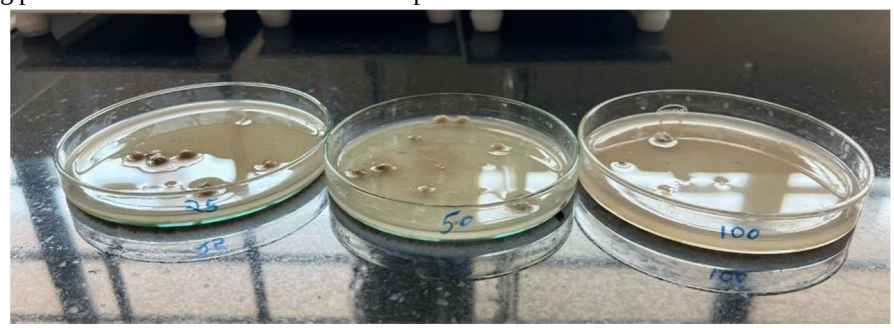

Fig 3.14: Pellets containing biomass after centrifugation

3) Lipid Extraction

Lipid extraction from the dried biomass was performed using the Folch method (chloroform : methanol, 2:1).
Screening of Chloroform and Methanol Efficiency
Chloroform dissolves non-polar lipids
Methanol disrupts cell membranes and solubilizes polar lipids and proteins. The combination helps in comprehensive lipid extraction.

Selective
Ratio affects lipid class extraction: more chloroform = more non-polar lipids; more methanol = better protein precipitation and polar lipid extraction.
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4) Transesterification Process For Biodiesel Production

The extracted lipids were converted into Fatty Acid Methyl Esters (FAME) via the transesterification process, using methanol as the alcohol and sodium hydroxide (NaOH) as the base catalyst.

5) Process Parameters

Methanol to lipid molar ratio: 6:1 Catalyst concentration: 5% (w/w of lipid)
The methoxide solution was prepared by mixing methanol and NaOH, then reacted with the lipid extract. The reaction mixture was allowed to settle, forming two distinct layers:
Top layer – Biodiesel (FAME) Bottom layer – Glycerol
The biodiesel produced.

IV. RESULTS AND DISCUSSION

A. Control Sample (Blank Tube)

This was the blank or control setup, where the brewery wastewater was used without any dilution. It helps to know how the microbes behave under the original conditions.

1) Physicochemical Parameters

The untreated wastewater used in the blank tube was characterized as follows:

- Chemical Oxygen Demand (COD): 2240 ppm
- Biological Oxygen Demand (BOD): 40–45 mg/L
- Total Dissolved Solids (TDS): 100–1100 mg/L
- pH: 4.98

2) Yield Summary

Parameter	Value
Volume of culture harvested	165 ml
Dry biomass obtained	0.046 g
Lipid extracted	0.0518 g
Lipid per gram of biomass	1.126 g/g

Formula used

Lipid per gram biomass(g/g) = Lipid extracted (g)/Biomass(g) = 0.0518/0.046 = 1.126g/g

This value being more than 1 indicates some experimental variation—maybe there was some leftover solvent or extracellular lipids that got counted in the lipid mass. But overall, this control tells us how things go without any modifications.

B. Changing Of Dissolved Oxygen

This section presents the experimental outcomes of the study evaluating the effect of dissolved oxygen (DO), manipulated through dilution with distilled water, on the production of oleaginous organisms using brewery wastewater as the culture medium. The initial COD of the undiluted brewery wastewater was measured at 2250 ppm, indicating a high organic load and low native DO levels, potentially limiting microbial growth.

1) Calculation of Cod After Dilution

To simulate increased DO availability, the wastewater was diluted with sterile distilled water in three ratios: 25 ml, 50 ml, and 100 ml per 100 ml of brewery wastewater. Since distilled water has negligible COD, the dilution directly reduces the COD of the final mixture.

The resulting COD after each dilution was calculated using the formula:

COD_diluted = (V_wastewater / V_total) × COD_original

Where:

- V_wastewater = 100 ml
- COD_original = 2250 ppm
- V_total = V_wastewater + V_distilled_water

Table 4.1: Change in COD values after the addition of distilled water

Sample	Distilled Water Added (ml)	Total Volume (ml)	COD (ppm)
A	25	125	1800
B	50	150	1500
C	100	200	1125

2) Dry Biomass And Lipid Yield Analysis

Following dilution, each culture was incubated under identical conditions, and the dry biomass and lipid content were measured. The biomass was recovered via filtration and drying, and lipid was extracted using solvent extraction methods.

Table 4.2: Lipid per biomass obtained

Sample	COD (ppm)	Dry Biomass (ml)	Lipid Yield (g)	Lipid Yield per ml Biomass (g/ml)
A	1800	112	0.048	0.00043
B	1500	140	0.054	0.00039
C	1125	196	0.061	0.00031

Formula Used:

Lipid per ml biomass = Lipid Yield (g)/Biomass Volume (ml)

3) What The Results Mean

As the dilution increased:

Biomass production increased (more growth). Lipid yield per mL biomass decreased.

This means that more oxygen availability (lower COD) helped microbes grow better, but they stored less lipid. On the other hand, high COD caused stress, which microbes responded to by storing more lipids. So, for good lipid production, a little bit of stress is actually useful.

4) Graphical Representation

The trend observed in dry biomass and lipid production is visualized in Figure 1. A dual-axis graph was employed to simultaneously represent the increase in biomass and lipid yield against the volume of distilled water added (i.e., the dilution factor).

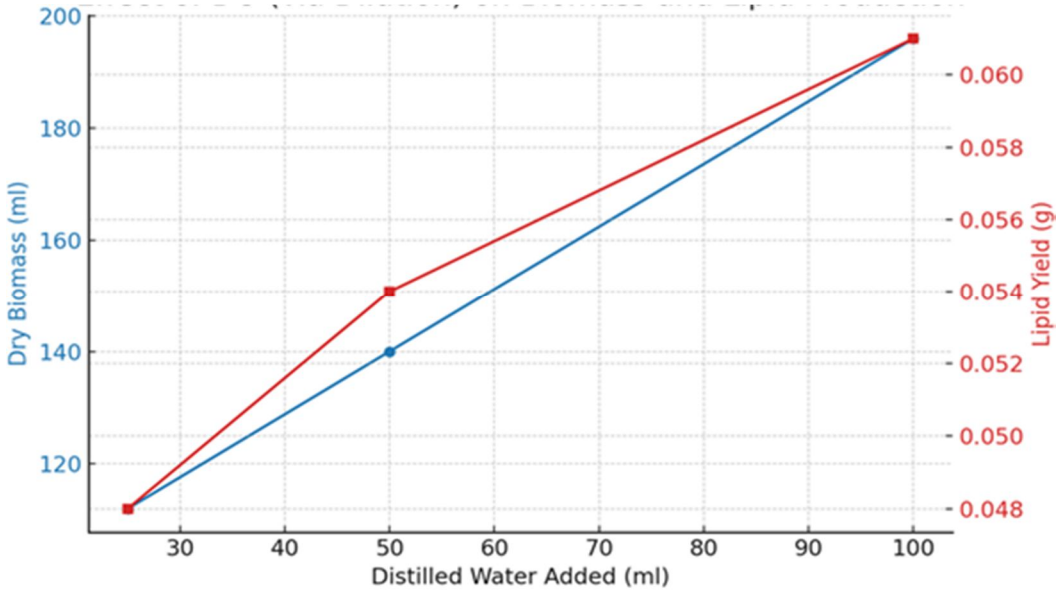

Figure 4.1: Effect of DO (via COD reduction) on Biomass and Lipid Production

Observations

- Dilution of brewery wastewater with distilled water effectively reduced COD from 2250 ppm to as low as 1125 ppm.
- Lower COD led to improved DO conditions, which significantly enhanced the growth of oleaginous organisms.
- Lipid yield increased by ~27% from the lowest to the highest dilution.
- These results confirm that oxygen availability is a limiting factor in microbial lipid production in high- COD environments like brewery wastewater.

C. Effect Of Carbon Sources

Here, different carbon sources were added—glucose, lactose, sucrose, and maltose—to the wastewater to see which one supports better lipid production.

Table 4.3: Amount of Lipid and Biomass obtained for different carbon sources

Carbon Source	Volume Harvested (mL)	Dry Biomass (g)	Lipid Yield (g)	Lipid per g Biomass (g/g)
Glucose	112	0.080	0.461	5.76
Lactose	225	0.183	0.620	3.39
Sucrose	162	0.080	0.330	4.13
Maltose	162	0.060	0.410	6.83

1) Formula Used

Lipid per g biomass = Lipid Yield (g)/ Dry Biomass (g)

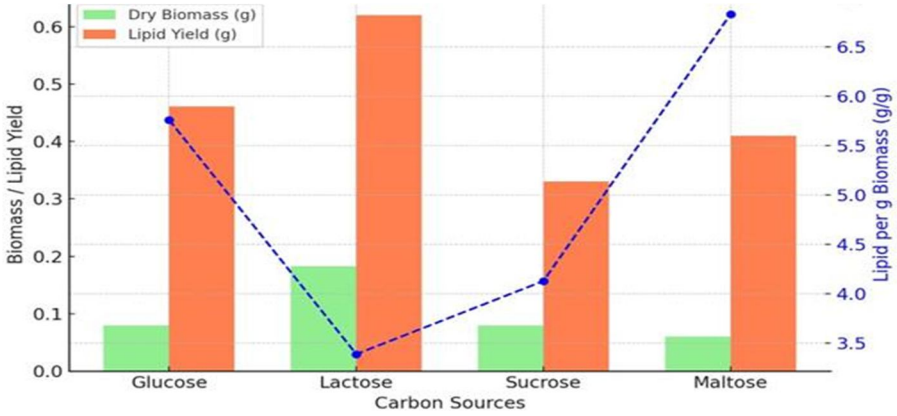

Fig 4.2: Effect of carbon source on biomass and lipid yield

2) Observation

Maltose gave the highest lipid per biomass, even though total biomass was low. It means maltose encourages the microbes to store more fat inside their cells.

Lactose helped grow the most biomass but had lower lipid conversion.

Glucose and sucrose were kind of average—moderate growth and good lipid yield.

So, just because biomass is high doesn't mean lipid yield will also be high. The type of sugar changes how the microbes function inside. Some sugars push the microbes to grow, while others push them to store lipids.

D. Summary Of All Results

Condition	Best biomass yield	Best lipid yield	Highest lipid/Biomass ratio
Control	-	-	1.126 g/g
COD Dilution	Sample C (196 ml)	Sample C (0.061 g)	Sample A (0.00043 g/ml)
Carbon source	Lactose (0.183 g)	Lactose (0.620 g)	Maltose (6.83 g/g)

E. Overall Discussion

The control sample had a high lipid-to-biomass ratio but very low total biomass, so it's not ideal for scaling up.

When COD was lowered (by adding water), biomass increased, but lipid yield per unit decreased. So, there's a trade-off: you either get more cells or more fat inside each cell, but not both at the same time. Adding sugars showed that the type of carbon source really matters. Even though lactose helped microbes grow the most, maltose helped them store the most lipid per gram of cells.

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V. CONCLUSION

The present study successfully demonstrated the isolation, screening, and utilisation of oleaginous microorganisms from brewery wastewater for lipid accumulation and biodiesel production. The characterisation of waste water was d successfully done by checking various factors like COD,BOD,TDS and TSS and it was suitable to harvest the oleaginous microorganisms. The Sudan Black B staining method effectively confirmed the presence of lipid-accumulating microbes, indicated by the appearance of black inclusions under microscopic observation and the rest of non lipid accumulating part will be of no colour.

Various carbon sources—glucose, sucrose, lactose, and maltose—were tested to evaluate their influence on microbial growth and lipid accumulation. Among these, lactose and glucose were found to be the most effective substrates, resulting in higher biomass yields and lipid content. This shows that simpler or more accessible metabolic pathways. The lipid extraction using a methanol–chloroform mixture was efficient, producing clear separation of lipid layers, which were further processed through transesterification.

Transesterification process which is the conversion of extracted lipids into biodiesel through methanol and sodium hydroxide catalysis was successful, cultures yielding the highest quantities of biodiesel. The quality of the biodiesel was confirmed through combustion, which produced a clean flame with slight black ash, indicating the presence of combustible esters.

In conclusion, this study demonstrates a sustainable and cost-effective method for biodiesel production using brewery wastewater and low-cost carbon sources. The process not only save the cost of treating the waste water but also supports renewable energy by converting microbial lipids into valuable biofuels.

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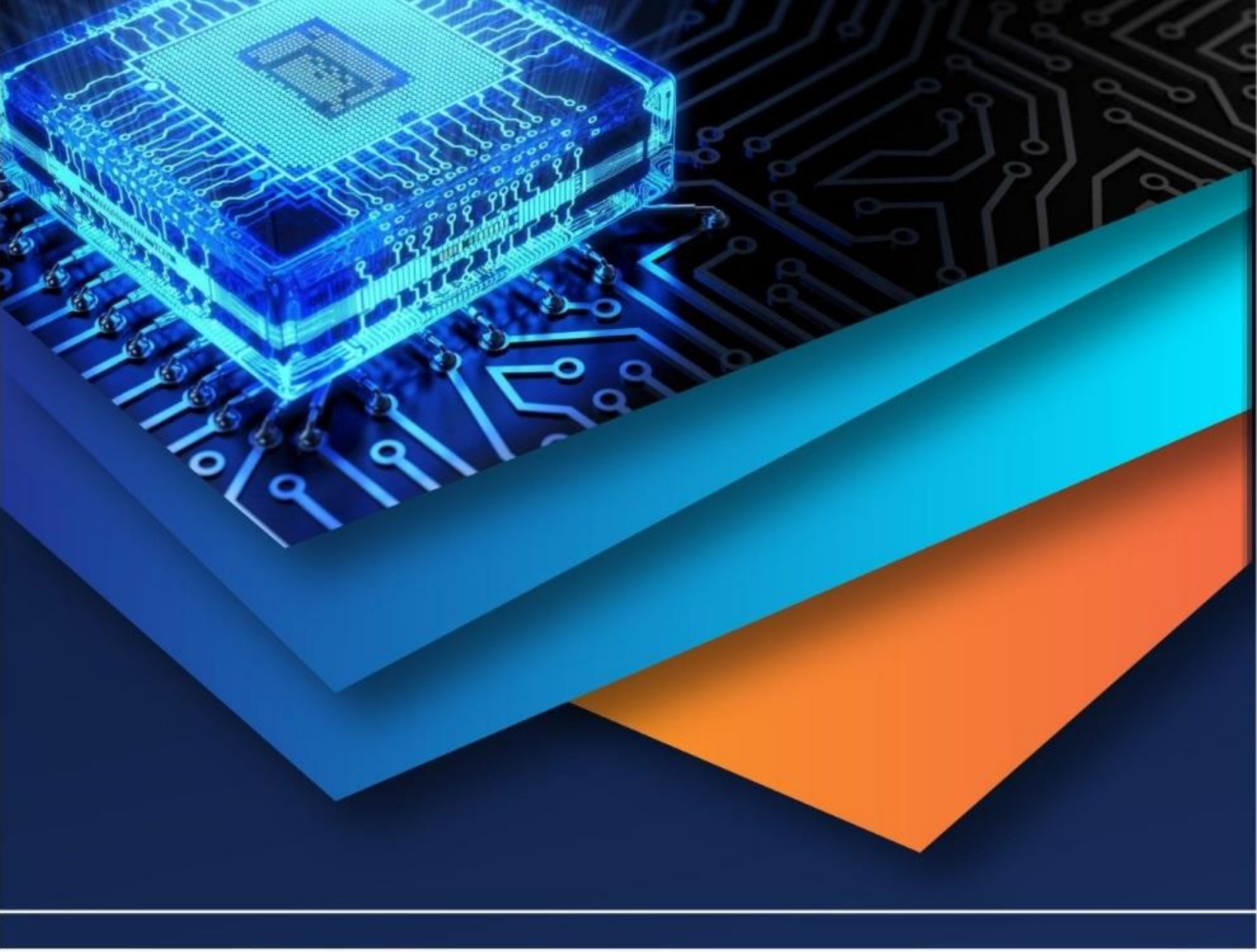

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