MUSIC

Market Uptake Support for Intermediate Bioenergy Carriers

WHITE PAPER: MICROBIAL OIL

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Contents

List of Acronyms

ARA - Arachidonic Acid

ASTM - American Society for Testing and Materials

BTX - Benzene Toluene Xylene

CBP - Consolidated Bio Processing

CLA - Conjugated Linoleic Acid

CO_2 - Carbon Dioxide

CORSIA - Carbon Offsetting and Reduction Scheme for International Aviation

DHA - Docosahexaenoic Acid

EC - European Commission

EPA - Eicosapentaenoic Acid

FA - Fatty Acid

FAEE - Fatty Acid Ethyl Ether

FAME - Fatty Acid Methyl Ester

FFA - Free Fatty Acid

FPBO - Fast Pyrolysis Bio-Oil

FRL - Fuel Readiness Level

GHG - Green House Gas

GLA - Gamma Linoleic Acid

HEFA - Hydroprocessed Esters and Fatty Acids

HFS-SIP - Synthesized Iso-Paraffins from Hydroprocessed Fermented Sugar

HMF - Hydroxymethylfurfural

HVO - Hydrotreated Vegetable Oil

IBC - Intermediate Bioenergy Carrier

ILUC - Indirect Land Use Change

ktoe - Thousands of tons of oil equivalent, a unit of measure

LA - Linoleic Acid

MVA – Mevalonic Acid

MO - Microbial Oil

MFSP - Minimum Fuel Selling Price

MUFA - Mono Unsaturated Fatty Acid

PHA - Polyhydroxyalkanoate

PUFA - Poly Unsaturated Fatty Acid

PVC - Poly Vinyl Chloride

RED II - Renewable Energy Directive II

RES - Renewable Energy Source

RJF - Renewable Jet Fuel

SAF - Sustainable Aviation Fuels

SCO - Single Cell Oil

SHLP - Separate Hydrolysis and Lipid Production

SmF - Submerged Fermentation

SSF - Solid State Fermentation

SSLP - Simultaneous Saccharification and Lipid Production

TAG - Triacylglyceride

TB - Torrefied Biomass

TRL - Technology Readiness Level

UCO - Used Cooking Oil

UV - UltraViolet

Figures

Tables

1 Introduction

Intermediate bioenergy carriers (IBCs) are biomass that are processed to energetically denser materials, analogous to oil, coal, and gaseous fossil energy carriers. This means they are easier to transport, store and use.

The **EU H2020 MUSIC** project supports the market uptake of Intermediate Bioenergy Carriers (IBCs) by developing feedstock mobilisation strategies, improved cost-effective logistics and trade centres.

IBCs are formed when biomass is processed to energetically denser, storable, and transportable intermediary products analogous to coal, oil and gaseous fossil energy carriers. They can be used directly for heat or power generation or further refined to final bioenergy or bio-based products. IBCs contribute to energy security, reduce greenhouse gas emissions, and provide a sustaiable alternative to fossil fuels in Europe.

Microbial Oil (MO) is one such IBC. It is produced by oleaginous yeasts from lignocellulosic biomass; presently it is at the early stages of development as potential feedstock for an EU bio-based economy, with a Technology Readiness Level (TRL) currently ranging between 4 and 5. However, MO has an immense potential as a substitute for vegetable oils and food-related lipid feedstocks, i.e., for commercial Hydrotreated Vegetable Oil (HVO) biorefineries. This is especially true since the Renewable Energy Directive II set a cap for such food- and feed-based biofuels and also defined targets to reduce the use of high Indirect Land Use Change (ILUC)-risk feedstock's - such as palm oil - starting in 2023 and with a complete phase-out by 2030. Furthermore, MO could also be of specific interest for the fossil refineries sector when used as co-feeding feedstock, supporting their transition towards a low-carbon economy.

This report provides information on Microbial Oil properties and the various phases of the production process, with a thorough overview of the main techniques and technologies used. Examples of application of Microbial Oil as intermediate bioenergy carrier beyond direct energy production are presented, with a focus on transport fuels applications: several case studies are evaluated related to biodiesel and Renewable Jet Fuels (RJF) production. Nutraceuticals and biochemical applications are also briefly assessed since the market price for microbial oils feedstock suitable for use in these sectors is considerably higher than the prices that could be paid for renewable fuels lipid feedstock. Considerations on the economics related to the production processes are carried out, and the corresponding Minimum Fuel Selling Price (MFSP) is calculated; to provide a better perspective on the topic, comparisons with vegetable oil-based alternatives and fossil fuels are provided. The possible uses of co-product streams generated during the microbial oil production process are also evaluated, together with the underlying economics; further stream valorisation would indeed reflect better overall economics for a biorefinery, thus reduction of MFSP. Specific focus is dedicated to lignin, given its abundance in the process and the number of research projects to transform it into useful and valuable biochemicals.

2 Oleaginous Microorganisms and Microbial Oil properties

Lipids can be acquired from several types of biogenic renewable resources such as oil plants, food processing side streams, and a variety of microorganisms. In this latter case, **archaea, bacteria, yeast, fungi,** and **microalgae** can be sourced for a significant amount of lipids, produced for essential structural and functional roles mainly in the form of **triacylglycerides** (TAGs) and **fatty acids** (FAs)[1].

These **microbial lipids** or **microbial oils** are also known by the name **single cell oils** (SCOs). **SCO** initially designated the triacylglycerol (TAG) fraction of the total cell lipids [2]; however, it is now used to include all types of fatty acid (FA) containing lipids, **produced by oleaginous microorganisms able to accumulate more than 20% of their cell dry weight as lipids** [3].

One of the main advantages of SCOs production processes is that they are **independent from seasonality and climate**; moreover, they can be **obtained from a wide range of carbon sources**, including renewable ones and organic wastes.

SCOs could have different FA compositions compared to plant seed or fish oils; thus, they can also be seen as new sources of nutraceuticals (a dietary supplement that provides health benefits and its fundamental nutritional value) precious for human life. SCOs could as well be considered as Intermediate Bioenergy Carriers (IBC), suitable for vegetable oils substitution for **biofuels production** [4]–[9].

Figure 1 below visually summarises a selection of valuable products derived from neutrals lipids (TAGs), free fatty acids and acyl-CoA, (acyl-Coenzyme A) which in turn are produced by oleaginous microorganisms. These beneficial products include **glycolipids**, **unsaturated** as well as **polyunsaturated fatty acids** and derived **esters**, **alcohols**, **aldehydes**, and **alkanes**. As already pointed out, they could be used as food supplements, cosmetics, fine chemicals, pharmaceuticals, and biofuels.

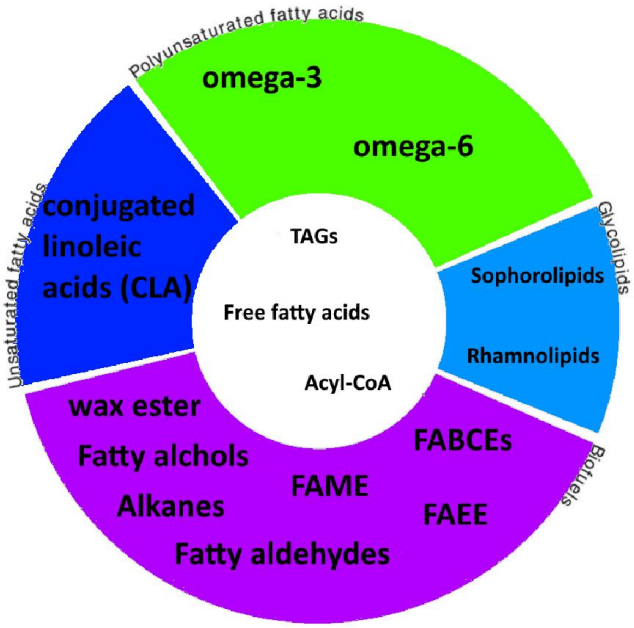

Figure 1: A selection of valuable products derived from neutrals lipids (TAGs), free fatty acids and acyl-CoA, produced by oleaginous yeasts (Author's elaboration from [3])

2.1 Oleaginous microorganisms and composition of Single Cell Oils

Oleaginous microorganisms can be found among various species of microalgae, fungi (filamentous and yeasts) and bacteria [1], [10], [11]:

- **Filamentous fungi and yeasts:** Yeast oil contents from literature range from 58% to 72% of dry cell weight, with a *Rhodotorula glutinis* strain accumulating the highest level; moulds oil contents are reported as ranging from 57% to 86%, with a strain of *Mortierella isabellina* presenting the highest level in the range. Data has been found on the following strains: *Umbelopsis* (*Mortierella*), *Microsphaeropsis, Fusarium, Candida, Meyerozyma, Rhodotorula, Rhodosporidium, Pichia, Cryptococcus, Lipomyces, Trichosporon* and *Yarrowia.*
- **Bacteria:** oil accumulations are reported as ranging from 24% to 78% of dry weight, with the highest levels reported for *Arthrobacter* sp. at >40%, and up to 78% from glucose feedstock [12]. Data has been found on the following strains: *Rhodococcus, Streptomyces, Nocardia, Mycobacterium, Dietzia* or *Gordonia.*
- **Microalgae:** the highest reported oil contents range from 20% to 77% of dry weight, with *Schizochytrium* ranging from 20% to 77%. The more investigated strains are: *Chlorella, Scenedesmus, Chlamydomonas, Nannochloropsis, Chlorococcum, Isochrysis, Cylindrotheca, Tetraselmis, Auxenochlorella, Botryococcus*.

The **FA profile of microbial oil** is usually quite similar to that of the oils produced by oleaginous plants (i.e. soybean, rapeseed, sunflower and palm oils); anyhow, it slightly variates according to the genus and species [10]:

- **Oleaginous yeasts and filamentous fungi** SCOs mainly consist of myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1), linoleic (C18:2) and α- and γ-linolenic (C18:3) acids, with palmitoleic, oleic and linoleic usually being the most abundant.
- The **oleaginous bacteria** are characterised by the presence of more saturated FAs, such as lauric acid (C12:0), C14:0, C16:0 and C18:0.
- **Microalgae species** can synthesise long-chain FAs with a higher number of double bonds, such as docosahexaenoic acid (DHA) (C22:6), eicosapentaenoic acid (EPA) (C20:5) and arachidonic acid (C20:4). These polyunsaturated fatty acids are primarily used to produce cosmetics, nutraceuticals and animal feed.

Besides fatty acid-derived alka(e)nes, isoprenoids constitute the second class of cellular metabolism-derived compounds with promising biofuels prospects. Most isoprenoids have nonessential secondary metabolites functions, especially in plants.

All isoprenoids are derived from the C_5 isoprene unit; depending on the number of incorporated isoprene units, they are classified as **monoterpenes** (C10 - i.e. pinene, sabinene and limonene), **sesquiterpenes** (C15 - i.e farnesene and bisabolene), etc.

Mono- and sesquiterpene hydrocarbons have properties (carbon chain length, density, freezing point, and heat of combustion) similar to conventional, petroleum-derived jet fuel; thus, they have been proposed as **alternative aviation fuels** or fuel additives. The possibilities to enhance the production of mono- and sesquiterpenes in microbial hosts such as *S. cerevisiae* and *E. coli* have been investigated by several studies. As *S. cerevisiae* could produce isoprenoids within the mevalonic acid (MVA) pathway, starting from acetyl-CoA, approaches similar to those used to increase the production of fatty acid-derived products in yeast have also been employed to increase isoprenoid synthesis [7].

2.2 Basics of lipid accumulation process and maximum theoretical yields in oleaginous microorganisms

In order to accumulate high lipid levels in a microorganism, its metabolic pathways must be manipulated, to stop cells from multiplying beyond a certain limit. A culture medium with a limited amount of available nitrogen is a commonly used method to obtain lipids accumulation; this is related to the fact that, when nitrogen is depleted, the cells become unable to synthesise different amounts of proteins and nucleic acids, that they require for their synthesis. Aside from nitrogen, carbon supply should always be available in the culture medium, and it is usually provided in glucose. However, many other carbohydrate feedstocks can be used aside from glucose, with the only obvious cost limitation [4], [5].

Under this framework, it is possible to divide the culture of an oleaginous microorganism into two distinct phases. When all the nutrients needed are available, the first phase sees a **balanced growth of the cells.** This phase finishes when the growth-limiting nutrient becomes exhausted

(i.e. Nitrogen). In this situation, cells are no longer able to multiply but are still metabolically active. Thus, in the **second phase** of the process, related to lipid accumulation, the cells continue to take up the carbon source in the medium and channel it into lipid biosynthesis, since they no longer need to produce a high amount of metabolically available energy [1], [2]. Figure 2 below visually summarises the ideal trends of nutrients available in the culture medium and biomass and lipids accumulation trends across the processing timeframe.

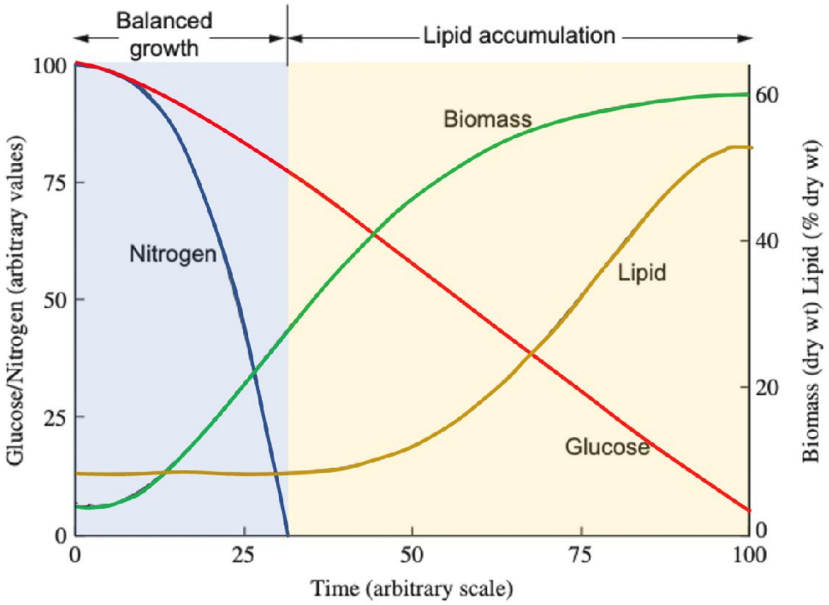

Figure 2: Lipid accumulation trends for oleaginous microorganism (author's elaboration on [2]).

Several potential metabolic pathways and by-products exist for the biological conversion of sugars to long-chain hydrocarbons, such as isoprenoids, fatty acids, triglycerides, and paraffins [5]. Each pathway exhibits **varying theoretical yields**, dictated by underlying metabolic mass and energy yields; i.e. for oleaginous microorganisms literature reports a theoretical maximum yield of **25g to 35g TAG from 100g glucose**, depending on the involved metabolic pathway [13], [14]. Table 1 opens this evaluation to various by-products pathways; in this framework, ethanol still proves to be a superior by-product from bioconversion of sugars in the context of fuel molecules, both in terms of theoretical mass and energy yield (e.g. heating value) of product relative to sugar. The energy yield for hydrocarbon by-products is close, but still remains 5% to 24% lower.

All these examples of by-products are diesel-range molecules; however, by-products such as gasoline or jet-range generally compare similarly in terms of the energy yields [5].

Table 1: Theoretical Metabolic Yields for Various By-Product Pathway [5]

By-Products	Mass yield	Carbon yield	Energy yield (HHV basis)
Ethanol	51%	67%	98%
Pentadecane	29%	62%	88%
Farnesene (DXP pathway)	29%	64%	85%
Farnesene (MVA pathway)	25%	56%	74%
Fatty Acid (Palmitic acid)	36%	67%	89%
FAEE (Ethyl palmitate)	35%	67%	90%
Fatty Alcohol (Hexadecanol)	34%	67%	93%

3 Overview of microbial oil production processes and technologies

The use of oleaginous yeasts for biofuels, nutraceutical or biochemical production could prove more advantageous than the use of microalgae or vegetable oils. Yeasts cultivation is not affected by environmental conditions, seasonal production or geographic location; other advantages of yeasts are related to their low duplication times and metabolic versatility. Moreover, they possibly present no competition with food or feed productions, given the fact that they can grow and accumulate MO on several renewable feedstock including **agricultural residues**, **industrial waste streams** and **non-food crops** [15]. In the following parts of this document we will focus on the use of yeasts and bacteria.

3.1 Suitable feedstock for Microbial Oil production

As heterotrophic organisms, yeasts metabolise carbon (C) from simple sugars or C-containing compounds such as glycerol. Thus, **fermentation feedstocks** can be monosaccharides such as **glucose**, or **C5 and C6 saccharide-containing hydrolysate** derived from the breakdown of lignocellulosic biomass [16]. Yeasts can utilise many different carbon sources (e.g., glucose, xylose, starch, cellulose hydrolysates, glycerol, industrial and municipal organic wastes). Reported carbon sources for lipid production in fungi are glucose, lactose, starches, oils, steep corn liquor, and agricultural waste [1], [15].

Looking at specific substrates, **sugarcane juice**, which contains approximately 15% (w/w) of fermentable sugars, is more than suitable for Microbial Oil production [17]. Also, crude glycerol is a promising low-cost, second-generation feedstock, available as a primary biodiesel production waste product [6]. Finally, **Used Cooking Oil** (UCO) is considered as a potential alternative feedstock for MO production, since several microorganisms are capable of utilising it as a carbon source [18], [19]. Such application is gaining interest since 29 million tons of UCO are generated each year globally, while a single litre of UCO can pollute up to 500 m^3 of water [19].

The theoretical sugar-to-oil yield of around 25-35% (depending on the considered metabolic pathway), leads to the consideration that 3 to 4 tons of sugar are needed to produce 1 ton of MO [2]; this clearly highlights the importance of using low-cost feedstocks, i.e. lignocellulosic materials (also as agro-residues) and organic wastes,, in order for MO to be cost competitive with a plant commodity oil.

Lignocellulose is a complex biopolymer composed of the polysaccharides **cellulose** and **hemi-cellulose**, the amorphous polymer **lignin** and a remaining smaller fraction which includes **pectin, proteins, extractives and ash**. Approximately two thirds of the biomass total dry weight is composed by the structural carbohydrates, which can be used as carbon source for MO production, after hydrolysis to fermentable sugars. Agricultural residues contain around 30% cellulose, while hardwood such as poplar, pinewood, and spruce reach 40% and more [20]. However, the digestibility of carbohydrates in lignocellulosic biomass by cellulases is low due

to its chemical composition (content of lignin, hemicellulose and acetyl groups bound to hemicellulose) and physical characteristics (accessible surface area, related to porosity, crystallinity and degree of cellulose polymerisation, the physical distribution of lignin in the biomass matrix and biomass particle size) [21].

The NREL report [5] evaluates the composition in terms of carbohydrate components (cellulose and hemicellulose), lignin, acetate and ash of a **blended feedstock** consisting of **agro-residues** such as multi-pass corn stover, single-pass corn stover, and switch grass (Table 2 below). The assumed moisture content is 20% and the non-structural component fractions obtained from the compositional analysis were combined under "extractives", primarily consisting of sugars, sugar alcohols, and organic acids, as well as of some non-structural inorganics [22].

Table 2: Typical agro-residues, lignocellulosic feedstock composition [5]

Component	Composition (dry wt.)
Glucan	35.1%
Xylan	19.5%
Arabinan	2.4%
Galactan	1.4%
Mannan	0.6%
Total structural carbohydrate	59.0%
Sucrose	0.8%
Total structural carbohydrate + sucrose	59.8%
Lignin	15.8%
Extractives	14.7%
Ash	4.9%
Protein	3.1%
Acetate	1.8%

3.2 Production process

The bioconversion of lignocellulose to microbial lipids includes following steps [5], [10], [20], [23]:

1. **Pre-treatment of lignocellulosic biomass:** This step allows to reduce biomass particles size, thus decreasing the degree of polymerisation and increasing surface area and porosity of biomass; as a result, the exposure to reagents is improved. Other processes such as chemical, physicochemical and biological could then be applied.
2. **Hydrolysis of structural carbohydrates to fermentable sugars:** During enzymatic hydrolysis of lignocellulose, cellulases and hemicellulases enzymes are used to convert, respectively, cellulose and hemicelluloses into glucose and a mixture of pentoses and hexoses.
3. **Microbial production of lipids:** The glucose and other hydrolysed sugars in the previous step are then conditioned to remove insoluble solids such as lignin, which are partially concentrated and converted into hydrocarbon molecules with bioconversion processes.
4. **Isolation and purification of the product:** Lipid recovery from fermentation broth involves microbial cells harvesting from the broth, drying cell biomass or forcing cell disruption, and successive lipid extraction. Centrifugation, filtration, and coagulation or flocculation are among the most commonly used cell-harvesting methods.

Below, these steps will be evaluated separately, highlighting the main processes and technologies used.

3.2.1 Lignocellulosic biomass pre-treatment

The first pre-treatment step consists of a reduction of particles size, usually done through a mechanical process that involves compression friction or shearing intending to grind from coarse (from cm to 500 μm) to fine (<100 μm) levels [10]. These processes have variable energy requirement that must be evaluated and are reported to be **affected** by the moisture content and chemical composition of the substrate [24], [25].

The main **chemical pre-treatment** processes are acid pre-treatment, alkaline pre-treatment and sequential acid-alkaline pre-treatment.

Acid pre-treatments effectively break the lignocellulosic matrix by solubilising more than 90% w/w of the hemicelluloses, reducing some of the cellulose, and removing part of the lignin. The structural characteristics of softwood lignin makes the acid pre-treatment not suitable for removal, while it works correctly with hardwoods and agricultural residues feedstocks [26].

The acids used can be either inorganic or organic [27], with sulphuric acid most commonly used. Acid pre-treatment could use either concentrated or diluted acid: the former has the advantage to obtain more than 50% of assimilable sugars at low temperature (<100 ◦C), thus with low energy costs, at the price of corrosion resistant equipment and hazardous reagents. The latter, instead, is less aggressive, more environmentally friendly, and less costly, given the lower acid

demand. The disadvantage here is the need for higher temperatures [28]. Both processes may need subsequent activities such as neutralisation, detoxification (related to by-products of sugar degradation) and the chemical recovery process [4].

Alkaline pre-treatment proves effective in extracting lignin and solubilising a part of the hemicelluloses, while causing negligible degradation of the cellulose. The most used bases are sodium, potassium, calcium hydroxide and ammonium hydroxide [28]. In general, alkaline pre-treatment is more effective on hardwoods and agricultural residues with low lignin content than on softwoods with high lignin content. It is considered of lower cost when compared to other pre-treatments; the low yield in monomeric sugar is one of the main disadvantages together with salts formation, since they are difficult to remove.

The combination of both acid and alkaline pre-treatment processes involve hydrolysis by using a dilute acid, followed by solid extraction and washing and then treatment with dilute alkaline solution. The acidic stage allows the recovery of most hemicelluloses (usually >70% w/w), with the alkaline stage allowing the extraction of a high percentage of lignin (>80% w/w). Cellulose recovering rate sets above 75%, highly hydrolysable. This combined pre-treatment minimises the generation of by-products, at the cost of higher investments in equipment and longer processing time [28].

Steam explosion, also called auto hydrolysis, is the most widely used physicochemical pre-treatment method. It consists of treating the biomass at high pressure and at high temperature using water vapour for a short period of time and then quickly depressurising. This generates a disruption of the biomass with partial elimination of the lignin. In addition, the acidic condition causes acetic acid, which allows an auto hydrolysis of the hemicelluloses and a partial delignification. The steam explosion can also be used in two stages, first under milder conditions to recover hemicelluloses and then under more severe conditions to destabilise the cellulose and recover higher percentages of glucose. Its advantages are found in a lower environmental impact and a lower production of toxic compounds, when compared to acid and alkaline processes; on the other hand, it is less effective for softwoods [10].

Organosolv pre-treatment involves the application of an organic solvent to the lignocellulosic biomass in order to separate lignin in the liquid fraction and cellulose in high concentrations in the solid residue. It produces a solid phase, consisting mainly of cellulose and hemicelluloses, and causing the dissolution of lignin fragments [29]. It is carried out using different organic solvents (ethanol, methanol, acetic acid, formic acid, acetone, glycerol or phenol) with highly varying concentrations (1% to over 80% w/w) and is applied with or without catalysts (among which: sulphuric acid, magnesium chloride, or sodium hydroxide). It can be applied to hard and soft woods and the solvents can be easily recovered, causing little environmental effect [29]. The main disadvantages are related to the danger of ignition by concentrated solvents and the generation of various inhibitory residues that may inhibit enzymatic hydrolysis [30].

In a bio-refinery, Organosolv is reported as one of the best pre-treatment options due to very good quality of cellulose and lignin residual obtained [10].

3.2.2 Hydrolysis of structural carbohydrates to fermentable sugars

The structural polysaccharides (e.g., cellulose and hemicellulose) in the pre-treated lignocellulosic biomass are hydrolysed – in the subsequent saccharification step – into sugar monomers (e.g., glucose and xylose), that are used as carbon sources for microbial cultivation [4]. This can be done by using cellulolytic enzymes such as cellulases and hemicellulase, which could act synergistically, increasing cellulose and hemicellulose conversion rate into free sugars. Another possible conversion route is through a thermochemical process, at elevated temperature and in the presence of concentrate acid catalyst. Enzymatic hydrolysis is usually preferred, since the reaction is carried out under mild conditions (pH and temperature) and in a non-corrosive environment. The major drawbacks of enzymatic hydrolysis are longer process time, the higher price of the enzyme and possible inhibition by end products concentration [20].

Moreover, an effective enzymatic hydrolysis requires high accessibility of cellulose and xylan; thus, biomass pre-treatment becomes a critical step in the production of fermentable sugars [4].

Once the hydrolysis phase is complete, a solid-liquid separation step is required (i.e. using a vacuum filter press) to remove the insoluble fraction of the hydrolysate material, containing residual solids (primarily lignin), leaving only the soluble sugars. Another fraction of soluble sugars is recovered with an additional wash step of the solids fraction. Then, the washed lignin-rich solids fraction is ready for further use and valorisation. Enzymes are also removed during the solids separation step, thus making it impossible to have other hydrolysis activity that could occur downstream of the hydrolysis step [5].

Enzymatic hydrolysis may be inefficient due to some existing factors that negatively affect enzyme activity. Among them it can be found the high concentration of assimilable sugars in the media, the long residence time (that increases irreversible binding of enzymes with residual lignin - softwood biomass is more prone to that, due to the high residual lignin content [10]) and the presence of inhibitory compounds generated as by-products during chemical or physical pre-treatments [31], as illustrated in Figure 3.

Such by-products may also reduce the efficiency of the growth and metabolism of most microorganisms used in successive fermentation step. Furan aldehydes are among the inhibitory compounds derived from sugars, namely furfural and 5-hydroxymethylfurfural (5-HMF), respectively generated from the dehydration of pentoses and hexoses (e.g., glucose, mannose and galactose) in acidic media [4]. They start to affect enzymatic hydrolysis at 2.0 g/L and 4.0 g/L concentrations for furfural and 5-HMF, respectively. Other inhibitory compounds derived from sugars and affecting microbial metabolism are acetic acid, formic acid and levulinic acid. These acids cause a pH decrease that inhibits product formation and eventually

causes cell growth inhibition or death [28]. These compounds are mainly produced during acid, alkaline and organosolv pre-treatment [32], commonly within a range of 0.1–5 g/L, while being toxic to microorganisms at concentrations starting from 0.3 g/L [33].

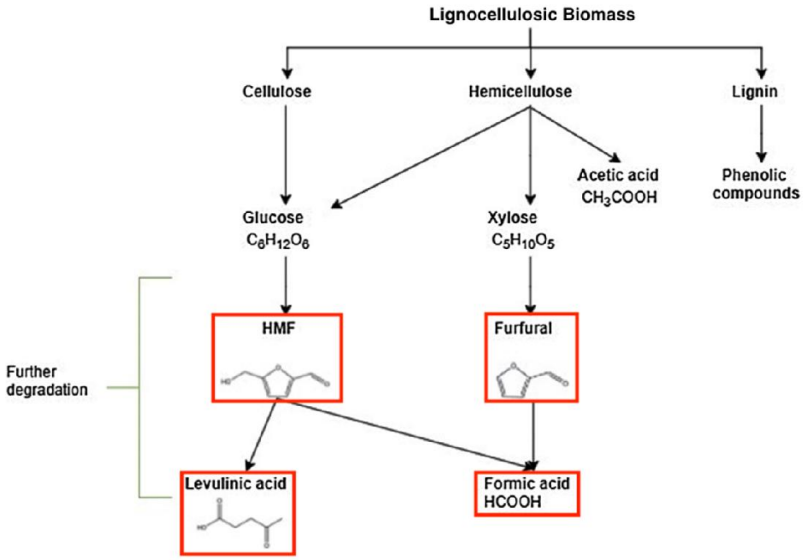

Figure 3: Depolymerisation process of lignocellulosic biomass polysaccharides into simpler sugars and related formation of degradation by-products (author's elaboration on [4]).

The effect of the pre-treatment on by-products has been widely studied in the case of ethanol-producing microorganisms. However, further investigation in oleaginous species is needed, since different yeasts have different responses to inhibitors [10]. One of the most common detoxification methods that can be used to remove these inhibitors is **overliming**: the pH of the hydrolysate is increased to 9–10 by the addition of $Ca(OH)_2$, and then decreased to 5.5 using H_2SO_4 [34]. This leads to the formation of precipitates that can be removed through filtration [4]. Thus, a biological detoxification method exists, but requires longer times [35].

3.2.3 Microbial production of lipids

Lipids or oils are synthesised in microorganisms through ***de novo*** or ***ex novo*** **processes** [36]. *De novo* lipid accumulation is an anabolic biochemical process, which occurs after nitrogen or, to a lesser extent, other essential nutrients (e.g., phosphorus) are depleted from the fermentation substrate [24]. This puts microorganisms under stress conditions and starts the process that end with the generation of cellular fatty acids that are then esterified with glycerol to form structural lipids (e.g., phospholipids, sphingolipids) and reserve lipids which are primarily TAG. ***Ex novo*** **lipid accumulation** occurs instead when fats or other hydrophobic compounds are used as the sole carbon source; in this case, lipids are synthesised regardless of the presence of nitrogen [24], [36].

In a ***de novo*** **process**, increasing the C/N ratio is one of the most common strategies used in industrial process to increase lipid synthesis. Molar C/N ratios from 20 to 100, even up to 368 with a carbon substrate range from 20 to 150 g/L in the case of glycerol and glucose were

published for *Y. lipolytica*, illustrating the strain dependence on the optimal C/N ratio and substrate concentration [37]. Also, a reduced oxygen supply (≤20% dissolved oxygen) is reported to promote lipid accumulation [38].

The microbial production of SCO can be either conducted through **submerged** (SmF) or **solid state fermentation** (SSF), also referred to as **Consolidate Bio-Process** (CBP).

SSF/CBP reproduces the natural microbiological processes used in food production, composting, and ensiling. It integrates in a single step cellulase production, carbohydrate hydrolysis and lipid production. A strain industrially viable for SSF/CBP has to efficiently secrete cellulases for hydrolysis of carbohydrates, besides providing high lipid productivity and titre. Suitable microorganism for the SSF/CBP could be isolated from nature or alternatively designed by genetic engineering [20].The advantages of SSF/CBP are higher productivity, higher product concentration, the possibility to use low-cost media, reduced energy, and costs related to waste water treatment. The disadvantages are i.a., difficulties in scale-up of the process, as well as in the control of process parameters (among which heat and mass transfer) and finally in the increased cost for product recovery [20], [39].

Production of lipids using lignocellulose biomass by SSF/CBP depends on the ability of oleaginous microorganisms to hydrolyse the carbohydrates from lignocellulosic biomass to fermentable sugars. Enhancement of cellulase activity is reported to be obtained by optimising the moisture content of the solid substrate, cultivation temperature, and adding complex substrates such as wheat bran and exogenous cellulase [40]. Another desirable characteristic of microorganisms is the ability is to grow on the insoluble substrate in the absence of free water. Several fungi strains were isolated with 20–35% (w/w) of accumulated lipids in cell dry weight.

The literature reports that lipid yields from **batch, fed-batch**, and **batch with repeated substrate replacement** processes. The latter strategy proved to be the most efficient one; repeated cycles of batch cultivations, each with replacement of 90% fermented substrate with a fresh one shortened the processing time [41]. Anyway, SSF bioprocess efficiency proved lower than in the SmF, with lipid yields at least two times lower, in the range of 0.02 – 0.09 $g/g_{LC\_biomass}$. Furthermore, metabolism products, formed in the layer of solid substrate during cultivation, were also reported to have negative and significant impact, since they inhibited the growth of microorganism and cellulase activity [20].

Submerged (SmF) culture is the dominant culture method reported in the literature for lipid production from lignocellulosic biomass: it is carried out using two different process configurations such as **Separate Hydrolysis and Lipid Production** (SHLP), **Simultaneous Saccharification and Lipid Production** (SSLP) (see Figure 4 below).

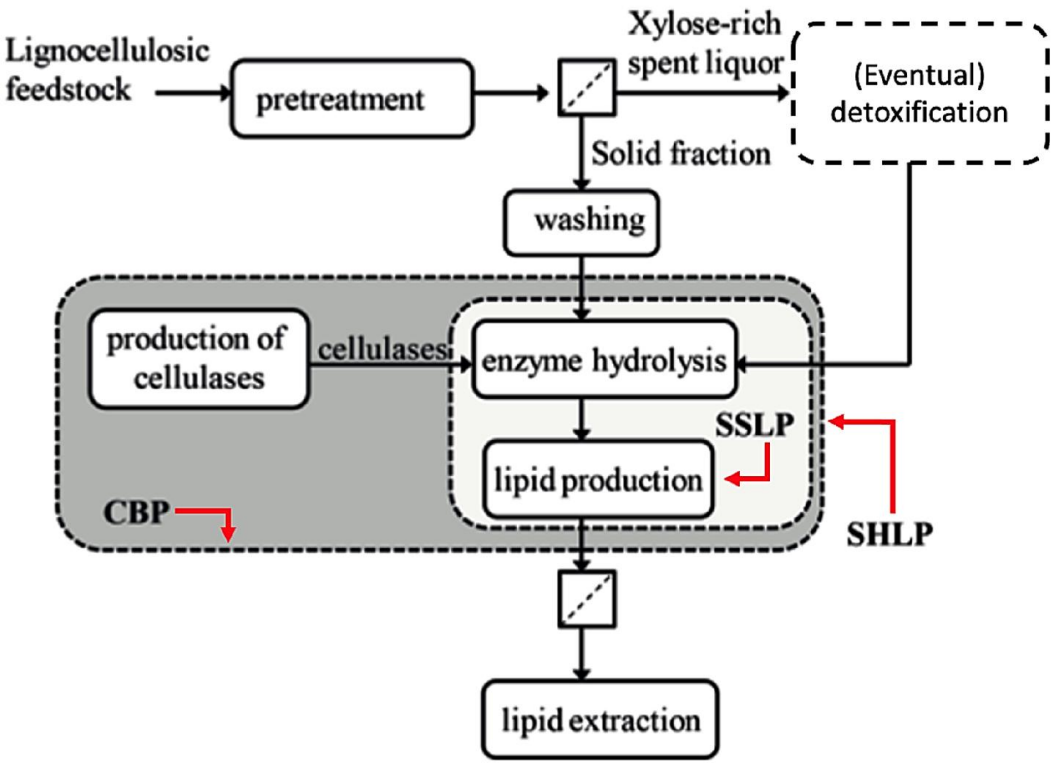

Figure 4: SHLP, SSLP and CBP production pathways for MO from lignocellulosic biomass (Author's elaboration from [20]).

In the former process enzymatic hydrolysis of lignocellulose and lipid production are performed as separate steps, thus in individual vessels; this allows to run the two steps under optimal conditions both for microorganism (pH = 4.8–6.0, T = 25–30°C) and cellulases (pH = 4.5–6,0; T = 50–60°C) [42]. However, inhibition of cellulase by accumulated glucose and cellobiose decreases fermentable sugars yield. As shown in Table 3 (in the Annex), in most of the batch SHLP under optimised culture conditions, lipid concentration and lipid productivity are reported below 20 g/L and 0.15 g/L h, respectively, while lipids yields are reported in the range 0.01 – 0.33 g/g_{sugar} (see the Annex for more details). On the other hand, the **SSLP** process integrates the two steps and simultaneously carries them out in one vessel. This has many advantages, such as decreasing the capital costs and making it possible for microorganism to assimilate sugars as soon as they are released by the hydrolysis, minimising the inhibition effect by the end-product. This in turn allows to enhance carbohydrate hydrolysis rate, shortening the processing time. The main SSLP disadvantage, when compared to SHLP is the necessity of running the process at a temperature favourable for the microbial growth such as 30–32°C, which is usually suboptimal for the cellulase hydrolysis [43]; as a result, the enzyme loading has to be increased in order to compensate lower activity at the process temperature. Lipid concentration and lipid productivity are reported below 17 g/L/h and 0.19 g/L/h, respectively, while lipids yields are reported in the range 0.11 – 0.21 $g/g_{L\text{-}C\_biomass}$.

Lignocellulosic hydrolysates mainly consist of multiple sugars substrates including hexoses (mainly glucose from cellulose of solid residue) and pentoses (primarily xylose from hemicellulose). For this reason, the simultaneous consumption of glucose and xylose by oleaginous strains is the key to efficient microbial oil production in the biochemical conversion process of lignocellulosic biomass [44]; the ability to consume minor sugars such as arabinose, mannose, or galactose is also desirable [45]. The simultaneous consumption of glucose and xylose is a challenging issue, since the recombinant strains often utilise xylose after the depletion of glucose [46]. In fact, yeast lacks xylose specific transporters, causing the sequential utilisation of xylose following the consumption of glucose.

Although *S. cerevisiae* has been engineered for xylose utilisation, industrially, no known xylose strain is reported to have reached utilisation levels as high as those of glucose [47].

3.2.4 Isolation and purification of the product

At the end of cultivation phase, oleaginous microorganisms are harvested from the cultivation medium. Typically, MO is accumulated intracellularly, thus it proves necessary to disrupt the microbial cell walls to obtain efficient oil extraction [48], [4]. Drying cell biomass after harvesting usually leads to higher lipid extraction yield, when compared with disrupted wet cell biomass [49]. On the other hand, drying is an energy-intensive process and cell harvest is less expensive when cell density is high in the fermentation broth.

Cells disruption methods could be categorised under mechanical, physical, chemical and enzymatic methods, while oil extraction methods could be divided into classical methods, pressurised liquid extraction and supercritical fluid extraction.

Looking into the **mechanical cell disruption category**, among the leading technologies, **bead milling** is simple and suitable for a wide range of microorganisms. Cells are disintegrated by compaction and shearing actions (and the resulting energy transfer) generated by the impact of grinding beads on biomass. Efficiency depends on parameters such as bead size and type, agitator velocity, flow rate, cell concentrations, bead loading, and type of microorganism (it is less suitable for bacteria, due to their small size).

Homogenisation process sees biomass forced under high pressure through an orifice. Disruption efficiency is dependent on applied pressure, number of passes and organisms and has been used successfully for yeast species [50], algae and bacteria [51]. In addition, several of the currently used equipment has been successfully adapted for cell disruption means from other commercial purposes, such as the homogenisation and size reduction of paint and milk.

Ultrasound cell disruption uses the effects of cavitation (growth and collapse of gas bubbles, results in shock waves creating liquid shear forces) on microorganisms. However, a general statement about the disruption suitability and efficiency of sonication is difficult, since the optimisation of parameters such as sonication time, cell density, power input, cycle number,

and operation mode (batch-wise or continuous) has to be performed on a microorganism-wise basis [52], [53].

Aside from the latter method, which presents difficulty in energy transmission to larger volumes, mechanical methods have great industrial potential and seem to be less dependent on species, thus are scalable up to industrial size [39]. On the other hand, a disadvantage of employing mechanical methods is the related heat generation that makes cooling necessary, in order to prevent damage to heat sensitive lipids [54].

Physical cell disruption methods include thermal treatment, decompression, osmotic shock, microwave-treatment, pulsed electrical fields, and drying (also freeze-drying). They usually require less energy, but application is often restricted by limitations in process economy and efficiency to small-scale processes. Thermal treatment is applied to microorganisms to disrupt cells, using pressure vessels. Reported temperature values range across 100 to 160°C, with pressure values of 1 to 6 bar. The following solvent extraction of MO is reported to reach almost 100% efficiency at around 140°C, with a solvent/cell slurry ratio of 1:1 (solvent is a mixture of Hexane and iPrOH with a 3:2 ratio) [55]. Decompression is achieved by mixing cell suspension with pressurised supercritical gas and subsequent release of the pressure. The gas which has entered the cells expands upon pressure release and causes cell disruption due to the high pressure. It is a gentle technique, minimising chemical and physical stresses and heat development; CO_2 proved to be highly efficient in disrupting wet yeast cells decompression, compared to Nitrogen, with the downside of influencing culture pH [56].

Microwaves are oscillating non-ionising electromagnetic waves with frequencies between 300 MHz and 300 GHz, generating heat in dielectric or polar material by electric field-induced polarisation and reorientation of molecules, which causes friction. The high potential of microwaves for cell disruption is based on their interaction with the abundant free water within cells, resulting in sudden, non-uniform, temperature rise especially where free water is available in more significant amounts. The volume expansion of the heated water increases intracellular pressure, causing spontaneous cell rupture [39].

Generally speaking, physical methods have the advantage of being gentler on the microorganisms, thus better preserving MO properties; on the other side, they appear to be less suitable for yeasts and have less potential for scalability [39], [56].

Chemical cell disruption methods (or permeabilisation) present different selectivity, efficiency and mode of action on cell wall components of various microorganisms depending on the chemical used, e.g., antibiotics, chelating agents, chaotropes, detergents, solvents, enzymes, alkalis, and acids. Therefore, MO used in food industry cannot be extracted with toxic solvents or should in the best case avoid any solvents to prevent solvent residues in food or contaminations with heavy metals. Moreover, harsh chemical conditions may also damage the oil [57].

Of interest is the fact that some chemical methods allow to combine cells disruption with i.e. lipid extraction, as in the case of the use of **solvents**. **Classical extraction methods** like **Bligh and Dyer** and **Folch** can be used for wet and dry biomass but uses large amounts of hazardous organic solvents, like chloroform and methanol. While solvent extraction is an efficient and mature technology, the solvent-recovery process is energy intensive. Instead of organic solvents, supercritical liquids (e.g., CO_2, N_2) have also been applied to lipid extractions to avoid energy intensive solvent recovery [23]. However, most microorganisms cell walls are reported to be impermeable to most solvents, therefore, a cell conditioning or pre-treatment has usually to be applied prior to solvent treatment to enhance solvent contact and extraction efficiency [58]. **Acid catalysed *in situ* transesterification** of either wet or dry biomass combines instead both cell disruption, lipid extraction, and transesterification to fatty acid methyl or ethyl esters (FAME or FAEE, respectively) for biodiesel production [59], [60]. It has been studied mostly on oleaginous microalgae, but it has been also applied to oleaginous yeast and fungi [59].

Enzymes specifically attack cell wall components leading to a release of intracellular products. **Cell disruption with lytic enzymes** possesses several advantages such as mild reaction conditions and therefore prevention of stresses, being environmental-friendly and safe for food applications. However, specific enzymatic cocktails are needed for various microorganisms, to grant effectiveness of cell disruption [39]. Enzymatic lysis for cell disruption has been extensively studied, especially for yeast and *E. coli* cells [61].

Chemical cell disruption methods are reported to be suitable for large scale applications, with the limit of possible high costs, especially for enzymatic methods.

Considering all limitations, the optimal **extraction method** should enable a rapid, reproducible, quantitative, cost-effective, and non-toxic removal of lipids under mild conditions to prevent oxidative damage to polyunsaturated fatty acids. Cell disruption by mechanical methods is often combined with solvent extraction. Contamination with chemicals of the product lipid is unlikely as long as no chemicals are used for pre-treatment.

4 Examples of application

Lipids are attractive feedstocks for production of renewable fuels due to their high carbon-to-heteroatom ratios. MO lipids are mainly in the form of Triacylglycerols (TAGs) and Free Fatty Acids (FFAs). Therefore, biodiesel from lipids is usually produced either via transesterification of TAGs or via hydro treatment (usually under the name of renewable diesel) [8].

Moreover, also food applications see MO as valuable feedstocks, when they contain essential FAs (EFAs) [14]. Important EFAs include gamma linoleic acid (GLA), eicosapentaenoic acid (EPA), arachidonic acid (ARA), and docosahexaenoic acid (DHA).

C3 commodity chemicals, currently produced from propylene in petrochemical processes, could have glycerol, produced as a by-product during lipid processing, as an alternative feedstock [10–12] [23].

Focus on these three specific applications, namely transport fuels, nutraceuticals and biochemicals are presented in the following sections, together with examples from existing processes and information on the underlying economics (whenever available).

4.1 Microbial oil as IBC for renewable transport fuels production

Even if the primary use of renewable lipids can be found in biodiesel and renewable diesel production, the conversion into advanced Renewable Jet Fuels (RJF) is gathering increasing interest, also due to the Sustainable Aviation Fuels mandate under evaluation for several EU Member States and the CORSIA scheme [62]. Besides fatty acid-derived alka(e)nes, iso-prenoids constitute the second class of compounds derived from cellular metabolism with promising prospects as biofuels. To date, the most advanced example of isoprenoid hydrocarbon production by far is β-farnesene, produced on an industrial scale by Amyris using a heavily engineered *S. cerevisiae* strain, fed with sugarcane-derived glucose, through the proprietary Biofene® technology [63]. Amyris and Total use farnesene to produce RJF under the Synthesised Iso-Paraffins from Hydroprocessed Fermented Sugars (HFS-SIP) production pathway that received ASTM certification in 2014, for a maximum 10% blend with fossil-based jet fuel.

4.1.1 Biodiesel from microbial lipids and lignocellulosic feedstock: the NREL study

The NREL report [5] describes in detail one potential conversion process to hydrocarbon products by way of biological conversion of lignocellulosic-derived sugars, providing a production cost for a cellulosic renewable diesel blend stock.

The process is divided into nine areas, each analysed in terms of technical solutions and economics, from feed handling to utilities. Here the focus will be set on Area 300, related to enzymatic hydrolysis, hydrolysate conditioning, and bioconversion and Area 500, encompassing product recovery and upgrading.

Area 300 is designed as a separate hydrolysis and fermentation (SHF) process, with the enzymatic hydrolysis portion that follows the same basic process schematic used in bioethanol plants: a hydrolysis process carried out at an elevated temperature, to provide higher enzyme activity and have faster reaction. Hydrolysis is split in two sequential reactions: a high-solids continuous flow reactor followed by batch hydrolysis in a stirred tank.

Enzymatic hydrolysis is initiated in a continuous, high-solids vertical tower reactor with the slurry - mixed with the cellulase enzyme - flowing down the reactor by gravity at 48°C; the residence time in the continuous reactor is 24 h and this is required as the feed material is not pumpable until the cellulose has been partially hydrolysed, with 20% or more solids in it. After this stage, the slurry is pumpable and is batched to one of six 3,800 m^3 (3,600 m^3 working volume) vessels, agitated and temperature controlled at 48°C using a pump-around loop with cooling water heat exchange, for another 60 h of enzymatic hydrolysis.

The enzyme loading was set to 26 mg/g, resulting in 89% cellulose conversion.

When the hydrolysis phase is completed, the hydrolysate material containing soluble sugars and insoluble residual solids (primarily lignin) is sent to a solid-liquid separation step where the insoluble fraction is removed.

Finally, the bioconversion of the released sugars occurs separately at lower temperature and in separate vessels. The NREL model utilises a fed-batch process (operating at 32°C) in the bioreactors that favours the inclusion of an initial sugar concentration step. In fact, before being sent to the biological conversion step, the sugar is further concentrated up to around 500 g/L, as reported in many other studies from literature on hydrocarbon biofuels production. Then, the bioreactors are filled to a 50% initial level (including inoculum), using the clarified dilute sugar material obtained after solids separation (representing 30% of the total hydrolysate liquor, with 13.8 wt% total sugar concentration) to commence bioconversion. Then, as the reaction proceeds, the remaining 70% of the hydrolysate liquor is delivered to the bioreactors after first being concentrated to the levels previously described (46.3 wt% concentration). This enables a high product titre. The maximum fill level is set at 80%, to ensure adequate space for vapour-liquid disengagement and to mitigate foaming issues. Thus, to calculate the number of required bioreactors, an average vessel working volume of 65% (650,000 L) is assumed over the duration of the fed-batch cycle and this leads to a number of 19 vessels. FFA productivity is expected for 1.3 g/L/h, with a bioconversion residence time of around 69 h.

In aerobic processes, oxygen is a nutrient that is used by microorganisms. Economically scaling up mass oxygen transfer to a commercial level sufficient for biofuels applications becomes a challenge. The ability to maintain effective gas-liquid mass transfer (i.e., sufficiently high volumetric oxygen transfer rates) will ultimately limit the size at which microbial fuel production can be operated. Moreover, this section's agitation and aeration power is expected to increase an order of magnitude compared to a bioethanol fermentation section, from 2.6 MW to around 20 MW. Figure 5 provides a flow diagram of the process.

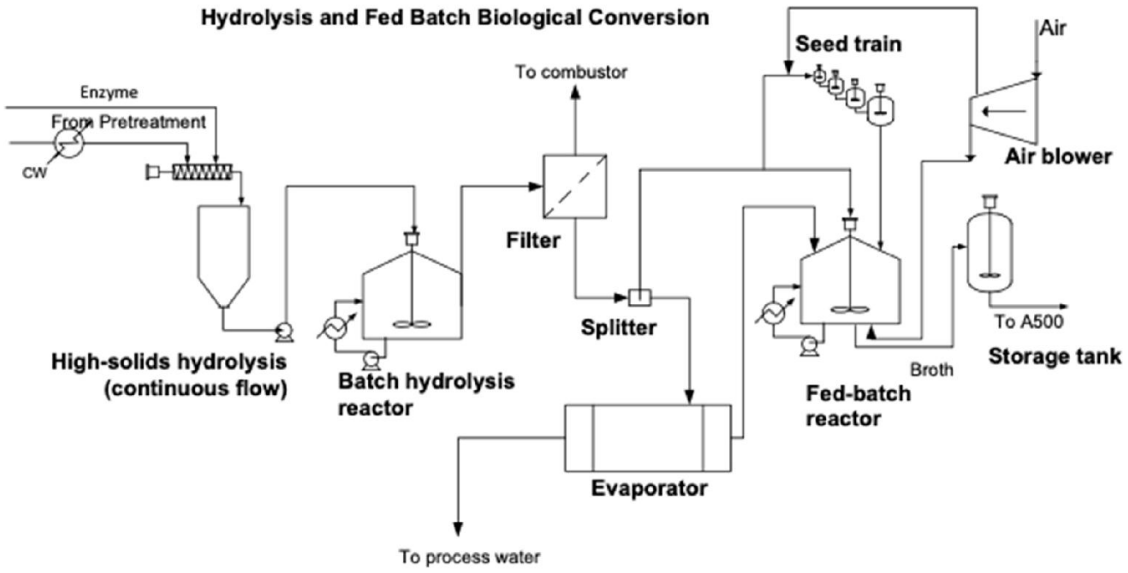

Figure 5: Flow diagram of the sections enzymatic hydrolysis, hydrolysate conditioning, and bioconversion process

Area 500 separates the bioreactor broth from Area 300 (containing approximately 9 wt% FFA) into a hydrocarbon (FFA) phase and an aqueous phase containing water, soluble solids (including unconverted sugars), and organism biomass. Aside from the bioconversion organism, fractions of the suspended solids of the broth are small as the lignin and other insoluble solids were already removed upstream. As reported in Figure 6, a set of 4 biodiesel-type oil water separation decanter carries out the primary concentration of the hydrocarbon phase, with a 1 h residence time estimated to be adequate for a 97% product recovery. It is followed by a subsequent centrifugation step in a disk stack centrifuge for a final concentration of more than 99%. Following FFA product concentration, the material is upgraded in a hydro-treater, with mild temperature and pressure conditions at 350°C and 35 atm., but with a high hydrogen feed ratio.

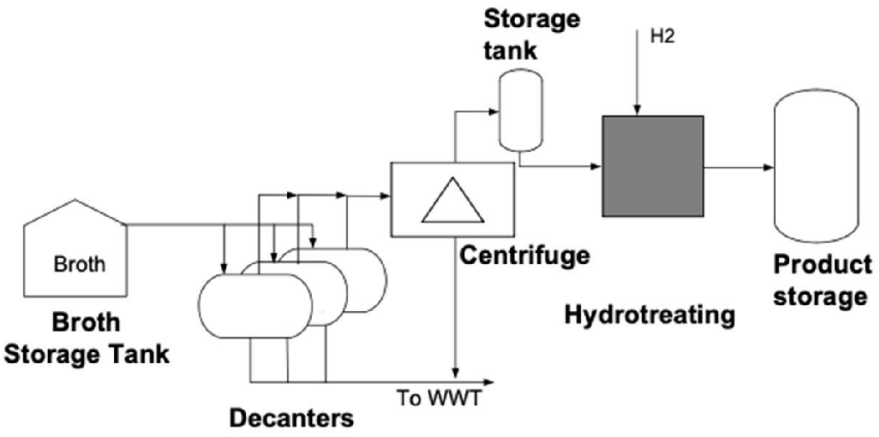

Figure 6: Flow diagram of sections product recovery and upgrading.

4.1.2 Renewable Jet Fuel from farnesene: the Amyris-Total process

Farnesene itself could be produced through either the mevalonic acid (MVA) pathway, the 1-deoxy-d-xylulose 5 phosphate (DXP) pathway, or anaerobically [64]. Amyris and Total process are based on fermentation of lignocellulosic sugars to isoprenoids, following the mevalonate pathway.

A pre-treatment step is necessary to separate cellulose and hemicellulose from lignin if the feedstock is raw biomass. Then the cellulose is transformed to sugars through hydrolysis process and, in the following step, sugars are converted through fermentation to farnesene. Such bioconversion of sugars to product occurs in a system of stirred-tank aerated vessels, using heavily engineered *S. cerevisiae* strain, developed by Amyris [65], and capable of converting both C5 and C6 sugars, with ammonium hydroxide and diammonium phosphate supplied as nutrients [66]. The very low solubility in water that farnesene has over short-chain alcohols comes as an advantage at this stage: the molecule forms a separate phase on the top of the fermentation broth, hence facilitating subsequent recovery and purification [67]. Moreover, centrifugation can be used to separate these compounds from the fermentation broth, resulting in considerable energy savings, compared with distillation [68]. A two-stage centrifugation process is used together with a de-emulsification process, with a reported 95% recovery of farnesene and 97% purity [64]. In the first stage, the yeast biomass and a large portion of the aqueous phase are removed. Before further centrifugation, the pH and salt concentration are adjusted to disrupt the emulsion created by the presence of extracellular material [66]. Finally, the olefin is mildly hydrotreated to the corresponding iso-paraffin called farnesane, by introducing 4% of its mass in H_2 [67]. Figure 7 describes farnesane production process, using sugarcane as feedstock, also highlighting the synergies between the various plant sections.

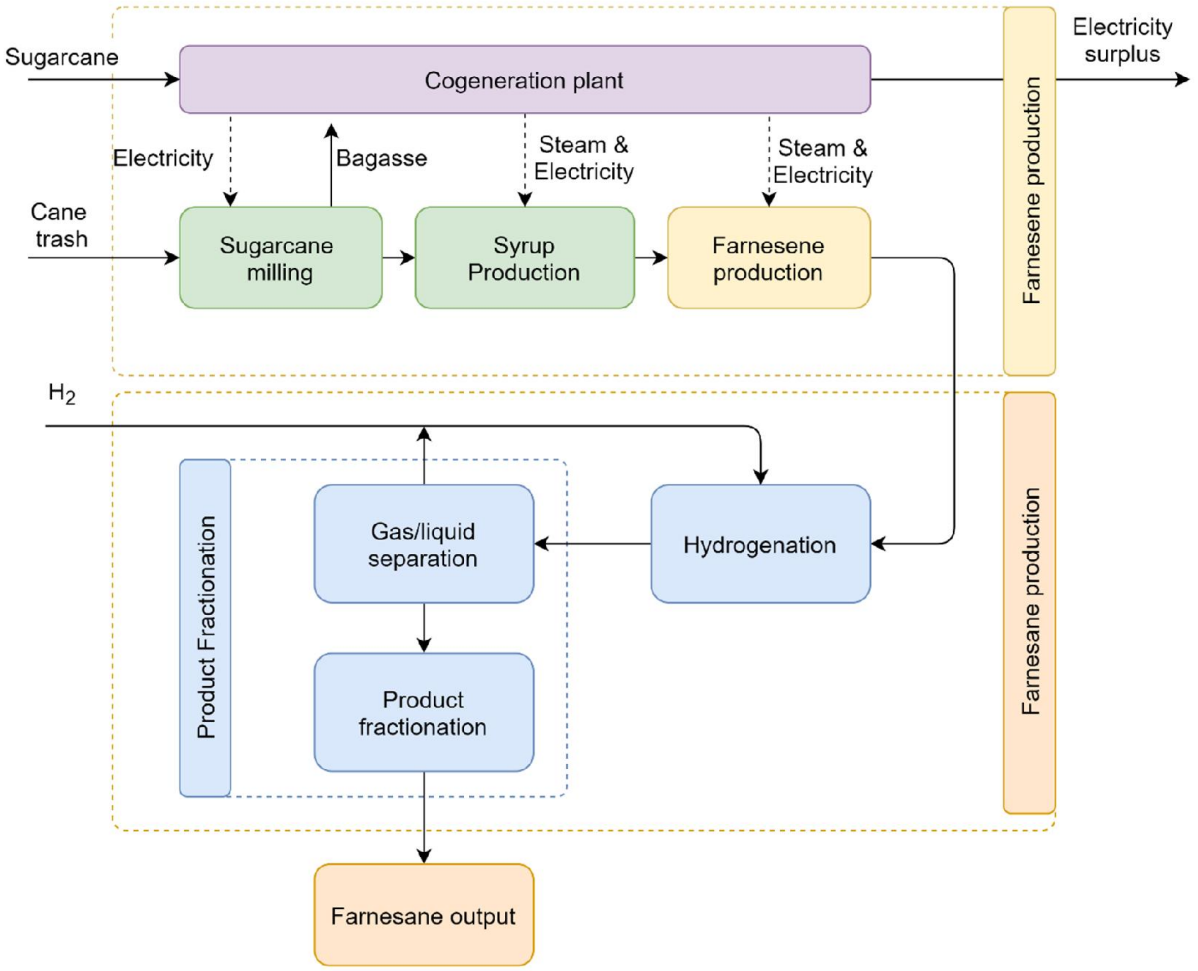

Figure 7: The HFS-SIP production process, broken down in farnesene and farnesane production steps.

Correspondingly farnesene yields are reported at 16.8 g farnesene/100 g of sugar, with an average productivity of 16.9 g/L/d. The theoretical mass yields of sesquiterpenes compounds, among which is farnesene, are approximately 30–45% lower than ethanol, with metabolic mass yields ranging across 25% and 35%, compared to 51%. Their energy densities are significantly higher than ethanol and as a result, the enthalpy of combustion yields for these diesel compounds approach 90%, against ethanol 97%. The farnesene made from the mevalonate pathway is an exception, with a lower yield of 75%, resulting from a 4% to 5% lower mass yield [62]. Several reports point out performance can achieve about 50-65% of the theoretical value; Amyris reported in 2010 a 17 wt% farnesene yields from glucose [69].

HFS-SIP development is being led by a joint venture between Amyris and Total which was restructured in 2015, with Total claiming a 75% stake in the partnership.

Their first commercial plant in Brota, Brazil, has been operational since December 2012 and has the capacity to produce up to 50 ML of farnesene per annum (38.6 kt/y), with six reactors

with 200 kL capacity each [67]. The facility has been certified by the Roundtable on Sustainable Biomaterials.

Total and Amyris' Biofene jet fuel has reached the following milestones, thus is considered to have a FRL[1] of 7, moving towards 8, since:

- It has achieved ASTM certification.
- A fuel purchase memorandum of understanding has been signed with an airline.
- A first commercial plant capable of producing jet fuel is operational.

A two-year programme between Amyris and Cathay Pacific began in May 2016, in which its 10% blended sugarcane-derived fuel would be used on all Airbus A350 delivery flights from Toulouse to Hong Kong. Airbus announced that such a programme would continue with A350-1000 delivery flights, which commenced in June 2018 [70].

LS9 is another company that worked on commercialising bio-jet fuel, with a pilot plant in 2008, but since being purchased by REG Life Sciences the company has changed focus to biodiesel and biochemicals. Other sugar-to-jet pathways have yet to be commercialised, including isoprene. The publicly available literature on HFS-SIP fuels produced by other companies is insufficient to assess their FRLs, but since none are pursuing ASTM certification, FRL 5 is assumed to be a maximum [71], [72].

4.1.3 Economics of renewable transport fuels production from Microbial Oil

Quite different results stem from the literature review carried out in this work: several reports describe highly unfavourable scenarios, with MO-based fuels minimum selling prices at least three to four times higher than those of current vegetable oils-based biodiesel (or fossil jet fuel, as with the case of farnesene). Some others, instead, report quite competitive prices; a possible explanation for such wide-ranging results could either be searched in the projected low-cost of biomass feedstock or in the scaled-up dimensions of the process considered in the analysis.

Koutinas et al. [15] studied the use of *R. toruloides* for the production of microbial oil and biodiesel from glucose, assuming a lipid yield of 0.23 g/g, and obtained an estimated production cost of USD 5,500/ton oil and USD 5,900/ton biodiesel. This result clearly proves to be not viable in a market with i.e. 2020 price levels, thus with vegetable oil prices around USD 800–900/ton and biodiesel at around USD 1,220/ton. If microbe meal coproduct is considered to be available for sale, with a price of around USD 400–800/ton, the economics would be slightly more favourable (USD 5,000/ton oil), but still hardly if not viable.

Soccol et al. [17] provide an estimation of biodiesel production cost from MO, split between raw materials and energy consumption. This analysis was conducted considering 1000 L of the

[1] The Fuel Readiness Levels (FRL) scale has been developed under the CAAFI (Commercial Aviation Alternative Fuels Initiative). It is a special TRL scale for fuel development; R&D phases are described by FRL ranging between 1-5, Certification phases by FRL 6-7, and Business & Economics phases byFRL 8-9. Further information can be found on https://www.caafi.org/information/pdf/FRL_CAAFI_Jan_2010_V16.pdf

fed-batch working volume, and a lipid productivity of 0.44 g/L/h, obtained using a low-cost medium composed by sugarcane juice and urea. The total cost of the culture medium was estimated to be USD 0.26/L of MO. Energy costs, including electrical energy and steam consumption for heating and cooling at all MO production process steps were estimated at USD 0.50/L. Thus, the final cost of microbial biodiesel was then estimated as USD 0.76/L, or, in other terms, around USD 900/ton.

Davis et al. [5] reports a projected Minimum Fuel Selling Price (MFSP) for renewable diesel from MO of around USD 1,800/ton, after the detailed process techno-economic analysis partially reported in section 4.1.1.

HFS-SIP is currently reported as one of the most expensive RJF production pathway because of the very high operational costs due to necessary processing steps [73] and the low yields: for the production of 1kg of farnesane, at least 5 kg of sugar is needed [67]. Klein-Marcuschamer et al. [66] reported that most of the facility-dependent cost is related to hydrocracking, accounting for around 85% of total, with fermentation equipment accounting approximately 10%. De Jong et al. [74] reported that a farnesane MFSP is obtained at USD 2450/ton. Modelling the process to use less expensive hydrogenation processes, lowers the MFSP to USD 1970/t. Finally, Bauen et al. [71] indicated projections for HFS-SIP costs to remain high at above € 4000 per ton.

4.2 Microbial oil use for nutraceuticals production

FAs differ by the length of the aliphatic chain, the degree of unsaturation, the location, and the conformation of double bonds. In general, the FAs are classified as:

- Saturated fatty acids, such as palmitic and stearic
- Mono-Unsaturated Fatty Acids (MUFAs) such as palmitoleic and oleic
- Poly-Unsaturated Fatty Acids (PUFAs) that, in turn, can be classified in several families such as:
    - Omega-3 (ω-3): includes ALA (C18:3 n-3), Eicosapentaenoic Acid (EPA) (C20:5 n-3), and Docosahexaenoic Acid (DHA) (C22:6 n-3)
    - Omega-6 (ω-6): gathers linoleic acid (LA) (C18:2 n-6), Gamma-Linolenic Acid (GLA) (C18:3 n-6), Arachidonic Acid (ARA) (C20:4 n-6), and conjugated linoleic acid (CLA).

PUFAs of the ω-3 and ω-6 families are essential for maintaining many functions in humans; mammals lack the ability to synthesise LA and ALA, thus they must be supplied by the diet from different food sources.

LA is practically found in all foods and is the predominant PUFA in land-based meats, dairy, vegetables, vegetable oils, cereals, fruits, nuts, legumes, seeds, and breads. GLA can also be found in some plant oils such as evening primrose and borage oils. DHA and ARA are found in mother's milk which provides their requirements for neural development and visual acuity to

newborns. However, since DHA and ARA are absent from cow's milk, these PUFAs should be added to the diet of babies to ensure a normal development, when it is used in place of mother's milk.

Fish oil is considered to be the best source of PUFAs, but its inclusion into infant milk formulas is not recommended, due to the presence of environmental pollutants.

Therefore, oleaginous microorganisms can provide an alternative and economically feasible source of PUFAs, provided that most of the PUFAs occur in TGAs which is the preferred form to take lipids within the diet.

As mentioned above, strictly, EPA and DHA are nonessential ω-3 FAs as the human body can convert essential ALA into EPA and DHA. However, this conversion is not efficient enough by humans to meet the EPA and DHA demand to impart beneficial health effects; thus, it is expected to obtain these fatty acids from dietary sources.

DSM and Nissui (in partnership with Suntory) are the most significant industrial producers of an ARA-rich oil, produced by submerged fermentation of the filamentous, zygomycete fungus *Mortierella alpina*. An agitated, fed-batch fermentation can produce more than 50 g/L of dry cell mass in 5-7 days. Then cells are harvested, dried, and the oil is extracted from the biomass and refined, using techniques similar to those used for extraction of oils from oilseed crops.

While there is no plant or microorganism that produces EPA as a single, dominant PUFA, DuPont at Wilmington, DE, decided to transform the oleaginous microorganism *Yarrowia lipolytica* to produce EPA via genetic engineering, since at the time it was the only oleaginous organism whose genome had been sequenced. The final recombinant strain was able to produce a lipid content in the cells of about 30% (w/w) with EPA accounting for 56% of the total fatty acids. However, the extracted EPA sales, as nutraceutical, proved disappointing, thus the entire EPA-rich yeast biomass was used as farmed salmon feeding, to significantly decrease the amount of fish oil and fish meal that needed to be fed to the salmon [2].

4.3 Upgrade of microbial oil to biochemicals

Fatty alcohols are used as feedstocks for the synthesis of antifoaming agents, cosmetics, detergents, pharmaceutical, surfactants and toiletries within the oleochemical industry. In the same industry, fatty amines present several applications, including the production of floatation, anticaking and water-repellent agents, corrosion inhibitors, lubricants and fuel additives. MO free fatty acids can be converted into various oleochemicals through a chemical process, i.e., through ozonolysis, monounsaturated free fatty acids can be converted into dicarboxylic acid, the intermediate for the formation of polyester and polyamide. Moreover, saturated free fatty acids can produce linear ω-unsaturated free fatty acids through steam cracking; then, the unsaturated fatty acids could further be used with alkanes for polyolefins synthesis. PUFA-rich oil can be utilized for the synthesis of epoxidised free fatty acid, which can be further used as UV-curable coatings and PVC stabilizers [4], [75].

5 Utilisation of microbial oil co-products

Coproduct valorisation in a lignocellulosic MO bio-refinery is important, to try offsetting the processing cost of biofuels. Lignin and microbe cake after lipid processing are prominent coproducts. Usually, lignin is burned, in order to supply energy for the SCO bio-refinery. However, there is scope for lignin to be valorised into fuels and chemicals, to generate additional revenue for the biorefinery [76]. Moreover, while a portion of the microbes is recycled after fermentation, the remaining microbes could be sold as animal feed that could easily displace the traditional soy meal or other oil cakes used for feeding animals. The microbe cake could also be further processed to produce amino acids or peptides, which have wide applications (e.g., biomaterials, bioplastic, biofoam) [77],[23].

5.1 Lignin co-product valorisation

Lignin is usually reported as the most abundant output product in a lignocellulosic bio-refinery, on a mass basis. Thus, cost-effective lignin integration and valorisation strategies offer significant opportunity for enhancing bio-refinery operations.

The heterogeneous nature of lignin (see Figure 8 below) defines the two primary challenges associated with its utilisation in the context of bio-refining and MO production:

- Separation of lignin from biomass without negatively impacting carbohydrate yields
- Conversion of lignin from a heterogeneous feedstock to a product of substantial purity, quality, and/or quantity

A co-design approach seems to be needed for a proper carbohydrate and lignin utilisation, accounting also for economic viability improvement of the hydrocarbon production process [5].

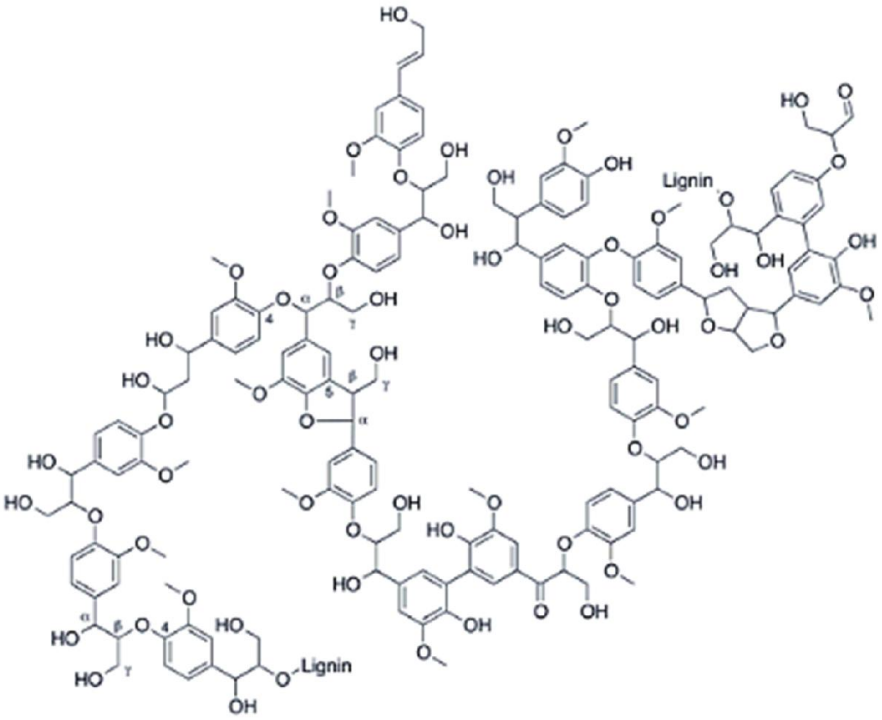

Figure 8: Intact lignin co-polymer.

Opportunities that arise from the possible use of lignin fit into three categories [78]:

- Power, fuel and syngas, where lignin is used purely as a carbon source and aggressive means are employed to break down its polymeric structure (generally near-term opportunities)
- Macromolecules, where the advantage of the macromolecular structure imparted by nature is retained in high-molecular weight applications (generally medium-term opportunities)
- Aromatics and miscellaneous monomers, where technologies are employed that would break up lignin's macromolecular structure but maintain the aromatic nature of the building block molecules (long-term opportunities)

The main products targeted to date are reported to include diacids (e.g., muconic acid, adipic acid, terephthalic acid, and itaconic acid), medium-chain alcohols, polyhydroxyalkanoates (PHAs), aromatics, hydrogen, and low molecular weight lignins for resin applications. The multiple end products considered reflect the complexity of the starting material and its potential value in many applications.

A broad range of conversion strategies and product targets are evaluated, with both physicochemical and biological pathways represented, including electrochemical approaches, improved and low-cost oxidation catalysts and engineered microorganisms with ability to funnel useable lignin monomers to specific chemicals [79].

One of the most rewarding possibilities but also a chief challenge in lignin application lies within high-value chemicals, such as benzene, toluene, xylene (BTX), and phenols. The annual demand of BTX exceeds 100 million tons, and the average price for BTX is around USD 1200/ton. Phenols are another kind of important platform chemical in industry, which are of particular interest that can be produced from lignin. Current phenol production volumes amount to 8 million tons per year. Phenol market value is around USD 1500 per ton. It is also very promising to convert lignin to value-added materials, including carbon fibre, activated carbon, and composite materials [80], [81].

6 Summary and conclusions

Microbial Oil production processes are still at the early stages of development, with a Technology Readiness Level (TRL) currently ranging between 4 and 5. However, MO has a huge potential as a substitute for vegetable oils (such as palm oil feedstock) and food-related lipid feedstocks, i.e., for commercial Hydrotreated Vegetable Oil (HVO) biorefineries. Moreover, MO benefits from the possibility of using several already developed technologies in the upstream phases of pre-processing and enzymatic hydrolysis, inherited from the bioethanol production processes and other well-developed industrial processes.

As is the case for most of the low-TRL technologies, MO profit margins are still too low to stimulate any current commercial interest [2], being still in a lab-to-pilot scale situation.

Help in stimulating further research could arrive from the chemical, pharmaceutical, and nutraceutical markets that could benefit from the use of MO feedstock in their processes, which pay, on average, much higher prices than the fuels market and require relatively lower feedstock volumes.

Another pathway for MO bio-refinery economics improvement comes from the upgrading of lignin and microbe meal to high-value products; however, further research and investments are still needed to reach this goal.

Annex – Reported Microbial Oil yields for oleaginous microorganisms

Table 3: Reported Microbial Oil yields for oleaginous microorganisms

Yeasts	Biomass	Pretreatment	System	X (g/L)	L% (w/w)	L (g/g)	Ref.
Candida albicans	Sugarcane bagasse	SE	Batch	6.2	31%	0.1	[10]
	Rice husk	SE	Batch	8.3	22%	0.09	
Candida tropicalis	Palm oil biomass	Alk	Batch (shake-flask)	-	-	0.14	[4]
	Palm oil biomass	Alk	Batch (shake-flask)	-	-	0.08	
	Palm oil biomass	Alk	Batch (shake-flask)	-	-	0.07	
Chlorella pyrenoidosa	Rice straw	Trifluoroacetate	Batch (flask)	-	56.3%	0.12	[23]
Cryptococcus curvatus	Wheat straw	DA	Batch (flask)	-	33.5%	0.20	
	Corn stover	Ionic liquid	Batch (flask)	-	43.4%	0.23	
	Pelletized wood	Pyrolysis	Batch (flask)	-	31.9%	0.11	
Cryptococcus sp.	Corn cob	DA	Batch (flask)	13	60%	0.13	[10]
Lipomyces kononenkoae	Corn stover	DA	Batch (flask)	48	59%	0.22	
Lipomyces starkeyi	Wheat straw	DA	Batch (flask)		31.2%	0.16	[23]
Lipomyces tetrasporus	Corn stover	DA	Batch (flask)	54	53%	0.15	[10]
Meyerozgpra gulliermondii	Sugarcane bagasse	SE	Batch	6.1	38%	0.05	
	Rice husk	SE	Batch	6.5	37%	0.04	
Mortierella isabellina	Corn stover	DA	Batch (flask)	-	34.5%	0.15	[23]
	Corn stover	DA	Batch (flask)	-	24.8%	0.12	
	Switchgrass	DA	Batch (flask)	-	35.6%	0.15	
	Miscanthus	DA	Batch (flask)	-	32.2%	0.13	
	Giant reed	DA	Batch (flask)	-	21.2%	0.11	
	Wheat straw	DA	Batch (flask)	-	34%	0.17	
	Corn stover	Dilute alkali (NaOH)	Batch (flask)	-	29.5%	0.09	
	Corn stover	DA, alkali	Batch (flask)	-	37%	0.15	
Mucor circinelloides	Avicel	None	Batch (flask)	-	3.32%	0.06	
Pichia kudriavzevii	Sugarcane bagasse	SE	Batch	6.2	31%	0.1	[10]
	Sugarcane bagasse	SE	Batch	6.1	30%	0.04	
	Rice husk	SE	Batch	8.2	24%	0.1	
	Rice husk	SE	Batch	8.4	29%	0.04	
Pichia manshurica	Sugarcane bagasse	SE	Batch	8.2	24%	0.09	
	Rice husk	SE	Batch	6.5	20%	0.09	
Rhodococcus opacus	Kraft hardwood pulp	None	Batch (flask)	-	45.8%	0.18	[23]

Yeasts	Biomass	Pretreatment	System	X (g/L)	L% (w/w)	L (g/g)	Ref.
Rhodororula glutinis	Corncob	NA	Batch (Bior5L)	15	36%	0.13	[10]
	Corncob	NA	Fed batch (Bior5L)	75	47%	0.15	
	Wheat straw	DA	Batch (flask)	-	25.0%	0.12	
Rhodotorula graminis	Corn stover	DA	Batch (flask)	-	34%	0.08	
Rhodosporidium paludigenum	Corncob	Alk	Batch (Bior3L)	28	70%	0.21	
	Corncob	Alk	Fed batch (Bior3L)	-	70%	0.28	
Rhodosporidium toruloides	Wheat straw	DA	Batch (flask)	-	24.6%	0.08	[23]
	Corn stover	Ionic liquid	Batch (flask)	-	36.4%	0.10	
	Corn stover	DA	Batch (flask)	43	61%	0.19	[10]
	Corn stover	NA	Batch	36	59%	0.19	
	Corn stover	NA	Fed batch	54	59%	0.29	
Rhodotorula mucilaginosa	Sugarcane bagasse	SE	Batch	6.7	30%	0.1	[10]
	Rice husk	SE	Batch	8	24%	0.1	
	Palm oil biomass	Da	Batch (shake-flask)	-	-	0.064	[4]
	Palm oil biomass	Da	Batch (shake-flask)	-	-	0.093	
Trichosporon coremiiforme	Corncob	DA	Batch (flask)	-	37.8%	0.17	[23]
Trichosporon cutaneum	Corncob	DA	Batch (flask)	38	32%	0.1	[10]
	Corn stover	DA	Fed batch (Bior3L)	19	39%	0.15	
	Corn stover	DA	Batch (flask)		39.2%	0.15	[23]
	Corn stover	DA	Batch (flask)		23.5%	0.06	
Trichosporon dermatis	Corncob	DA	Batch (flask)	17	40%	0.16	[10]
	Corncob	DA	Batch	-	24%	0.1	
	Corncob	DA	Batch	-	45%	0.16	
	Corncob	DA	Batch (flask)	24	40%	0.16	
	Corncob	Alk	Batch	-	28%	0.1	
	Corncob	Alk	Batch	-	56%	0.19	
	Corncob	Organic solvents	Batch (flask)	-	40.1%	0.17	[23]
Trichosporon fermentans	Rice straw	DA	Batch (flask)	-	40.1%	0.1	
	Sugarcane bagasse	DA	Batch (flask)	-	N/A	0.14	
Yarrowia lipolytica	Wheat straw	DA	Batch (flask)	-	4.6%	0.01	
	Sugarcane bagasse	Hydrochloric acid hydrolysis	Batch (flask)	-	58.5%	0.33	
	Defatted rice bran	Sulfuric acid hydrolysis	Batch (flask)	-	48.0%	0.10	

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