2
Review of Literature

2.1 INTRODUCTION

Continuous and indiscriminate use of fossil fuels reserves with concomitant (Figure 2.1), an increase in population (count to be hit 9.8 billion by the next three decades (2050), according to UN) across the globe, has resulted in a drastic rise in GHG emissions as well as the petroleum prices. Some of the studies have predicted that the world will face a shortage of fossil fuel and petroleum in the next five decades [Shields-Menard *et al.*, 2018]. To address these problems, the scientific/industrial community across the world is researching alternate energy sources [Kumar, 2018; Sitepu *et al.*, 2014a]. Currently, world energy demands are met primarily by non-renewable fossil fuels [Qadeer *et al.*, 2017]. Amongst the various renewable energy sources, bioenergy sources are particularly promising in providing energy security for the future. Biofuels are the energy sources made from living things or the waste of it. The biofuels derived from the plant and biological debris are the only fuels that are available in liquid, solid or gaseous form and can replace the conventional transport fuels. Transportation is the second-largest sector at consuming the energy [U.S. E.I.A., 2012]

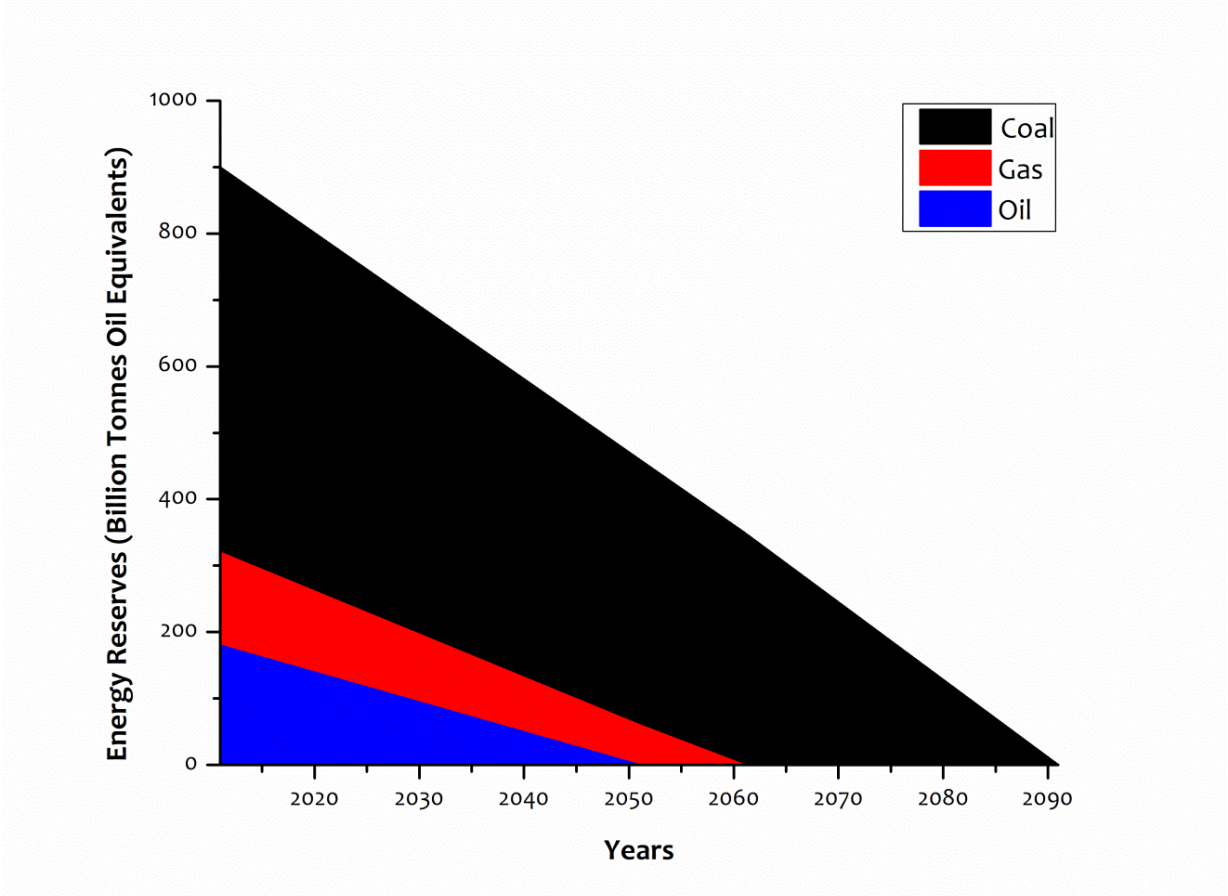

Figure 2.1: Prediction for the depletion of fossil fuels; Data source: CIA world factbook (2011)

Biofuels are excellent alternatives to conventional fuels, but several factors affect their availability for widespread use [Ghosh *et al*., 2019]. Sustainable biofuel production process would involve social relevance/acceptability, economic viability, and beneficial impact on the environment (Figure 2.2) [Ghosh *et al*., 2019]. Currently, biofuels account for only 4 % of the total global transport fuels [Leong *et al*., 2018].

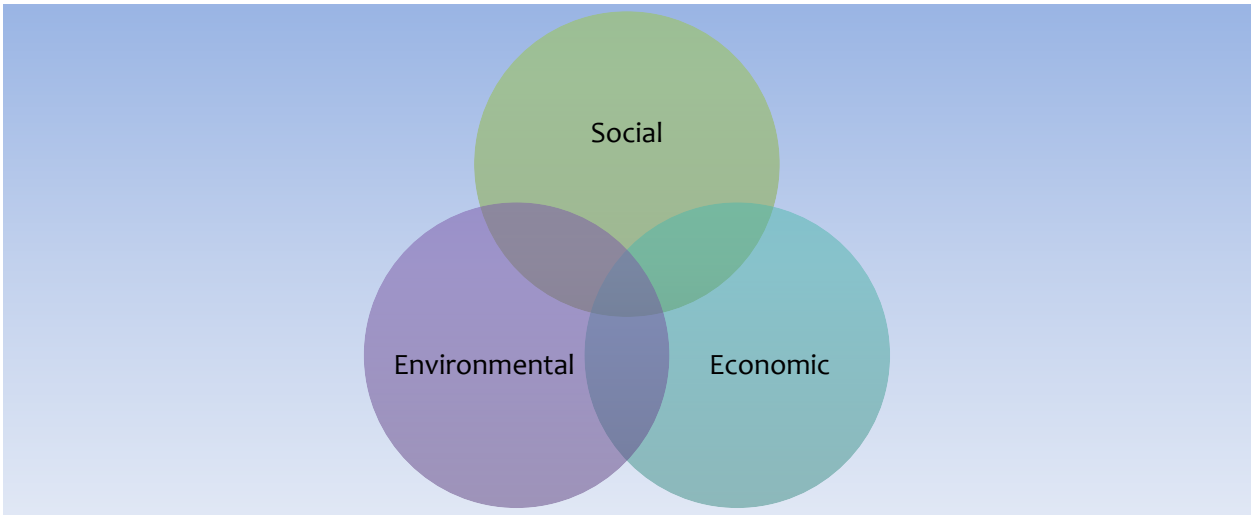

Figure 2.2: Definition of sustainability

According to Energy independence and security act 2007, by 2022, the biofuel production will be around 36 billion gallons in the U.S. [U.S. Congress, 2007]. A goal to reach up to a 10 % share of biofuels in the transportation sector by the year 2020 was set by the European Union (EU) [EPCU, 2009]. Keeping in view the promise associated with the use of biofuels as well as the projected targets, research on biofuels has gained tremendous impetus in the last decade. The popularity of biofuels such as biohydrogen, bioethanol, biodiesel has significantly increased while the scientists are figuring out a sustainable platform for their production.

2.2 BIODIESEL: DEFINITION, GLOBAL STATUS, AND CLASSIFICATION

Biodiesel is alkyl esters of long-chain fatty acids derivated from plant, animal, microbial-based oils. Biodiesel offers several advantages. It emits low GHG & other toxic gases like nitrous oxides and sulfur, has superior lubricity, is non-hazardous, has a higher flash point, and is compatible with the conventional diesel engines [Alptekin, 2017; Gujjala *et al*., 2019; Leong *et al*., 2018; Meira *et al*., 2015]. Biodiesel can be used directly or as blends with the Petro-diesels. In the retail market, it is marked with the B Factor. If 20 % of biodiesel is blended with 80 % of the petrol-diesel, it is marked as B20. The biodiesel without any blend in its pure form is characterized as B100. Organization for Economic Co-operation and Development -Food and Agriculture Organization (OECD-FAO) predicted that global biodiesel production would increase at a rate of 14 % from 797 million liters in 2000 to reach up to 37.9 billion liters by 2020 [OECD/FAO, 2016]. Worldwide, 32.8 billion liters of biodiesel were produced during 2014, whereas India's total biodiesel production was around 10000-250000 metric tons in 2017

[Aradhey and Sindelar, 2017]. The government of India (GOI) has proposed to reduce its import of crude oil by 10 % by the year 2022 [Aradhey and Sindelar, 2017]. India has approximately 85.75 million tons of high-speed diesel consumption per year. The national policy of biofuels (2018) expects 5 % blending in the high-speed diesel by the year 2030, which will need approximately 5.45 million tons per year [PIB-GOI, 2019]. It warrants an urgent need for suitable feedstock and process for biodiesel production.

According to the difference in the sources of the feedstock, the biodiesel can be divided into four generations as below (Figure 2.3).

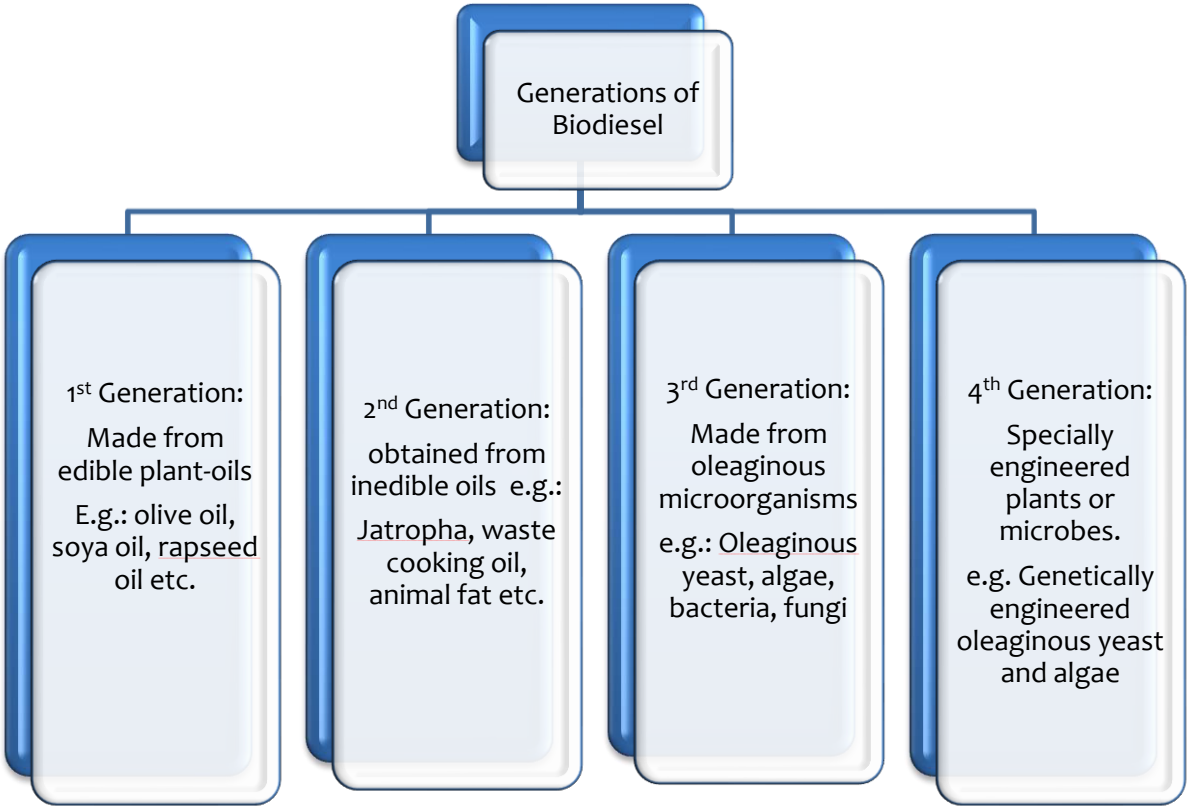

Figure 2.3: Generations of biodiesel

2.2.1 First-generation biodiesel

The first-ever compression ignition engine fueled by peanut oil was demonstrated by Rudolf diesel in 1898 [Crew, 1940]. All the vegetable and plant-derived oils have triacylglycerides (TAGs) as one of the main components. Biodiesel (fatty acid ethyl/methyl esters) is produced by the trans-esterification between TAGs and alcohol using a suitable catalyst (acid/base/enzyme) [Sitepu *et al.*, 2014a] (Figure 2.4). Out of 140 million tons of vegetable oils produced in the year 2009-10 worldwide, the majority came from rapeseed, palm, soybean, and sunflower oils [OECD/FAO, 2016].

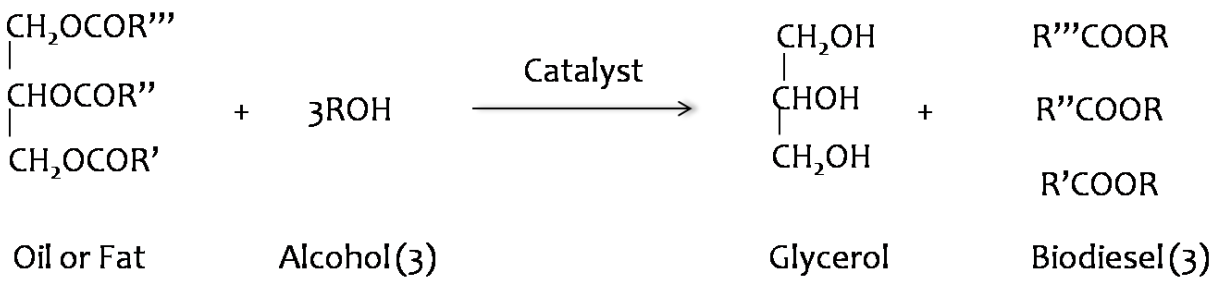

Figure 2.4: Transesterification of lipids

Biodiesel based on soybean oil emits 40 % less GHG and pollutant then the petrol-diesel [Hill *et al*., 2006]. Despite several benefits, the first generation biofuels are largely unsustainable due to ever-reducing agricultural land triggering 'food vs. fuel' debate [Cherubini, 2010; Leiva-Candia *et al*., 2014; Nigam and Singh, 2011]. The European parliament has decided to remove all the crop-based biofuels by 2021 from all the member states in 2017 [Masri *et al*., 2019] due to food security issues. It warrants the search for other utilizable feedstock for biodiesel production.

2.2.2 Second generation biodiesel

The second generation biodiesel is suitable alternatives to first-generation biodiesel, particularly when it comes to feedstocks [Sitepu et al., 2014a]. The feedstock used for it are inedible oils such as jatropha, sea mango, Karanja, jojoba, castor, Kusum, the waste cooking oil (WCO), and animal-derived fats, tallow, and grease [Gujjala *et al*., 2019]. Some of the plants cultivated for this purpose include *Jatropha curcas, Croton megalocarpus, Cerbera mangas* [Leong *et al*., 2018]. While the second-generation biodiesel overcomes the limitation of the first generation, the biodiesel derived from these feedstocks has higher viscosity due to the presence of high free fatty acids [Pinzi *et al*., 2014]. Also, feedstocks such as WCO are available in a limit. The aerial productivity of bio-oils from inedible plants, i.e., jatropha is also very low, rendering this generation of biodiesel even as unsustainable.

2.2.3 Third generation biodiesel

Third generation biodiesel employs the use of single-cell oils derived from bacteria, yeast, fungi, and algae [Chaturvedi *et al*., 2018a; Sitepu *et al*., 2014a]. Microorganisms that can produce lipids more than 20 % (w/w) of their cellular dry weights are termed as oleaginous microorganisms [Ratledge *et al*., 1988]. These organisms can divert their cellular metabolisms towards lipid accumulation, particularly during nitrogen/nutrient limitation. The aerial/volumetric productivities realized by third-generation biodiesel are way higher than the second and first-generation. Also, the estimates show that this generation of biodiesel can meet global demands for energy. The lipids produced by microbes require minimum space and can be provided around the year irrespective of the climate conditions [Sitepu *et al*., 2014a].

Third-generation biofuels have emerged as promising alternatives to the existing transport fuel. However, the process and the production cost are still higher when compared to conventional fossil fuels [Leong *et al*., 2018]. The challenges related to its production can be addressed by searching for productive species and strains or engineering the existing ones. The traits needed include high volumetric oil productivity, the ability to utilize wastewater/biomasses, and faster growth rates.

2.2.4 Fourth generation biodiesel

Fourth generation biodiesels are derived using the genetically modified microorganisms, mainly algae [Abdullah *et al*., 2019]. The goals for genetic modification include improving the photosynthetic efficiency, enhancing intracellular lipid accumulation, production of *in vivo* drop-in biofuels, intracellular transesterification for fatty acid ethyl ester production, minimizing the photo-inhibition, etc., [Tandon and Jin, 2017]. This approach is at a very nascent stage and promising. As these GMOs will be cultivated in the open ponds, there are concerns over their utilization of environmental health & potential risk. The use of GMOs requires stringent policies and guidelines framed after deliberations between scientists and the governments. It would involve elaborate ecological monitoring and risk assessment [Abdullah *et al*., 2019].

2.3 OLEAGINOUS MICROORGANISMS

Microorganisms that produce more than 20 % (w/w) of lipids of their dry weights are termed as oleaginous microorganisms [Ratledge and Wilkinson, 1988]. The history of these micro-organisms dates back to the early era of world wars. Single-cell oils (SCO) producing microbes (especially *Lipomyces* and *Rhodotorula* yeasts) were explored as production alternatives to the commodity oil (for food, fuel, and feed) in Germany during both the world wars [Sitepu *et al*., 2014a]. However, the interest in SCO diminished subsequently due to the availability of vegetable oils in abundance [Karamerou and Webb, 2019]. In the last few decades, the rising environmental pollution and depleting fossil reserves has rejuvenated the research on SCO.

Oleaginous microorganisms are widely distributed among various classes of microorganisms, such as bacteria, yeasts, fungi, and algae [Cohen and Ratledge, 2010]. These organisms accumulate lipids comprising of water-insoluble triglycerides (TAG) (made up of C16 and C18 chain fatty acids), and some steryl esters. TAGs are stored form of energy for cells and appear as lipid droplets known as SCO. The oleaginous microbes are industrially crucial as they can produce valuables along with TAGs [Kumar *et al*., 2017].

2.3.1 Bacteria

There are limited reports on bacteria producing single cell oils (SCO) [Cho and Park, 2018]. The structures of bacterial lipids are different from eukaryotes such as algae, fungi, and yeasts. Most of the bacteria produce lipids in the form of lipoprotein, glycolipids, and wax esters [Shields-Menard *et al*., 2018]. The bacteria are easy to cultivate and exhibit fast growth rates. The lipid content of oleaginous bacterial cells roughly varies from 20-80 % (w/w) [Meng *et al*., 2009]. *Rhodococcus*, *Nocardia, Streptomyces, Gordonia, Bacillus, Acinetobacter, Arthrobacter,* etc., are examples of oleaginous bacteria [Cho and Park, 2018].

Among all the oleaginous bacteria, *R. opacus* is a widely studied one. It can utilize substrates such as aromatic hydrocarbons [Goswami *et al*., 2017, 2018, 2019]. It can also use corn-stover alkaline pretreated waste [Le *et al*., 2017], and wastewater generated from dairy [Kumar *et al*., 2015], etc. It can produce up to 80 % lipid of their dry weight. *R. opacus* PD360 [Alvarez *et al*., 1996] was able to grow and produce lipids in the presence of various inhibitors released from the lignocellulosic biomass pre-treatment [Wang *et al*., 2014]. Co-culture of *R. opacus* PD360 and *Gordonia* sp. grown on various wastes from agro-industries accumulated more than 50 % of the lipids [Gouda *et al*., 2008]. Similarly, reports on the use of *Serratia* and *Bacillus subtilis* growing and accumulating lipids on sludge waste and lignocellulosic biomass are reported previously [Kumar and Thakur, 2018; Zhang *et al*., 2014]. Bacterial lipids, in general, are not suitable for biodiesel [Li *et al*., 2008]. Moreover, the lipid content is not very high, and the lipid recovery is challenging. The lipids from bacteria, however, can be cultivated to produce higher-value containing low titers products such as polyunsaturated fatty acids (PUFA), etc.

2.3.2 Filamentous Fungi

Fungi are very diverse group of heterotrophic organisms, with over 1.4 million species identified [Hawksworth, 1991]. Fungi, among all the groups of microorganisms, are only capable of complete utilization of lignocellulosic biomass and can grow under low water activity. Fungi produce variable amounts of TAGs and high-value lipids such as Eicosapentaenoic acid (EPA), γ- linolenic acid (GLA), Arachidonic acid ($C_{20:0}$), Docosahexaenoic acid (DHA), and Polyunsaturated fatty acids (PUFA) [Bellou *et al*., 2016]. The fungi can withstand dry and acidic conditions, and the lipid recovery is easy due to their filamentous nature.

Filamentous fungi belonging to genera such as *Aspergillus, Mortierella, Penicillium, Trichoderma, Mucor, Geotrichum, Collelotrichum, Alternaria, etc.,* have been explored for their potential for lipid and biofuel production [Khot *et al*., 2018; Leong *et al*., 2018]. Fungi can readily grow on simple sugars to complex industrial wastes substrates [Khot *et al*., 2018]. Many of the filamentous fungi have the natural cellulolytic ability. There are reports on the direct accumulation of lipids using different cellulosic substrates, also termed as consolidated bio-processing (CBP) [Bardhan *et al*., 2019; Hui *et al*., 2010].

The genus *Aspergillus* has been widely studied for lipid accumulation using various carbon substrates. It could accumulate 3.1-3.5 g/L of lipids on waste glycerol [André *et al*., 2010]. *A. candidus* grew on the cheese whey, accumulated oil, which upon transesterification exhibited fuel properties as per the guidelines of the standards [Kakkad *et al*., 2015]. Solid-state fermentation (SSF) of wheat straw using the cellulolytic fungus *A. oryzae* A-4 gave the lipid yield of 62.87 mg/g of a dry substrate [Hui *et al*., 2010]. *A. tubingensis* TSIP9 could collect 39.5 ± 2.2 mg lipids/ gm substrate in an SSF. The substrate consisted of the dry lignocellulosic substrate and palm oil mill effluent (POME). The lipid accumulation improved by up to 79.9 ± 3.5 mg/ds upon the addition of co-substrates (empty fruit bunch and palm kernel cake) [Cheirsilp and Kitcha, 2015]. Although, fungi have several unique properties which make them suitable for SCO production, yet due to their slow growth rates and different form of growth in shake flask, Bioreactor, and solid-state fermentation the process is limited by them. These are also the reasons for the underutilization of fungi for industrial applications.

Table 2.1: Oleaginous microorganisms

Bacteria						
Species name	**Substrate used**	**Mode of cultivation**	**Lipid Output (g/L)**	**Lipid productivity (g/L. h)**	**Lipid content (% w/w)**	**Reference**
Rhodococcus opacus DSM 43205	1. Bushnell-haass medium + PAHs	Shake flask	0.045-0.030	N.M.	65.2 -74.2	[Goswami *et al*., 2019, 2017, 2018]
Rhodococcus rhodochrous	Glucose	14-L BIOFLO410 fermenter	3.01	0.0206	43.00	[Shields *et al*., 2015]

Rhodococcus erythropolis IGTS8	Glycerol	Shake flask 2-L stirred tank Bioreactor	1.84 1.64	0.0191 0.0170	45.80 14.00	[Sriwongchai, 2012]
Serratia sp. ISTD04	Municipal secondary sludge	Shake flask	3.07 ± 0.19	0.0639	12.14	[Kumar and Thakur, 2018]
Bacillus subtilis HB1310	Cotton stalk hydrolysate	Shake flask	2.3	0.0479	39.8	[Zhang *et al*., 2014]
Filamentous fungi						
Aspergillus oryzae A-4	Wheat straw bran	Shake flask	N.M.	N.M.	15-18.5	[Hui *et al*., 2010]
Mortierella isabellina NRRL 1757	Ricotta cheese whey	3-L stirred tank reactor	3.62 ± 0.06	0.04 ± 0.00	49.58	[Carota *et al*, 2018]
Penicillium brevicompactum NRC 829	Sunflower oil cake	Static flask	8.014 ± 0.06	0.0556	57.60	[Ali *et al*., 2017]
Penicillium citrinum PKB20	Glucose	Shake flask	4.45 ±0.12	0.0309	60.61 ± 2.08	[Bardhan *et al*., 2019]
Trichoderma reesi	Glucose	Shake flask	9.72	0.135	32.40	[Bharathiraja *et al*., 2017]
Aspergillus tubingensis TSIP9	*Palm empty fruit bunch and palm kernel cake*	Solid-state fermentation	91.9 (mg/g ds)	N.M.	N.M.	[Cheirsilp and Kitcha, 2015]
Penicillium commune NRC2016	Sweet sorghum	Shake flask	99.1 mg/g ds	N.M.	N.M.	[Abdelhamid *et al*., 2019]
Microalgae						
Arthrospira platensis	Dairy farm wastewater	Photobioreactor	1.50	N.M.	30.23	[Hena *et al*., 2018]
Scenedesmus	Synthetic secondary effluent	2-L Photobioreactor	0.0341	0.00071	66.05 ± 8.55	[Zhan *et al*., 2016]

quadricauda	(Modified BG-11 Medium and A5+Co solution)					
Chlorella vulgaris	Artificial wastewater (Glucose and A5 +Co solution)	2-L column aeration photo-bioreactor	N.M.	0.0061	42	[Feng, Li, and Zhang, 2011]
Neochloris oleoabundans	Anaerobic effluents from pig waste	4-L flat-plate bioreactor	0.141	N.M.	22.4	[Olguín *et al*., 2015]
Yeast						
Lipomyces starkeyi AS 2.1560	Glucose	7-L stirred tank reactor- 2 stage fermentation	67.9	1.60	64.9	[Lin *et al*., 2011]
Rhodotorula glutinis	Glucose	5-L stirred tank reactor	74.0	1.028	40.0	[Pan *et al*., 1986]
Trichosporon fermentans	Molasses	Shake flask	17.5	0.104	62.4	[Zhu *et al*., 2008]

(Where N.M.: Not mentioned; PAHs- Polyaromatic hydrocarbons)

2.3.3 Microalgae

Algae are the microorganisms that are photosynthetic in nature. They require water, sunlight, and inorganic carbon to thrive. The most notable of the algae are *Chlorella, Navicula, Crypteheconidium*, *Dunaliella* etc., [Chisti, 2008]. The lipid content in algae can go up to 70 % of cell dry weight (w/w) [Meng *et al*., 2009]. In addition to the lipids, algae represent a useful source of biomass that can be converted to hydrogen, methane, ethanol, etc. Out of 33,000 known species of algae, only about 40 are known to be oleaginous [Griffiths and Harrison, 2009]. Algae can be cultivated in photo-bioreactors or open ponds. Algae cultivation for biofuel production is challenging as the growth rates realized in the real outdoor conditions is small and requires a leap of studies enabling efficient algae cultivation. Several new methods and models for the cultivation of algae have been developed in the past decade to increase algae growth rates and oil productivities. When compared to vegetable oils, the aerial productivities of algae (amount of lipid per hectare) is much higher [Cho *et al*., 2015; Fei *et al*., 2015; Kim *et al*., 2014; Leong *et al*., 2018; Ma *et al*., 2018]. Alga can also grow on various types of agricultural, municipal, and industrial wastewaters or seawater [Cho and Park, 2018]. Certain microalgae are mixotrophs and can be cultivated both heterotrophically and autotrophically [Miao and Wu, 2006].

The mixotrophic cultivation enables simultaneous wastewater treatment and bioenergy production. For example, *C. vulgaris* grown on artificial wastewater could accumulate 42 % of lipid while removing COD (86 %), NH_4^+ (97 %), and phosphate (96 %) [Feng *et al*., 2011]. Similarly, other microalgae such as *A. platensis (*Lipid content: 30.23 %*), Neochloropsis oleabundans* (22.4 %) and *S. quadricauda* (66.05 %) can grow on different types of wastewaters while accumulating lipids (Table 2.1) [Hena *et al*., 2018; Olguín *et al*., 2015; Zhan *et al*., 2016]. Algae cultivation is also beneficial for the environment. As per the estimates, production of about 100 tons of algal biomass fixes around 183 tons of carbon dioxide [Chisti, 2008]. Apart from biodiesel production, algae lipids have importance in nutraceuticals, pharmaceuticals, and food industry applications. The techno-economic analysis for the algae has been well studied. Biodiesel production from algae has been estimated to cost between $ 1-80 per gallon [Davis *et al*., 2011]. Biofuel production from algae involves the following steps: cultivation, biomass harvesting, drying, lipid extraction, anaerobic digestion, water use, and recycling. All these steps have several challenges and need to be addressed before algae-based biofuel can be commercially made available.

2.3.4 Yeast

Yeasts are defined as "fungi that asexually reproduce by budding or fission, which results in growth mainly comprising of the single cells" [Kurtzman *et al*., 2011]. They mainly belong to the fungal phyla Ascomycota and Basidiomycota. Currently, More than 1600 yeast species are identified to date [Kurtzman *et al*., 2011], out of which more than 70 species are characterized as oleaginous yeasts. The different strains of the same species may vary in the content of lipids [Sitepu *et al*., 2014a]. Yeasts have been well studied for SCO productions and can accumulate up to 70 % lipids per dry weight of cells. It has several advantages over algae, which include higher specific growth rates, readily amenable to genetic manipulations, less susceptibility to phage infection, and bacterial contamination as they prefer acidic pH [Sitepu *et al*., 2014a]. Many preliminary discoveries on SCO have pushed the modern-day studies on oleaginous yeasts. Oleaginous yeasts have immense potential to serve as a source of oils for future fuels, food, feed, and chemicals [Blomqvist *et al*., 2018]. The lipid profile of oleaginous yeast is similar to palm and other vegetable oils [Schilter, 2019].

Yeast lipids consist of triacylglycerols (TAG), diacylglycerols (DAG), monoacylglycerols (MAG), fatty acid, streyl esters, free sterols, and others such as glycerophospholipids, sphingolipids, hydrocarbons (comprising straight-chain hydrocarbons, squalene, and carotenoids), cardiolipins, glycolipids, long-chain alcohols, waxes, polyprenols, isoprenoid quinones, and others [Sitepu *et al*., 2014a]. The TAGs are about 90 % of total lipids and are essential for biodiesel production [Ratledge, 1993; Xue *et al*., 2018]. Yeast lipids have high oleic acid, which further makes it a suitable alternative to the vegetable oils [Vasconcelos *et al*., 2019].

The representative genera of oleaginous yeast include such as *Yarrowia, Rhodosporidium, Rhodotorula, Cryptococcus, Trichosporon, Candida, Sporobolomyces, Meyerozyma, Lipomyces, Debaryomyces*. The majority of the studies have been done on *R. glutinis*, *R. toruloides*, *R. kratochviloe, Y. lipolytica*, *C. curvatus, T. oleaginous, L. starkeyi,* etc. [Ageitos *et al*., 2011; Sitepu *et al*., 2014a]. The lipid production by oleaginous yeasts is reported as lipid content (Weight of extractable lipid relative to weight of dry cell mass), lipid output (g lipid per liter of culture), lipid co-efficient (g lipid per g or 100 g carbohydrate consumed or per g) or lipid productivity (Mass or volume lipid per volume culture per time) [Sitepu *et al*., 2014a]. The highest lipid productivity of 1.6 g/L. h so far has been reported using *L. starkeyi* AS 2.1560 cultivated on molasses [Lin *et al*., 2011]. The lipid productivity of 1.028 g/L. h by *R. glutinis* was reported on glucose as a substrate [Pan *et al*., 1986]. The lipid productivities as well composition differ with the culture conditions and substrates used [Karamerou and Webb, 2019].

2.4 OLEAGINOUS YEASTS AS OIL PRODUCERS

Oleaginous yeasts are promising oil-producing microbes, and some of their useful traits are discussed in the sections below:

2.4.1 Heterotrophic nature and diverse physiological pathways

Yeast is heterotrophic and therefore exhibit high growth rates over autotrophic algae. Yeats can utilize simple to complex substrates [Sitepu *et al*., 2014a]. The heterotrophic nature of yeast allows the utilization of various complex substrates such as agro-industrial waste and other waste biomasses. Yeasts can assimilate monomer substrates such as xylose, arabinose, glycerol, etc. The xylose is derived in abundance after the acid treatment of the lignocellulosic biomass. The xylose is assimilated via the phosphoketolase pathway, and the carbon is channeled into lipid synthesis [Evans *et al*., 1984]. Similarly, hydrophobic substrates such as glycerol, fatty acid, alkanes, etc. are also utilized as growth substrates by yeasts [Thevenieau *et al*., 2010]. Yeasts possess ex-novo and de-novo pathways for lipid accumulation. In the case of the de-novo pathway, lipid production occurs during the secondary metabolism after the nitrogen or phosphorous depletion. While in the case of ex-novo pathway, lipid synthesis occurs irrespective of nitrogen concentration in the external medium [Patel and Matsakas, 2019; Papanikolau and aggelis, 2011].

2.4.2 Growth rate and cultivation

Yeast has a much shorter generation time of 1 to 1.5 h when compared with microalgae (3.5 h) [Chisti, 2008]. They prefer acidic pH and can divide into a broad range of temperatures [Chaturvedi *et al*., 2018a]. Industrial yeast cultivation is mostly done using fermenter, in batch/ fed-batch/continuous/repeated batch mode. Therefore, bioprocess principles are easily applicable to yeast growth optimization and scale-up [Karamerou and Webb, 2019]. The oleaginous yeasts have also been successfully cultivated under non-sterile conditions. This helps reduce the operational costs of the process [Ling *et al.,* 2013; Taskin *et al*., 2016; Taskin *et al*., 2015].

2.4.3 Genetic manipulation

The yeasts are amenable to genetic manipulations. Several commercial genetic tools are available for engineering *Y. lipolytica*. Other oleaginous yeasts such as *Rhodotorula, Trichosporon, Lipomyces* have immense potential, but genetic tools for their manipulation are not available. Some of the studies in the literature discuss genetic manipulation of *Rhodotorula*, one of the most promising oleaginous yeast, but with limited success. The primary reasons for the problematic genetic manipulation of *Rhodotorula* include high GC content and resulting codon bias [Johns *et al*., 2016; Liu *et al*., 2016]. The genomes of *Rhodotorula* and *Rhodosporidum* strains have been sequenced, and advances in system and synthetic biology approaches have paved the way towards the development of genetic tools for these organisms as well [Tiukova *et al*., 2019; Zhu *et al*., 2012]. The primary objectives of genetic engineering include enhancing lipid content, enhancing C16, C18 fatty acids in TAGs, increasing degree of unsaturation in fatty acids. This also includes *in vivo* transesterification, in vivo hydrogenation, and expression of hydrolytic enzymes for CBP. The model organisms such as *Saccharomyces cerevisiae* and *Escherichia coli* and offer convenient platforms for genetic engineering [Steen *et al*., 2010]. Still, other oleaginous species also offer attractive platforms, particularly for consolidated bioprocessing.

2.4.4 Use of By-Products

Yeasts are a source of several other industrially relevant chemicals and biomolecules. This enables their use in bio-refineries wherein products such as biofuels, oleochemicals, and other value-added products can be simultaneously produced. Lipids derived from yeasts have applications i.e., food & nutrition, pharmaceuticals, cosmetics, lubricants, and biopolymer industries (Figure 2.5) [Vasconcelos *et al*., 2019]. For example, the cocoa butter is obtained from

the beans of *Theobroma cacao*. Specific oleaginous yeasts are reported to produce cocoa-butter like lipids [Hassan *et al*., 1994; Papanikolaou *et al*., 2003; Wei *et al*., 2017] which are used as raw materials in chocolate and biscuit industries. Yeasts also produce essential metabolites, including pigments, proteins, carbohydrates, sophorolipids, fatty alcohols, alkanes, enzymes, and other nutrients [Meng *et al*., 2009].

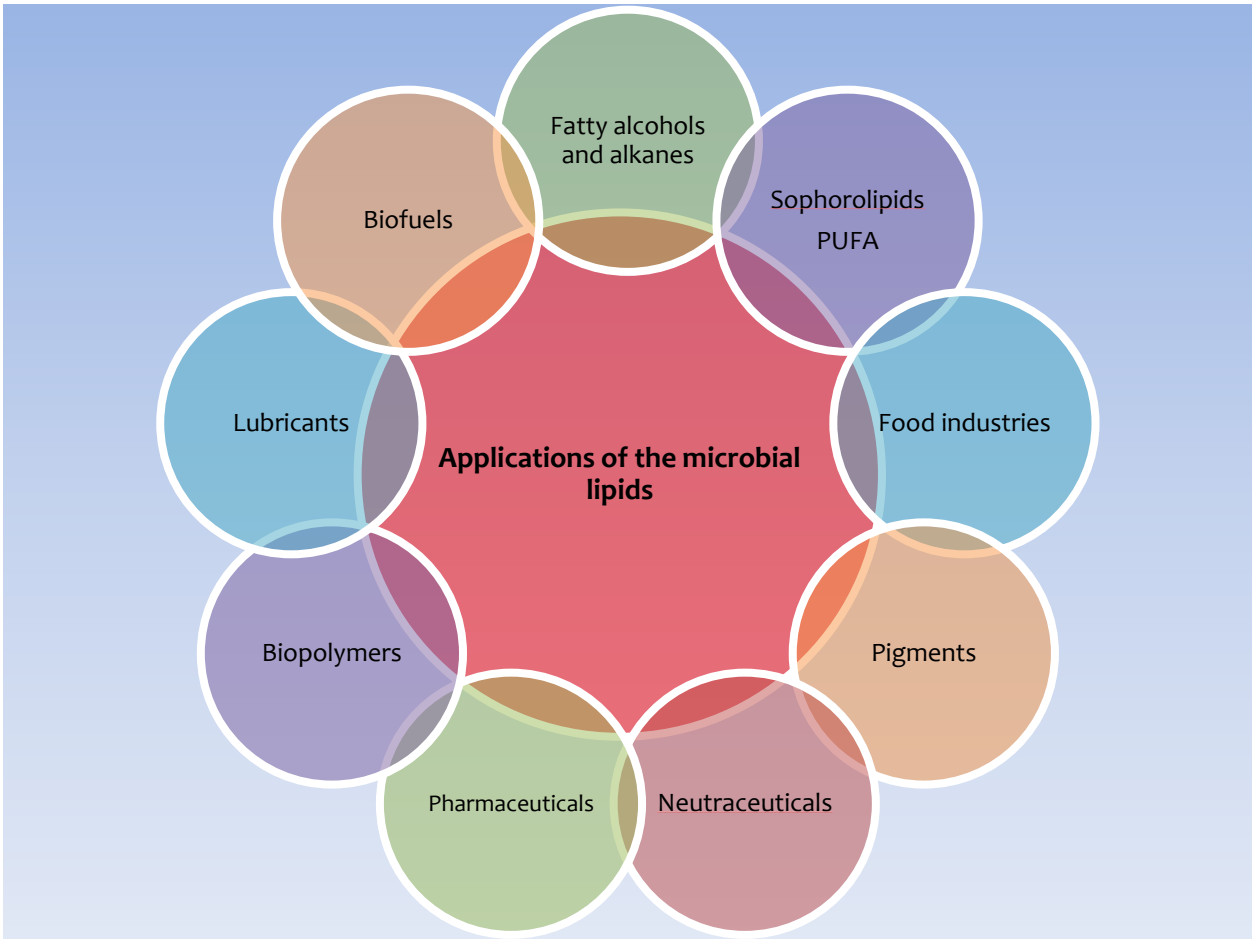

Figure 2.5: Applications of the microbial lipids

2.5 COMPREHENSIVE PRODUCTION OF BIODIESEL USING OLEAGINOUS YEASTS

2.5.1 Characteristics of desirable yeast

The characteristics of the yeast species for biofuel production include broad substrate range, high inhibitor tolerance, osmotolerance, easy cultivation, higher specific growth rate, and high lipid productivities and biomass. Most of the oleaginous yeasts accumulate lipid in the late log phase (with few exceptions such as *C. terricola*) [Pedersen *et al*., 1961; Boulton and Ratledge, 1984]. Besides the points mentioned above, the lipid profile of the organism should be suitable for biofuel production [Patel *et al*., 2017a].

2.5.2 Extracellular enzymes produced by the oleaginous yeast

The ability to utilize a wide variety of substrates is one of the desirable characteristics [Sitepu *et al*., 2014a]. Yeasts can grow on different types of monosaccharides, disaccharides, polysaccharides, and other organic carbon sources. Biofuel production requires a high volume

of substrates. Therefore, the most abundant cellulosic substrates are targeted for raw materials. Cellulose is the most abundant carbohydrate on earth [Van Soest, 1982] and often the most desirable raw material for biofuels. Lignocellulosic biomasses contain a mixture of lignin, cellulose, and hemicelluloses. The cellulosic and hemicellulosic fractions are utilizable while the lignin fraction is not. Also, the pretreatment methods for lignocelluloses results in the generation of phenolic and non-phenolic compounds which act as inhibitors of fermentation. As mentioned earlier, fungi, particularly the white-rot fungi, can completely degrade the lignocellulosic biomass. Also, this group of microbes is the most potent producer of cellulases, hemicellulases, and ligninases. These enzymes have been well characterized, and their applications have been studied in detail.

Similarly, a lot is known about bacterial cellulases and their applications [Juturu and Wu, 2014]. Yeasts, on the other hand, are not the potent producers of cellulolytic enzymes. Fewer reports discuss the expression of cellulases (except β-glucosidases) by yeast. So far, yeast belonging to genera T*richosporon, Rhodotorula, Cystobasidium, Cryptococcus, Geotricum, and Candida* have been found to produce a low amount of cellulases [Štursová et al., 2012]. Cellulose is a linear chain of a crystalline form of polymer consisting of the D-glucose monomer subunits linked by β 1-4 glycosidic bonds [Hon, 1994]. It is an essential component of cell wall imparting rigidity to the plant cells, algae, and certain prokaryotes [O'Sullivan, 1997]. Hemicellulose is a different polymer with a C5 sugar backbone and is the next most abundant carbohydrate after cellulose. This adds another required trait in the desirable yeast, which is the simultaneous utilization of C5 and C6 sugars. Yeasts have been studied for the production of various hydrolytic enzymes. Table 2.2 summarizes some of the essential studies.

Table 2.2: Expression of hydrolytic enzymes by oleaginous yeasts in the literature

Species name	**Source of the sample**	**Carbon source**	**Name of the enzyme**	**Enzyme activity (IU/mL)**	**Lipid content (% w/w)**	**References**
Candida orthopsilosis Y09GS34	Indonesian resources	CMC	CMCase	1.19 (IU/hour)	63.75	[Kanti and sudiana, 2015]
Rhodotorula mucilaginosa,	Antarctic oligochaete *Grania* sp.	CMC	Cellulase	N.M.	N.M.	[Herrera et al., 2017]
Rhodotorula glutinis	Antarctic oligochaete *Grania* sp.	CMC	Cellulase	N.M.	N.M.	[Herrera et al., 2017]
Cystobasidium slooffiae	Antarctic oligochaete *Grania* sp.	CMC	Cellulase	N.M.	N.M.	[Herrera et al., 2017]
Trichosporon asahii Y-SL1	Industrial wastes-Deli product	soapstock of pomace olive oil refining	CMCase *β*-glucosidase	0.11 0.15	60.16 ± 1.86	[Ayadi *et al*., 2018]
Rhodotorula mucilaginosa	Laboratory culture	Starchy waste	FPase Amylase	1.63 ± 0.12 0.16 ± 0.13	25.48 ± 16.89	[Chaturvedi *et al*., 2019a]

Y-1	collection		Xylanase	0.50 ± 0.01		
Rhodotorula glutinis NRRL Y-1091	Laboratory culture collection	Starchy waste	FPase Amylase Xylanase	1.87 ± 0.20 1.38 ± 0.11 0.59 ± 0.06	20.36 ± 2.13	[Chaturvedi *et al.*, 2019a]
Cryptococcus curvatus DSM 70022	Laboratory culture collection	Starchy waste	FPase Amylase Xylanase	2.46 ± 0.27 6.68 ± 0.16 0.82 ± 0.07	26.78 ± 18.41	[Chaturvedi *et al.*, 2019a]
Lipomyces satrkeyi CBC 1807	Laboratory culture collection	Starchy waste	FPase Amylase Xylanase	1.14 ± 0.01 1.12 ± 0.06 0.50 ± 0.01	52.04 ± 8.61	[Chaturvedi *et al.*, 2019a]
Trichosporon cutaneum AS2.571	Laboratory culture collection	Starchy waste	FPase Amylase Xylanase	1.57 ± 0.11 15.23 ± 0.83 0.29 ± 0.10	10.85 ± 2.55	[Chaturvedi *et al.*, 2019a]

(Where N.M.: Not mentioned; CMC-Carboxymethylcellulose, CMCase-Carboxymethylcellulose, FPase- Filter paper enzyme activity, IU/mL: International Units per mL)

2.5.3 Isolation and identification of oleaginous yeasts

A large number of oil-producing yeast is known already, and more and more are discovered [Johnson and Echavarri-Erasun, 2011]. The natural environments are screened to find yeasts with all the desirable characteristics, as mentioned in the earlier section. The search not only intends to find the newer species but also study the known species for oil accumulation. Studies indicate that the probability of finding oleaginous yeast in the randomly selected sample is 3-10 % [Sitepu *et al.*, 2014a]. The systematic screening strategies (summarized in one of the reviews) enable the discovery of novel oleaginous yeasts. It also helps find oleaginous nature in already known species [Sitepu *et al.*, 2014a]. The screening procedure must test the ability of the isolate to grow on complex substrates, inhibitor tolerance, fatty acid profile, and lipid productivity [Patel *et al.*, 2017a]. Yeasts are found everywhere, and a large number of natural environments can be screened for their isolation.

There are many reports on isolation and identification of different oleaginous yeasts, out of which some of the recent reports are summarized in Table 2.3.

Table 2.3: Isolation and identification of oleaginous yeasts

Total no. of yeast isolated	**Oleaginous yeast**	**Source**	**Yeast species**	**References**
479	79	Himalayan permafrost soil	*Rhodosporidium kratochvilovae* HIMPA1	[Patel *et al.*, 2014]
340	18	Ethiopia	*Cutaneotrichosporon sp.*, *Rhodotorula sp.*	[Jiru *et al.*, 2016]
61	28	Sub-arctic region (king George island)	*Cryptococcus*, *Rhodotorula*, *Candida*, *Metschnikowia*,	[Martinez *et al.*, 2016]

			Debaryomyces	
17	12	Antarctica	*Rhodotorula laryngis*	[Vinarta *et al*., 2016]
88	21	Fruit surfaces	*Rhodotorula glutinis* *Lipomyces starkeyi* *Trichosporon fermentans*	[Maina *et al*., 2017]
N.M.	12	paper pulp industry waste and rotten wood samples	*Trichosporon* sp.	[Brar *et al*., 2017]
59	18	Phaff Yeast Culture Collection (University of California Davis)	*Rhodosporidium* aff. *Paludigenum* *Rhodotorula araucariae* *Rhodotorula* aff. *Lusitaniae* *Sporidiobolus johnsonii* *Sporidiobolus pararoseus etc.*,	[Garay *et al*., 2016]
28	1	CBS and other culture collection	*Schwanniomyces occidentalis* (*Debaromyces occidentalis*) CBS2864	[Lamers *et al*., 2016]

2.5.4 Lipogenesis in oleaginous yeast

The yeast is made up of C (~60 %), H (~9-10 %), O (20-22 %), N (~1-2 %), P, S (~ 5000 ppm concentration), and other trace minerals (~in ppm; ca, 860; Cu, 7.8; Fe, 130; K, 7000; Mg, 1300; Na, 880) [Braun *et al*., 2019; Espinosa-Gonzalez *et al*., 2014]. Under nitrogen limiting conditions, fatty acid synthase enzyme is activated, and this channels the excess carbon to lipids, which accumulate as oil droplets inside the cell [Ageitos *et al*., 2011; Schilter, 2019]. The lipid biosynthesis pathways are similar in both oleaginous as well as non-oleaginous yeasts [Ageitos *et al*., 2011; Botham and Ratledge, 1979]. The non-oleaginous yeast ceases to grow at high C/N ratios. Adenosine monophosphate (AMP) deaminase converts AMP into Inosine monophosphate (IMP) [Evans and Ratledge, 1983] (Figure 2.6). The key enzyme is ATP citrate lyase, which converts citrate to Acetyl Co-A and oxaloacetate [Boulton and Ratledge, 1983]. This enzyme is generally absent in non-oleaginous yeasts [Evans and Ratledge, 1983]. Acetyl CoA and Malonyl CoA serve as ingredients for fatty acid synthesis, and the reaction is catalyzed by fatty acid synthase (FAS). The complete pathway for lipogenesis is shown in Figure 2.6.

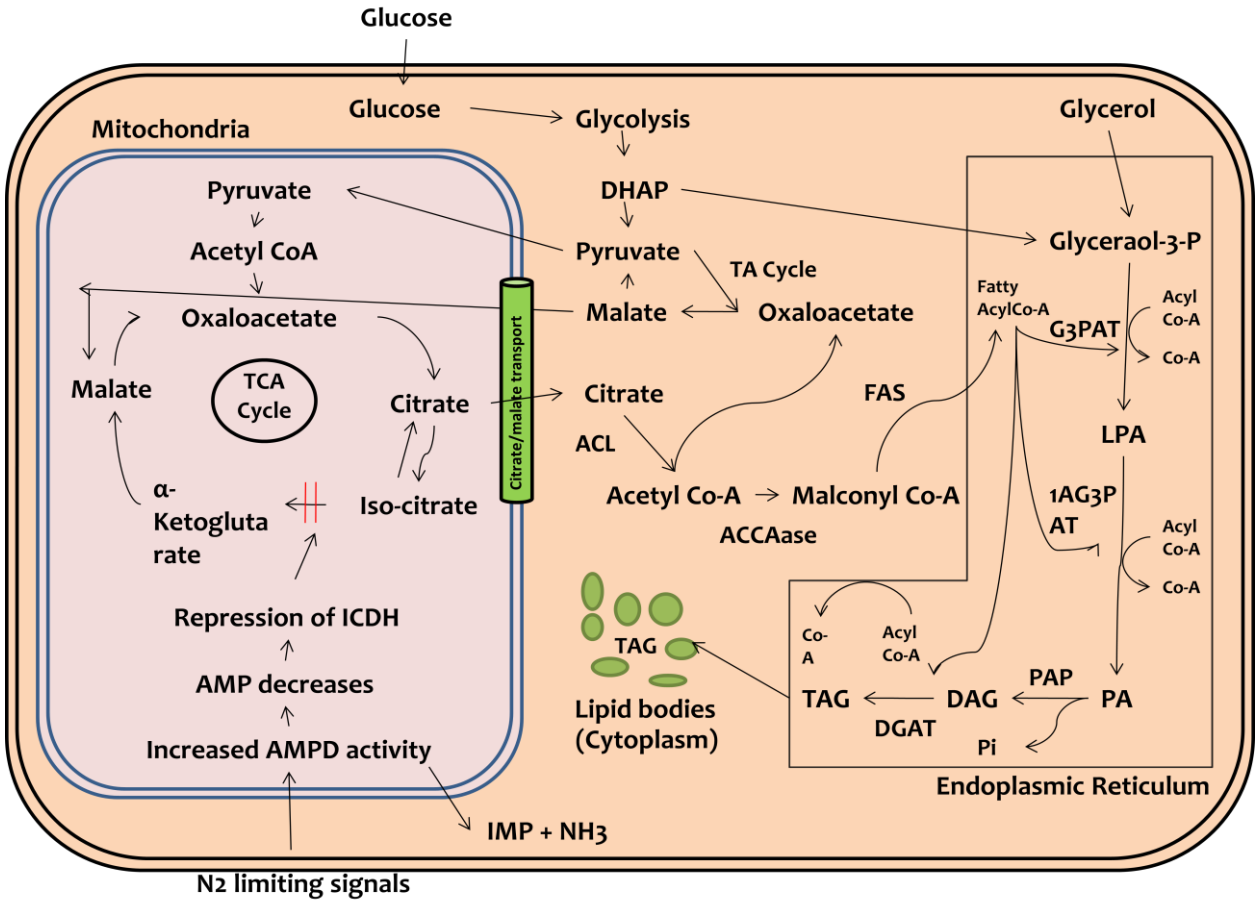

Figure 2.6: TAG Biosynthesis in oleaginous yeasts [Where ICDH- Isocitrate Dehydrogenase; AMPD-Adenosine monophosphate deaminase; ACL-ATP Citrate lyase; ACCase-Acetyl Co-A carboxylase; FAS-Fatty acid synthase; G3PAT- Glycerol-3-phosphate 1-acyl transferase; 1AG3PAT-1-acylglycerol-3-phosphate O-acyltransferase; PAP-Phosphatidic acid phosphatase; DGAT- Diglyceride acyl transferase; DHAP-Dihydroxyacetone phosphate; IMP-Inosine monophosphate; AMP- Adenosine monophosphate; LPA-Lysophosphatidic acid; PA-Phosphatidic acid; DAG- Diacylglycerol; TAG-Triacylglycerol; TA cycle-Transaldolase cycle; TCA cycle-Tricarboxylic acid cycle]

2.5.5 Factors affecting the growth of oleaginous yeasts

Both the yeast growth and lipogenesis is affected by the physical and chemical parameters of cultivations and the mode of culture (Figure 2.7) [Ageitos *et al.*, 2011].

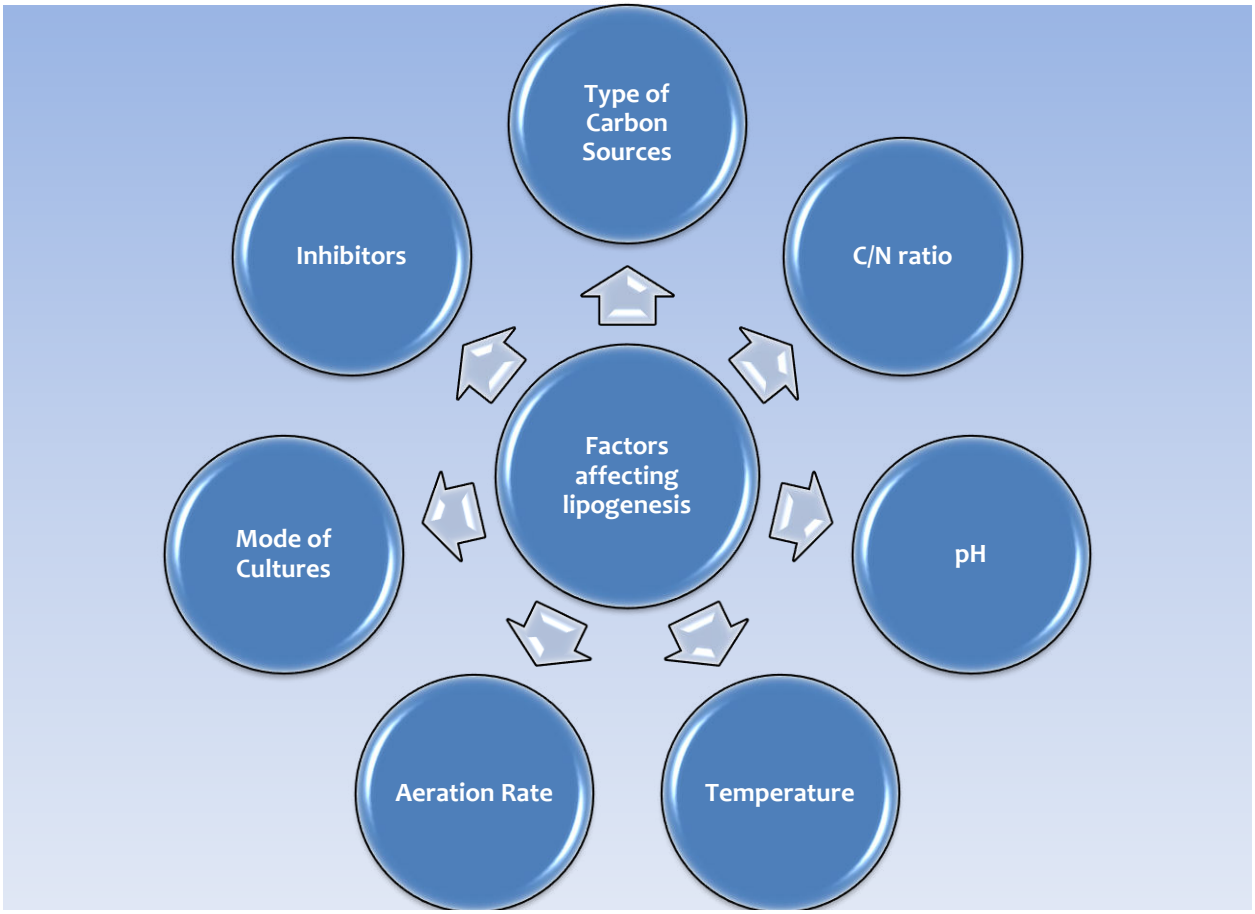

Figure 2.7: Factors affecting lipogenesis

2.5.5.1 Types of carbon sources

The ability of the isolate to utilize different types of carbon sources reflects upon its ability to use industrial wastes such as those obtained from biodiesel transesterification plants, dairy industry, starch processing industries, sugar mill effluents, etc. Most of the basidiomycetes yeasts can grow and accumulate lipid on C_5 as well as C_6 sugars [Sitepu *et al.*, 2013]. Yeasts belonging to the genera, *Rhodotorula*, *Rhodosporidium,* and *Yarrowia* were able to be cultivated on the carbon sources derived from the lignocellulosic biomass [Leiva-Candia *et al.*, 2014]. Glycerol, is a by-product obtained from the biodiesel industry, is widely employed as a substrate for lipid accumulation. It is important to note that the lipid profile of oleaginous yeast alters with the modification in the carbon source and other fermentation parameters [Sitepu *et al.*, 2013]. The carbon sources other than glucose are assimilated via different metabolic pathways and thus lead to various fatty acid compositions [Fakas *et al.*, 2009].

2.5.5.2 C/N ratio

The lipid accumulation in oleaginous yeast is induced at a C/N ratio > 20 [Papanikolaou and Aggelis, 2011; Annamalai *et al.*, 2018; Braunwald *et al.*, 2013; Ivančić Šantek *et al.*, 2017; Seo *et al.*, 2013]. The C/N ratio needed for optimal cell growth and lipogenesis is different. Therefore, the C/N ratio selected is a tradeoff between cell growth and lipid accumulation. The process can also be sequential wherein high biomass yield is achieved first, followed by lipid accumulation. Apart from nitrogen, phosphate, sulfate, and magnesium limitations too have been found to help with lipid accumulation [Bellou *et al.*, 2016; Kolouchova *et al.*, 2016; Wu *et al.*, 2011]

2.5.5.3 pH

Yeast prefers acidic pH and can tolerate a wide range of pH (2.0-7.5) [Osorio-González *et al*., 2019]. Acidic pH helps overcome bacterial contamination under non-sterile conditions. Deviations from the optimum pH lead to lower growth and lipid productivities, thereby making pH an essential parameter for bioprocess operation [Huang *et al*., 2012; Saenge *et al*., 2011a]. For example, *R. toruloides* NCYC 921 was able to grow in the pH range of 3.5-6.0 with the optimum pH for lipid accumulation as 4.0 [Dias *et al*., 2016]. Similar observations were made later [Wang *et al*., 2017]. In one of the studies, lipid accumulation was studied to be cultivated at different pH, and it was found that the lipid accumulation increase with the increase in the pH value ≥ 7.0 and decreased when the pH increased further [Mirza *et al*., 2019]. While most of the yeast prefers acidic pH, some of them, such as *T. cutaneum* CH002, could grow and accumulate lipids at pH values up to 8.0 [Chen *et al*., 2013]

2.5.5.4 Temperature

Yeast can be cultivated on a broad range of temperatures, while the majority of them are mesophilic (20-45 ^{0}C). Also, the lipid content of the yeast varies with the variation in temperature with the specific temperature supporting the highest lipid content. For example, *T. cutaneum* CH002 cultivated at 25 to 32 ^{0}C showed the most elevated lipids at 28 ^{0}C [Chen *et al*., 2013]. Psychrophilic (-20 to 10 ^{0}C; cold-adapted) yeasts present specific advantage by growing at low temperatures that prevent bacterial contamination and enable cultivation under non-sterile conditions [Rossi *et al*., 2009]. For example, cold-adapted yeast *Y. lipolytica* B9 exhibited optimum growth and highest lipid content at 15 ^{0}C when grown in non-sterile conditions at temperatures (4-35 ^{0}C) using cheese whey as a substrate [Taskin *et al*., 2015]. Similarly, *Metschnikowia pulcherrima* could accumulate high lipids at 15-20 ^{0}C, and both lipid content and cell growth rates dropped at temperatures >20 ^{0}C [Santamauro *et al*., 2014].

2.5.5.5 Aeration

Aerobic organisms realize high growth rates compared to anaerobes, and most of the yeasts are obligate aerobes. Therefore, high oxygen transfer rates support high biomass productivities. On the other hand, lipid accumulation is favored under oxygen stress conditions [Yen and Zhang, 2011]. Therefore, high biomass growth can be preceded by lipid accumulation by regulating the dissolved oxygen (DO) levels in the reactor. The solubility of oxygen is affected by temperature, shaking speed, design of the impellor, presence/absence of aerators, medium strength, and viscosity. The high DO concentrations favored the biomass growth while lower-level favored lipid accumulation in *R. glutinis.* Therefore balancing the biomass and lipid content concerning DO level is necessary. In another study, the biomass and lipid accumulation both increased upon an increase in the DO in the shake-flask experiment for the cultivation of *Pichia guilliermondii* [Chopra and Sen, 2018]. Aeration of the medium, in addition to stirring increments, the process cost. Co-cultivating yeast and algae help increase the DO levels of the medium without incurring additional expenses [Arora *et al*., 2019].

2.5.5.6 Modes of culture

Microbial fermentation can be classified as a batch, fed-batch, semi-continuous, continuous, repeated batch, and two-stage processes [Karamerou and Webb, 2019]. Batch fermentation is often the first and the most common mode of cultivation. Batch cultures reach very high process efficiencies but are limited to lab-scale only. Batch cultures do not realize steady-state, and the microbial growth rate as well as substrate utilization rate is primarily a function of batch cycle length. Also, the production phases are limited, owing to the continuously changing nutrient availabilities [Karamerou and Webb, 2019]. The fed-batch cultivation, on the other hand, is scalable and useful for the biofuel industry. It involves substrate/nutrient supplementation intermittently during fermentation. For example, the process can begin with C/N ratio < 20, which supports biomass growth, and after adequate growth, additional carbon source can be supplemented to enhance the C/N ratio enabling lipid

accumulation [Zhang *et al*., 2011]. Researchers have widely employed this mode of cultivation for optimizing lipid accumulation. For example, *R. toruloides* Y4 cultivated in a fed-batch style (glucose as carbon source) attained 151.5 g/L of lipid (48 % of dry cell weight) after 25 days. Further, lipid productivity of 0.54 g/L. h was achieved using a 15-L stirred tank reactor [Li *et al*., 2007]. In another study, fed-batch cultivation of *C. freyschussii* on glycerol enabled six-fold higher lipids compared to batch cultivation [Raimondi *et al*., 2014]. Continuous cultivation allows the achievement of steady-state and supports high lipid productivities for a longer time. The continuous cultivation with cell recycle is particularly useful for lowering the hydraulic retention time and enhancing the process reaction rates. For example, the continuous cultivation of *R. toruloides* AS 2.1389 at the dilution rate (D) of 0.02 /h gave lipid yield of 0.19 g/g of glucose and lipid productivity of 0.058 g/g of cell/ h [Shen *et al*., 2013]. The two-stage cultivation involving sequential reactors, one supporting high biomass, and the other supporting lipogenesis is also widely used by researchers for lipid production [Tang *et al*., 2018; Xu *et al*., 2017].

2.5.5.7 Presence of inhibitors

Inhibitors released from pretreated lignocellulosic biomass includes acetic acid, furans, furfural, 5-hydroxymethylfurfural, phenolic compounds, etc., [Osorio-González *et al*., 2019]. These compounds act as growth inhibitors and enzyme inhibitors [Osorio-González *et al*., 2019]. Yeast exhibit variable tolerance to these inhibitors, with some being highly sensitive and the others being highly resistant. The latter is desirable for biofuel production as the inhibitor removal step can be circumvented. Screening the yeast for inhibitor tolerance is one of the crucial experiments when finding a suitable biofuel producer. Several studies of this kind are reported in the literature. Yeast such as *R. toruloids*, *C. curvatus*, *T. cutaneum* were able tolerate the inhibitors at different concentrations [Osorio-González *et al*., 2019; Wang *et al*., 2016; Yu *et al*., 2014; Zhao *et al*., 2012]. The yeast can naturally tolerate inhibitors or can be engineered to tolerate them.

2.5.6 Estimation and recovery of lipid

The downstream processing for lipid extraction is one of the most critical and challenging steps [Patel *et al*., 2018]. It is tough obtaining pure lipids from cells. Lipids are isolated along with other lipophilic molecules such as aromatic compounds, pigments, etc., [Jacob, 1992]. Also, yeasts, especially basidiomycetous yeasts, have thicker cell walls made up of chitin, glucan, mannan, and proteins [Pomraning *et al*., 2015; Khot *et al*., 2020]. The first step towards lipid recovery is cell lysis and involves chemical, physical, or enzymatic methods [Ageitos *et al*., 2011]. Cell lysis methods include sonication, autoclaving, homogenization, microwave irradiation, maceration, bead beating, thermolysis, acid catalysis, hot water treatment, pulse-field electric, and laser treatment [D'Hondt *et al*., 2017; Patel *et al*., 2018]. Each method has its limitations and advantages. For example, ultrasonication is associated with the generation of free radicals, which lowers the lipid yield [Chemat *et al*., 2011], microwave irradiation and enzymatic treatment is expensive but may result in high returns [Patel *et al*., 2018; Jin *et al*., 2012]. Lipid recovery requires dry biomass, which adds cost to the process and poses difficulties in process scale-up [Patel *et al*., 2018]. The lipid recovery from wet oleaginous biomass is desirable [Dong *et al*., 2016]. Also, extracellular lipid production is another alternative. For example, yeast such as *Rhodosporidiobolus fluvialis* DMKU-SP31 and *R. fluviale* DMKU-RK253 can produce extracellular lipids and wax-like compounds in the medium, but extracellular lipid secretion is not very common. It may require the genetics/metabolic engineering of lipid producers. Nevertheless, the search for innovative methods for lipid recovery is essential when it comes to the sustainable biofuel production process [Polburee *et al*., 2016; Poontawee *et al*., 2018].

Lipid quantification during or after fermentation involves modified methods of Folch or Bligh and Dyer [Bligh and Dyer, 1959]. These methods are still the most popular and accepted

methods of lipid quantification and recovery. The solvent-based lipid extraction procedures are very long and time-consuming. Mainly, when the aim is to quantify lipids during fermentation, calorimetric and microscopic methods are desirable. Lipid quantification through fluorescence microscopy, fluorescence spectrometry, fluorescence-activated cell-sorting (FACS) (employing Nile red or BODIPY dye) [Bruder *et al*., 2018; Patel *et al*., 2019] is also popular. Fluorescence microscopy helps to visualize intracellular lipid droplets after staining with lipophilic fluorescent dyes like Nile red and BODIPY. It is also possible to quantify lipids based on the estimated size of lipid droplets [Patel *et al*., 2014]. Quantification using Nile red spectrofluorimetry shows a better correlation with gravimetric analysis (r=0.71) [Sitepu *et al*., 2012]. Other methods for monitoring lipids include light microscopy (Sudan black B dye), FT-IR, FT-NIR. Calorimetric methods based on sulpho-phospho vanillin are also reported in the literature [Frings and Dunn, 1970; Guo *et al*., 2019; Yamada *et al*., 2017].

2.5.7 Transesterification

Biodiesel is produced by the transesterification reaction between TAGs, ethanol/methanol in the presence of a suitable catalyst [Yusuf *et al*., 2011]. Fatty acid methyl/ethyl esters and glycerol are the products of such a reaction. The transesterification reaction can be catalyzed using homogenous/heterogeneous catalysts, or enzymes. Homogenous methods include base or acid catalysis. Base catalyzed transesterification involves the use of NaOH and KOH in methanol. The reaction proceeds at a faster rate and gives a good yield of FAME [Yusuf et al., 2011]. The presence of free fatty acids/water in feedstock results in the production of soaps during base catalysis, which interferes with FAME separation. Such a feedstock can be processed using acid catalysis. This involves the use of sulphuric acid, hydrochloric acid, or boron trifluoride [Morrison and Smith, 1964]. The acid catalysis is, however, slow making it a less preferred method for transesterification. Heterogeneous catalysts such as calcium oxide give high reaction rates, are recyclable, and allow easy product recovery. Heterogeneous catalysts are preferred over homogenous catalysts and adopted nowadays as a part of green chemistry.

Lipases are the enzyme belonging to the class hydrolases (EC 3.1.1.3). These enzymes can be utilized as bio-catalyst for the transesterification. Lipase catalyzed transesterification offers an eco-friendly solution and is being examined by the scientific community for its effectiveness. Almost all biological organisms produce lipases. Also, several yeasts, fungi, and bacteria are known to secrete it extracellularly. Some examples of lipase producers include *C. antarctica*, *C. rugosa*, *C. lipolytica*, and *P. roqueforti*, etc., [Norjannah *et al*., 2016]. Lipases exhibit a wide range of kinetic parameters affecting their substrate affinity, reaction rates, catalytic efficiency, turnover number, etc. The Novozyme 435 is a commercial lipase preparation from *C. antarctica* immobilized on the acrylic resin is widely being used for enzyme-catalyzed transesterification [Ortiz *et al*., 2019]. Researchers have tested the suitability of different lipase preparations for transesterification reactions. For example, *C. rugosa* lipase processed waste palm oil palm to FAME in a non-polar medium with 78% conversion and 4 h reaction time [Lara and Park, 2004]. The crude lipase preparation from *Cryptococcus* sp-2 catalyzed transesterification in the aqueous medium (water content 60-100 % w/w), giving 80.2 % conversion and 120 h reaction time [Kamini and Iefuji, 2001]. Immobilized lipase from *T. asahii* MSR54 gave 87.6 % conversion of lipids to FAME [Kumari and Gupta, 2012]. Certain studies also utilized intracellular lipases using whole microbial cell catalysts [Aarthy *et al*., 2014]. For example, 60-62 % of palmitic and oleic acid underwent conversion to FAME in 96 h using the whole-cell catalyst (*R. glutinis)*. The cells were also used in repeated batch mode with a 10% loss in activity per batch [Hatzinikolaou *et al*., 1999]. Similarly, Whole cells of *R. mucilaginosa* P11I89 were utilized for the conversion of palm oil and methanol (1:6) in the aqueous system enabling FAME production in 72 h with 83.29 % conversion [Srimhan *et al*., 2011].

2.5.8 Biodiesel potential of the lipid extracts using analytical techniques

The lipid profile varies in different strains of the yeasts [Sitepu *et al*., 2013]. It also changes with changes in a growth phase, carbon source, environmental conditions, and presence/absence of nutrients & micronutrients [Sitepu *et al*., 2014a]. Yeast lipid extract is primarily composed of stearic acid (C_{18}:0), palmitic acid (C_{16}: 0), unsaturated fatty acids such as oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$ώ6), linolenic acid ($C_{18:3}$ώ3). It also contains minor amounts of myristic acid ($C_{14:0}$), arachidic acids ($C_{20:0}$), behenic acid ($C_{22:0}$), lignoceric acid ($C_{24:0}$) [Sitepu *et al*., 2014a].

Several standards to advanced quantitative techniques are available to decipher the fatty acid profiles. The methods include thin-layer chromatography (TLC), High-Performance Liquid Chromatography (HPLC), Fourier transform infrared spectroscopy (FT-IR), Nuclear magnetic resonance (NMR) spectroscopy, Gas chromatography (GC), Mass spectroscopy (MS), Raman spectroscopy, and dielectric spectroscopy, GC-MS etc. [Patel *et al*., 2019]

Thin-layer chromatography (TLC) uses pre-coated silica gel on the aluminum/glass/ plastic base as a stationary phase and a mixture of standard solvents (such as 85:15:1 v/v/v hexane; diethyl ether; acetic acid) as a mobile phase. The technique can be used for qualitative as well as quantitative analysis of the lipids in the sample [Patel *et al*., 2019]. High-performance thin-layer chromatography (HP-TLC) enhances the resolution and quantification of lipids. Samples resolve as spatially located spots which can be detected using either iodine vapor or chemical-based heat treatment (methanolic $MnCl_2$). Gas chromatography coupled with various detectors (FID/MS/TOF analyzer) is one the most reliable method to identify and quantify the lipids present in the sample [Patel *et al*., 2019; Morrison and Smith, 1964]. Infrared spectroscopy (IR) measures the analyte based on IR absorption and resulting molecular vibrations thereof. FT-IR analysis is often used as a non-invasive method for the qualitative analysis of the lipid extract. Lipid quantification, based on IR, has been developed recently [Chmielarz *et al*., 2019]. NMR spectroscopy also aids in the qualitative assessment of the lipid extract. It also provides structural information on the molecules analyzed. Sarpal *et al*., developed a proton, NMR based method for the qualitative evaluation of lipids from cellular biomass. This included lipid quantification, determination of the degree of saturation, iodine value, etc., [Sarpal *et al*., 2014]. Raman spectroscopy and Dielectric spectroscopy methods are also applied recently for qualitative analysis of lipids [Patel *et al*., 2019].

The biodiesel characteristics differ significantly with carbon chain length and degree of saturation [Sargeant *et al*., 2017]. This necessitates maintaining bioprocess conditions for reproducible lipid profiles [Khot *et al*., 2018]. Lipid profile determines the fuel properties such as density, oxidative stability (OS). Kinematic viscosity (KV), Cold filter plug points (CFPP), flash point, iodine value (IV), cetane number (CN), etc. [Patel *et al*., 2017a]. These properties are marked by specific numbers, the values of which should fall in a particular range as prescribed by ASTM standard D 6751, and EN 14214 for biodiesel [Meng *et al*., 2009]. These properties are used to assess the suitability of the feedstock as automotive fuel, and the values must be within the prescribed ranges. The higher CN, more extended oxidative stability and low cold filter plug point are the criteria for excellent engine performance [Deeba *et al*., 2018; Patel *et al*., 2017a]. The CN is a measurement of fuel's autoignition quality [Deeba *et al*., 2018] with a value above 50 desirable in reducing white smoke emission [Knothe, 2014]. OS, KV, and CFPP determine the shelf-life of the fuel [Balat and Balat, 2010], the ability of the fuel to flow, and the freezing point of the fuel, respectively [Patel *et al*., 2017a].

2.5.9 Techno-economic analysis

Techno-economic analysis (TEA) on the use of oleaginous yeast for the production of SCO [Leong *et al*., 2018] is essential to check the sustainability of the process. Ratledge and cohen (2008) estimated that the SCO obtained from yeast as a feedstock was almost at a double cost than commodity oils. The SCO from yeast can't achieve viability until crude oil price is

hiked in the future [Ratledge and Cohen, 2008]. The oil prices for soybean, rapeseed, and jatropha were estimated at $ 1.64, 0.34, and 0.39 per kg, respectively [Meira *et al.*, 2015]. The estimated price of yeast oil was 1.6 $ /kg; this was cheaper than palm oil, which cost around 2.1 $/kg [Schilter, 2019]. The high cost of yeast oil is due to capital investment and electricity consumption for the fermentation operations [Koutinas *et al.*, 2014]. Yeast based biofuels can be made sustainable if it can grow on cheaper substrates under non-sterile conditions or have high lipid productivity under controlled fermentation conditions. Figure 2.8 shows the overall steps involved in biodiesel production using oleaginous yeasts.

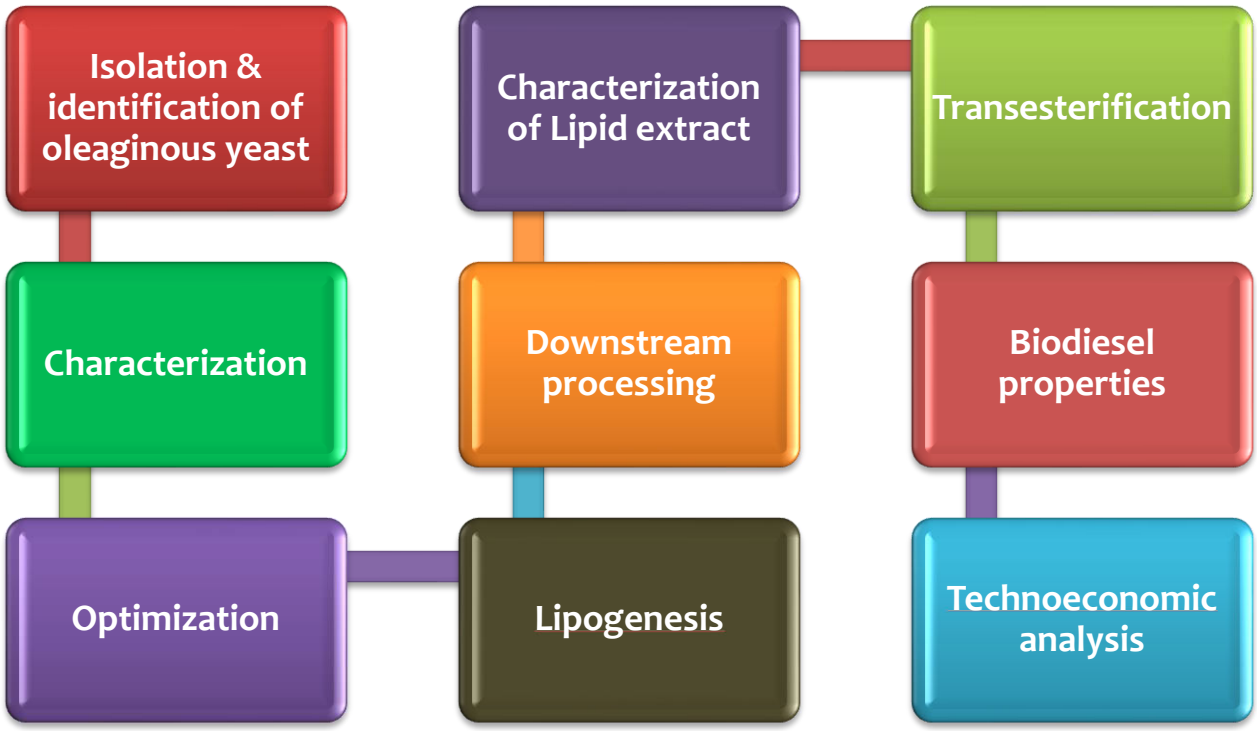

Figure 2.8: Flow chart of the steps involved in biodiesel production using oleaginous yeast

2.6 UTILIZATION OF INDUSTRIAL WASTEWATER FOR BIODIESEL

Glucose costs nearly 250 $/t in the international market and contributes 60-80% of the fermentation costs associated with yeast lipid production [Patel *et al.*, 2018; Qin *et al.*, 2017]. This mandates the use of readily available waste substrates and the optimization of the process parameters for their effective utilization [Khot *et al.*, 2018]. Wastewater generated from different sources, particularly food processing, paper & pulp, municipal wastewater is rich in organics. It can serve as a feedstock for oil production [Patel *et al.*, 2018]. These wastewaters need treatment before they are discharged into natural water bodies [Huang *et al.*, 2017]. Simultaneous wastewater treatment and lipid generation by oleaginous yeasts can be an effective method [Martínez *et al.*, 2015; Vasconcelos *et al.*, 2019]. A wide range of simple to complex substrates have been utilized for lipid production using oleaginous yeast [Leiva-Candia *et al.*, 2014]. Utilization of some of these substrates is briefly discussed in the sections below:

Table 2.4: Utilization of Industrial wastewater for biodiesel production by the oleaginous yeast

Fermentation (distillery) industry wastewater							
Yeast strain	**Substrate**	**Culture mode**	**Time (h)**	**Total dry weight (g/L)**	**Lipid production (g/L)**	**Lipid content (% w/w)**	**References**
Rhodosporidium toruloides	Distillery wastewater + domestic wastewater	Batch-Shake flask	72	8.12 ± 0.23	3.54 ± 0.04	43.65 ± 1.74	[Ling *et al.*, 2013]
***Trichosporon cutaneum* CH002**	Acetone-butanol-ethanol (ABE) wastewater	Batch-Shake flask	48	4.9	0.72	14.7	[Xiong *et al.*, 2015]
***Trichosporon cutaneum* ACCC 20271**	cellulosic ethanol fermentation wastewater	Batch -3-L bioreactor	120	16.20	2.16	13.33	[Wang *et al.*, 2017]
Biodiesel industry wastewater							
***Yarrowia lipolytica* LGAMS(7)1**	Industrial Crude glycerol	Continuous culture	N.M.	8.1	3.5	43	[Papanikolaou and Aggelis, 2002]
***Rhodosporidium toruloides*Y4**	Crude glycerol	Two-stage fermentation-shake flask	120	24.9 ± 0.5	12.2 ± 0.2	48.9 ± 0.6	[Yang *et al.*, 2014]
***Lipomyces lipofer* NRRL Y-115512**	Crude glycerol	Batch-Shake flask	264	9.48 ± 0.27	5.46 ± 0.34	57.64± 2.81	[Spier *et al.*, 2015]
Rhodotorula glutinis	Pure Glycerol	Fed-batch - 2-L bioreactor	N.M.	16.8	5.07	34.62	[Karamerou and Webb, 2016]
***Yarrrowia lipolytica* 347**	Raw glycerol	Batch-Shake flask	144	11.5 ± 0.0	2.60 ± 0.18	22.6 ± 1.5	[Canonico *et al.*, 2016]
***Rhodosporidium toruloides* 32489**	Crude glycerol	Batch-Shake flask	168	14.85 ± 0.29	6.20 ± 0.27	41.76 ± 1.54	[Gao *et al.*, 2016]
***Yarrowia lipolytica* A101**	Crude glycerol – saponification industry	Batch-Shake flask Batch-5-L jar bioreactor	120 96	6.87 ± 0.21 N.M.	1.69 ± 0.09 4.72	24.9 N.M.	[Dobrowolski *et al.*, 2016]

***Lipomyces starkeyi* AS 2.1560**	Crude glycerol	Batch-Shake flask Batch-5-L bioreactor		21.1 29.2	7.5 12.5	35.7 49.2	[Liu *et al*., 2017]
Trichosporon oleaginosus	Crude glycerol	Fed-Batch- 15-L fermenter Fed-Batch-15-L fermenter	56 52	24.80 43.82	12.14 21.87	48.95 49.89	[Chen *et al*., 2017]
Food industry wastewater							
***Cryptococcus curvatus* NRRL Y-1511**	Ricotta cheese whey	Batch-3-L stirred tank bioreactor	72	10.77 ± 0.21	6.83 ± 0.14	63.41	[Carota *et al*., 2017]
Cryptococcus curvatus	Pretreated cheese whey (HC treatment)	Batch-Shake flask	24	7.2	4.68	65	[Seo *et al*., 2014]
***Yarrowia Lipolytica* B9**	Deproteinized cheese whey	Batch-Shake flask (Non-sterile)	120	7.00 ± 0.18	2.73 ± 0.13	39	[Taskin *et al*., 2015]
Debaryomyces etchellsii	Deproteinized cheese whey	Batch-Shake flask	96	2.80 ± 0.11	0.40 ± 0.05	15.90 ± 0.93	[Arous *et al*., 2016]
Wickerhamomyces anomalus	Deproteinized cheese whey	Batch-Shake flask	96	2.61 ± 0.03	0.65 ± 0.01	24.00 ± 0.24	[Arous *et al*., 2017]
Candida tropicalis X37 Yarrowia lipolytica* TISTR 5151 *Mixed culture	Palm oil mill effluent supplemented with 3% glycerol	Batch-Shake flask	N.M.	N.M.	N.M.	33.5 ± 0.8 52.7 ± 2.8 47.7 ± 1.1	[Louhasakul *et al*., 2019]
***Yarrowia lipolytica* TISTR 5151**	Palm oil mill effluent	Batch-Shake flask	36	5.68 ± 0.32	1.64 ± 0.03	28.87	[louhasakul *et al*., 2016]
Lipomyces starkeyi	Palm oil mill effluent	Batch-Shake flask	N.M.	7.61 ± 0.23	1.62	21.32 ± 0.82	[Islam *et al*., 2018]
Rhodotorula	Starch	Batch-		0.18			[Chaturvedi *et*

mucilaginosa ***Rhodotorula glutinis***		Shake flask		0.11			*al*., 2018b]
Municipal and sludge wastewater							
***Lipomyces starkeyi* MTCC-1400**	Municipal waste activated sludge	Batch-Shake flask	N.M.	N.M.	N.M.	64.3	[Selvakumar and Sivashanmugam, 2017]
***Naganishia liquefaciens* NITTS2**	Municipal waste activated sludge	Batch-Shake flask	144	16.80 ± 1.42	9.35	55.7 ± 1.5	[Selvakumar and Sivashanmugam, 2017]
Paper & pulp industry wastewater							
Rhodotorula glutinis	Paper and pulp industry effluent Supplemented with Glucose xylose glycerol	Batch-3-L Bioflo 310 fermenter	168	N.M.	1.3-2.9	N.M.	[Shields-Menard *et al*., 2015]
***Rhodosporidium kratochvilovae* HIMPA1**	Paper and pulp industry effluent	Batch-Shake flask	144	13.87	8.56	61.71	[Patel *et al*., 2017b]
***Cryptococcus vishniaccii* (MTCC 232)**	Paper and pulp industry effluent	Batch-Shake flask	144	14.6	7.8	53.40	[Deeba *et al*., 2016]

(Where N.M.: Not mentioned)

2.6.1 Fermentation (distillery) industry wastewater

A large amount of wastewater containing high organics is generated from stillage during the alcohol fermentation [Wang *et al*., 2017]. Oleaginous yeast can be cultivated on such wastewaters. *R. toruloides* grown on distillery wastewater diluted with domestic wastewater (1:1 ratio) produced 3.54 ± 0.04 g/L (43.65 ± 1.74%) lipid after three days of fermentation with 86.11 ± 0.41% COD removal [Ling *et al*., 2013]. In one of the studies, acetone-butanol-ethanol (ABE) industry wastewater was used for cultivating *T. cutaneum* CH002 leading to the production of 0.72 g/L (14.7 % w/w) lipids after 48 h of fermentation. The process led to a 68 % COD removal at a rate of 6.025 g/L. d [Xiong *et al*., 2015]. *T. cutaneum* ACCC 20271 cultivated on cellulosic ethanol fermentation wastewater produced 2.16 g/L of lipid and removed 55.05% COD at the rate of 13.054 g/L. d [Wang *et al*., 2017].

2.6.2 Biodiesel industry wastewater

Crude glycerol from the biodiesel industry is another targeted substrate for oleaginous yeast cultivation. With the boom in the biodiesel industries, the volume of crude glycerol waste will increase. Crude glycerol contains various impurities such as methanol, sulfuric acid, soaps, etc. which make it challenging to utilize substrate [Uprety *et al*., 2017]. Several studies have been

conducted to find a feasible route for the utilization of crude glycerol. Cultivation of robust oleaginous yeast is one of the promising courses, as indicated by the studies in the literature. There are many reports on the utilization of crude glycerol (biodiesel industry waste) as substrates for lipid accumulation by oleaginous yeast, as shown in Table 2.4.

2.6.3 Food industry wastewater

Waste streams from food industries offer readily utilizable substrates for lipid accumulation by oleaginous microbes. Cheese whey is a greenish-yellow liquid residue, a by-product from the cheese-making process of dairy industries. It is typically composed of 92-95 % water, and solid content is 5-8 % (w/v) of which lactose accounts for 60-80 % (w/w), proteins account for 10 % (w/w), and trace elements, vitamins, fat, and other important elements account for the remaining 10 % (w/w). It contains 50-102 g/L, chemical oxygen demand (COD), and 27-60 g/L biological oxygen demand (BOD) [Leong *et al*., 2018]. Cheese whey can serve as feedstock for the production of biomolecules like ethanol, pigments, enzymes, lipids, single-cell proteins, etc., [Seo *et al*., 2014; Taskin *et al*., 2015]. Cheese whey is often pretreated before being used as feedstock for fermentation. For example, chemical treatment of cheese whey combined with hydrodynamic cavitations (HC) enabled the growth of *C. curvatus* [Seo *et al*., 2014]. In another study, *C. curvatus* NRRL Y-1511 grew on ricotta cheese whey obtained after acid, salt, and deproteinization pretreatment [Carota *et al*., 2017]. An oleaginous yeast *Y. lipolytica* B9 strain grew on non-sterile deproteinized cheese whey (DCW) and attained a lipid content of 2.73 ± 0.13 g/L [39 % (w/w)]. External supplementation of lactose and ammonium sulfate increased the lipid content to 4.29 g/L (58 % w/w) [Taskin *et al*., 2015]. *W. anomalus* accumulated 0.65 ± 0.01 g/L (24.00 ± 0.24 % of w/w) after 96 h of incubation [Arous *et al*., 2017]. However, *D. etchellsii* could not assimilate lactose in the DCW but utilized it as a nitrogen source while accumulating 0.40 ± 0.05 g/L of lipid [15.90 ± 0.93 % (w/w)] [Arous *et al*., 2016].

Palm oil mill effluent (POME) presents a high strength of organic waste (50-100 g/L COD) [Islam *et al*., 2018] utilizable for lipid accumulation by yeast. *Y. lipolytica* TISTR 5151 grown on palm oil mill effluent accumulated $>$ 33 % (1.64 ± 0.03 g/L) lipids, and 3353 ± 27 U/L of cell-bound lipase [Louhasakul *et al*., 2016]. *D. etchellsii* BM1 grown on composite wastewaters comprising of deproteinized cheese whey expired soft drinks, and olive oil mill wastewater in different proportions, realized 14.90-28.90 % w/w lipid content [Arous *et al*., 2016] with 41% to 58% COD removal [Arous *et al*., 2016]. In another study, different dilutions of POME were used to cultivate *L. starkeyi* and with 50% concentration supporting high lipid accumulation. Seventy-five percent of COD inherent in diluted POME was removed during the process [Islam *et al*., 2018]. *C. tropicalis* X37 and *Y. lipolytica* TISTR 5151 accumulated $>$ 30 % lipid using POME supplemented with 3 % glycerol and removed 87% COD [Louhasakul *et al*., 2019]. *R. glutinis* accumulated lipids using industrial waste cassava peel as a substrate [Chaturvedi *et al*., 2019b].

2.6.4 Municipal and sludge wastewater

A tremendous amount of sludge accompanies municipal wastewater treatment, and sludge volume is particularly high during aerobic treatments. The sludge needs to be continuously removed and disposed of separately. This, too, has been tested as feedstock for oil accumulation by oleaginous yeast. *L. starkey*i MTCC-1400 cultivated on municipal waste activated sludge (MWAS) resulted in 64.3 % (w/w) lipids [Selvakumar and Sivashanmugam, 2017]. *N. liquefaciens* NITTS2 isolated from municipal wastewater treatment plant when cultivated on MWAS produced 55.47 % (w/w) of lipid after optimization using the response surface method (RSM). The lipids obtained were also converted into FAME using garbage lipase with a yield of 88.34 ± 1.20 % (w/w) [Selvakumar and Sivashanmugam, 2018].

2.6.5 Paper & pulp industry wastewater

The Paper & pulp industry is one of the largest producers of wastewater [Amirsadeghi *et al*., 2015; Patel *et al*., 2017b]. The wastewater is not nutrient-rich and needs supplementation with different carbon/nutrient sources. In one of the studied wastewater supplemented with glucose, xylose, and glycerol enabled lipid accumulation using *R. glutinis*. Lipid accumulation up to 1.3-2.9 g/L was observed in a 3-L fermenter after 168 h [Amirsadeghi *et al*., 2015]. Pretreated paper mill sludge, when used by *C. vishniaccii* (MTCC 232), resulted in 7.80 ± 0.57 g/L (53.40% w/w) (higher than glucose medium) lipid in shake flask cultures [Deeba *et al*., 2016]. *R. kratochvilovae* HIMPA1 cultivated on paper and pulp industry effluent supplemented with 7 % glucose accumulated 8.56 g/L [61.71 %(w/w)] lipids after 144 h with 94.22 % COD removal at the rate of 0.106 g/L. d [Patel *et al*., 2017b]. Table 2.5 shows the comparison of COD removal rates for various industrial wastewater using oleaginous yeasts.

Table 2.5: Simultaneous COD removal and lipid generation by oleaginous yeasts

Sr No.	Organism name	Type of the wastewater	Cell dry weight X (g/L)	Lipid content (%)	Initial COD (g/L)	Final COD (g/L)	COD removal (%)	Reference
1.	***Rhodosporidium toruloides***	Distillery wastewater	8.12 ± 0.23	43.65 ± 1.74	3.54 ± 0.04	3.3	86.11 ± 0.41	[Ling *et al*., 2013]
2.	***Trichosporon cutaneum* CH002**	Acetone-Butanol-ethanol (ABE) Fermentation wastewater	4.9	14.7	18.05	6.00	68	[Xiong *et al*., 2015]
3.	***Debaryomyces etchellsii***	50 % Expired soft drink + 50 % DCW	7.90	14.90	96.36	40.13	58.35	[Arous *et al*., 2016]
		62.4 % expired soft drinks + 37.6 % olive mill wastewater	4.60	28.90	113.26	66.48	41.30	
		19.7 % DCW + 80.3 % Expired soft drink	6.80	18.40	120.6	78.14	35.25	
4.	***Rhodosporidium kratochvilovae* HIMPA1**	Paper and pulp industry wastewater	13.87	61.71	0.675	0.039	94.22	[Patel *et al*., 2017b]
5.	***Trichosporon cutaneum* ACCC20271**	Cellulosic ethanol fermentation wastewater	16.20	13.33	118.58	53.31	55.05	[Wang *et al*., 2017]
6.	***Lipomyces***	50 % Palm oil	7.61 ±	21.32 ±	26.176	6.541	75.01	[Islam *et*

	starkeyi	mill effluent (POME)	0.23	0.82	± 0.038	±0.006		*al.*, 2018]
7.	***Candida tropicalis*X37 + *Yarrowia lipolytica*TISTR 5151**	POME	7.37	47.7 ± 1.1	N.M.	N.M.	87.2	[Louhasakul *et al.*, 2019]

(Where N.M.: Not mentioned; POME: Palm oil mill effluent)

2.7 OBJECTIVES

It is necessary to search natural environments for novel yeast with all desirable characteristics considering the promise they hold towards biofuel production. The criteria would involve the ability of the isolate to grow and accumulate lipids on cellulosic substrates like CMC while simultaneously producing lipase to enable consolidated bio-processing. It is also imperative to test the tolerance of the isolated yeast to common inhibitors and its ability to utilize agro-industrial waste/waste hydrolysates. The thesis intends to find yeast with desirable characteristics of a biofuel producer and to test its ability to accumulate lipids sustainably.

Keeping all these points in consideration and after reviewing the state of the art, the following objectives are defined for this study:

1. Isolation, identification, and characterization of oleaginous yeast capable of cellulase and lipase production
2. Assessment of the biodiesel potential of the selected yeast using industrial wastewater.
3. Study of the effect of common inhibitors on cell growth and lipid accumulation and ability of the yeast to grow on agro-industrial wastes.

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