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Review Paper

New trends in microbial lipid-based biorefinery for fermentative bioenergy production from lignocellulosic biomass

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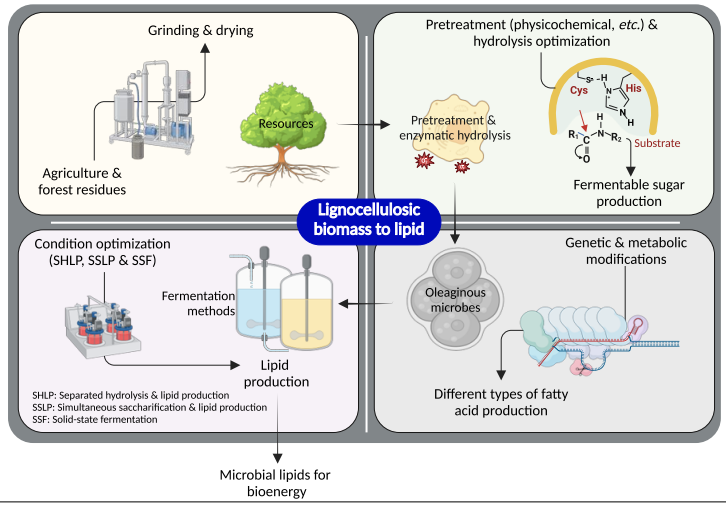
HIGHLIGHTS

- Chemical factors, pretreatment, and enzymatic hydrolysis in lipid synthesis are studied.
- The cellular and molecular mechanisms of intracellular lipid metabolism are analyzed.
- Novel fermentation strategies for increased lipid production are scrutinized.
- Genetic and metabolic engineering for increased lipid biosynthesis is analyzed.
- The routes of converting microbial lipids into novel fermentative products are discussed.

GRAPHICAL ABSTRACT



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ABSTRACT

Using oleaginous microbial lipid-based biorefinery from lignocellulosic biomass (LCB) to produce fermentative bioenergy (*i.e.*, biodiesel) represents an innovative second-generation fuel production technology. These lipids are predominantly intracellular triglycerides that accumulate through the metabolism of sugars in fermentation following pretreatment and enzymatic hydrolysis of LCB. This review investigates the recent advances in the microbial lipid production from LCB, focusing on the factors influencing the lead microbial lipid producers, different pretreatment methods (*i.e.*, physical, chemical, biological, and combined pretreatment), enzymatic hydrolysis approaches, novel bioprocessing strategies (*i.e.*, microbes-specific and fermentation model specific), and engineering techniques of the oleaginous microbes (*i.e.*, genetic and metabolic alterations). The study demonstrates that oleaginous yeasts can synthesize significantly higher quantities of lipids when incorporated into the system, known as separated hydrolysis and lipid production, following various combined pretreatment methods. Interestingly, CRISPR is found to be the most suitable way of engineering microbes genetically and metabolically for increased lipid synthesis. The study also explores economically viable strategies for fermentative lipid production, addressing associated challenges, and outlines future directions, including comprehensive techno-economic and life cycle assessments. This review offers invaluable insights into microbial lipid production from LCB, highlighting the potential for significant technological and environmental enhancements through ongoing research and development efforts.

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Contents

Abbreviations

BC	Biomass concentration	SCOs	Single-cell oils
C/N	Carbon-to-nitrogen ratio	SHELP	Separated hydrolysis and enhanced lipid production
CL	Content of lipid / g of biomass	SHLP	Separated hydrolysis and lipid production
CRISPR	Clustered regularly interspaced short palindromic repeats	SLAPT	Spent liquor from acid pretreatment
CDW	Cellular dry weight	SS	Single-staged
DAH	Detoxified acid-hydrolysate	SSELP	Simultaneous saccharification and enhanced lipid production
DSLAPT	Detoxified spent liquor from acid pretreatment	SSF	Solid-state fermentation
FAs	Fatty acids	SSLP	Simultaneous saccharification and lipid production
FPU	Filter paper unit	TAGs	Triacylglycerols
GWP	global warming potential	TALEN	Transcription activator-like effector nuclease
G3P	Glycerol-3-phosphate	TCA	Tricarboxylic acid
LC	Lipid concentration	TE	Thioesterase
LCA	Life cycle assessment	TEA	Techno-economic assessment
LCB	Lignocellulosic biomass	TS	Two-staged
LP	Lipid productivity	VA	Variable Amount
LYS	Lipid yield / g of pretreated LCB	VFAs	Volatile fatty acids
MUFAs	Monounsaturated volatile fatty acids	LCB	Lignocellulosic biomass
PUFAs	Polyunsaturated volatile fatty acids	USLAPT	Undetoxified spent liquor from acid pretreatment
PHAs	polyhydroxyalkanoates	ZFN	Zinc finger nuclease
RNAi	RNA interface		

1. Introduction

Replacing fossil fuels with sustainable and environmentally friendly biofuels, such as biodiesel, is the first step in alleviating global warming and the environmental crisis. Industrial-scale biodiesel production using selective vegetable oils (*i.e.*, sunflower, soybean, palm, and rapeseed) has been globalized for many years (Bradley and Maga, 2019). However, increasing biofuel demand necessitates alternative feedstocks (Ji and Ledesma-Amaro, 2020). Fermentative production of microbial lipids offers a promising solution, as these intracellular lipids can replace traditional oils for biodiesel production. Oleaginous microorganisms, abundant in nature, can contribute to increased intracellular lipid synthesis (Mahmud et al., 2022). These lipids, including microbial triacylglycerols (TAGs), can be derived from lignocellulosic biomass (LCB) and offer versatility in lipid composition. The availability and type of feedstocks influence the microbial intracellular lipid accumulation rate, with LCB, rich in sugar components, being an ideal source (Di Fidio et al., 2020; Uthandi et al., 2022). This process confirms the feasibility of smart biorefinery practices. The fermentative conversion of LCB into carbon-neutral bioenergy through microbial lipid biosynthesis represents the second generation of carbon-neutral renewable energy (Shields-Menard et al., 2018).

The microbial biorefinery of lipids from LCB is expected to increase from 25% in 2017 to 85% by 2050. Consequently, the global market value of microbial single-cell organisms (SCOs) is expected to grow from 143 billion USD in 2023 to 340 billion USD by 2033, with an annual growth rate of around 9% (Kumar et al., 2019). In mitigating the ever-increasing

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global demand for renewable energy, a wide range of oleaginous microorganisms have been used as the lead lipid producers, accumulating 20 wt% of lipids inside their cells. Oleaginous microorganisms, encompassing yeasts, bacteria, and fungi, exhibit diverse fatty acids (FAs) synthesis capabilities, producing both long-chain and short-chain hydrocarbons, including saturated FAs (SFAs), monounsaturated FAs (MUFAs), and polyunsaturated FAs (Kumar et al., 2019). Large-scale lipid production centers on microorganisms that can accumulate lipids ranging from 20% to 80% of their dry weight (Annamalai et al., 2018). Notable strains with high lipid yields, evaluated based on total lipid content and profiles, include *Mortierella isabellina*, *Cryptococcus albidus*, and *Rhodococcus opacus*, the latter of which boasts the highest reported lipid content at 87% (Arous et al., 2019).

A group of factors has been identified in provoking the productivity of lipids in microbial lipid-based biorefinery, such as chemical factors (*i.e.*, C/N, N3/N2, C/S ratios, pH, and glycerol concentration) (Elfeky et al., 2019; Llamas et al., 2020). Besides, as reported previously, overexpression of fungal lipid synthesis can be manipulated using amino acid supplements (Dzurendova et al., 2020). Similarly, diversified pretreatment methods, such as physical (heating, ultrasonication, irradiation, and cold plasma) (Sidana and Yadav, 2022), chemical (acidic, alkaline, and organic solvents) (Rezania et al., 2020), biological (microbial and enzymatic) (Zabed et al., 2019), and combined approaches have been introduced for LCB fractionation for high-efficient enzymatic hydrolysis (Basak et al., 2023), which is the main precursor of yielding functional sugars as the substrates of microbial lipid metabolism (Deng et al., 2019). In the lipid-based biorefinery of LCB, innovative bioprocessing technologies have been introduced to regulate the yield of SCOs. The fermenter model and specific microbial strains are crucial in this regulation (Kumar et al., 2022). Furthermore, to attain maximum productivity of fermentative lipids, there is a strong emphasis on engineered microbes, which are often genetically and metabolically modified (Jagadevan et al., 2018).

In light of recent advancements, a thorough review of microbial lipid-based biorefinery for fermentative bioenergy production from LCB is essential. This review delves into various pretreatment and enzymatic hydrolysis methods applicable to diverse oleaginous microbes. It considers factors influencing LCB fractionation and explores microorganisms synthesizing SCOs. The study provides insights into lipid synthesis bioprocessing, emphasizing genetically and metabolically modified microbial strains. Additionally, it discusses current trends in converting microbial lipids into value-added products, addressing challenges and prospects. The exploration of numerous oleaginous microbes systematically refining LCB into SCOs establishes microbial lipid-based biorefinery as an advanced approach, ensuring green energy, a circular economy, and environmental sustainability. A comprehensive overview of the systematic and rational integration of significant parameters for sustainable SCO-based biorefinery systems is provided in **Table 1**. These parameters have been carefully considered based on the latest and most promising review papers in the field.

2. Lead microbial lipid producers from lignocellulosic biomass

The lipid biosynthesis potential of oleaginous and non-oleaginous microorganisms has been extensively reviewed for maximum utilization (Carsanba et al., 2018). These microbes can accumulate up to 20 wt% of lipids inside their cells. They have shown a remarkable capacity to produce many FAs, including long-chain hydro-carbonated (C36) and short-chain hydro-carbonated (C6), with high efficiency. Additionally, depending on the length of the hydrocarbon chain and the number of double bonds, these microbes can synthesize a range of FAs, SFAs, MUFAs, and PUFAs (Saini et al., 2021).

Various microorganisms, including yeasts, bacteria, and fungi, exhibit the potential of high-yield lipid producers (Arora et al., 2019; Jones et al., 2019). The oleaginous microorganisms demonstrate varying capacities for intracellular lipid accumulation based on the metabolic assimilation of consumed substrate types. Notwithstanding the process of biosynthesis and the substantial accumulation of various lipid types in the extensively reviewed industrial microbes, there is a remarkable multitude of oleaginous microorganisms for which the precise percentage of diversified lipid productivity remains undefined, reviewed in this review (**Table 2**). Scientifically, oleaginous microorganisms that can accumulate lipids up to 20–80% of their total dry weight are only considered oleaginous for large-scale production (Annamalai et al., 2018).

The microbial strains with the highest lipid yield are evaluated based on their total lipid content and lipid profiles, including the presence of myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3) (Guo et al., 2019; Patel et al., 2020). Several microorganisms have been identified as highly significant lipid producers based on their intracellular lipid accumulation during fermentation. These include *Mortierella isabellina* (43.9%), *Cryptococcus albidus* (60.1%), *Claviceps purpurea* (60%), *Rhizopus oryzae* (57.1%), *Rhizopus arrhizus* (57.2%), and *Trichosporon pullulans* (65%) (Mahajan et al., 2019). Interestingly, *Rhodococcus opacus* has the highest reported lipid content of 87% (Alves et al., 2020). For high-end applications, various microorganisms have been identified based on their lipid content and type, including *Rhizopus arrhizus*

Table 1.
Comparison between previously published review papers and current review in microbial lipid production from LCB.

Lead Lipid Producers	Factors Influencing Lipid Production	Lipid Accumulation Mechanisms	Microbial Strain Improvement	Synthetic And Metabolic Engineering	Novel Fermentation Strategies	Lipid Conversion Routes	Sustainability Analysis	Challenges & Future Perspectives	Reference
✗	✓	✗	✗	✗	✗	✓	✗	✓	Jin et al. (2015)
✓	✓	✗	✓	✗	✗	✗	✗	✗	Soccol et al. (2022)
✗	✓	✗	✓	✓	✗	✗	✗	✓	Saha and Mukhopadhyay (2021)
✗	✓	✗	✗	✗	✗	✗	✓	✓	Sun et al. (2023b)
✗	✓	✗	✗	✓	✗	✗	✗	✓	Mondal et al. (2023)
✗	✓	✓	✗	✗	✗	✗	✗	✓	Robles-Iglesias et al. (2023)
✗	✗	✓	✗	✓	✗	✗	✗	✓	Zhang et al. (2020)
✓	✗	√	✓	✓	✗	✗	✗	✗	Awad et al. (2020)
✗	✗	✓	✗	✗	✗	✗	✗	✓	Sundarsingh et al. (2024)
✓	✗	✗	✗	✗	✗	✗	✗	✓	Uthandi et al. (2022)
✗	✗	✗	✗	✗	✗	✗	✗	✗	Jin et al. (2015)
✓	✓	✓	✗	✗	✗	✗	✓	✓	Jiao et al. (2021)
✗	✗	✗	✗	✗	✗	✗	✓	✓	Shapiro et al. (2023)
✗	✓	✗	✗	✗	✓	✗	✗	✗	Yu et al. (2022)
✓	**✓**	**✓**	**✓**	**✓**	**✓**	**✓**	**✓**	**✓**	**Present Review**

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(12%) for linolenic acid, *Yarrowia lipolytica* (51%) for linoleic acid, *Cryptococcus albidus* (73%) for oleic acid, *Rodotorula glutinis* (66%) for stearic acid, *Mucor circinelloides* (38%) for palmitoleic acid, *Lipomyces lipofer* (37.2%) for palmitic acid, and *Lipomyces starkeyi* (34.1%) for myristic acid (Mahajan et al., 2019; Patel et al., 2020; Alves et al., 2021).

Regarding both qualitative and quantitative lipid production, yeasts and fungi outperform bacteria. Notably, certain fungal species, including *Rhodosporidium toruloides* (66.2%), *Rodotorula glutinis* (72.4%), and *Rhodococcus opacus* (87%), exhibit even higher lipid yields than oleaginous yeasts like *Lipomyces lipofer* (64.1%), as indicated in previous studies. Surprisingly, specific lipid concentrations, such as linolenic acid, myristic acid, and palmitoleic acid, remain undefined for many microorganisms, particularly oleaginous fungi (**Table 2**)

Table 2.
Outstanding lipid-producing microorganisms based on their total lipid content (%) and individual lipid profiles.

Microbial kingdom	Industrial microorganisms	Content of lipid (%)	Segmented lipid profiles							Reference
			Linolenic acid	Linoleic acid	Oleic acid	Stearic acid	Palmitoleic acid	Palmitic acid	Myristic acid	
			C18:3	C18:2	C18:1	C18:0	C16:1	C16:0	C14:0	
Bacteria	*Cryptococcus albidus*	60.1	ND*	12.2	73.0	3.2	1.0	12.1	ND	Alves et al. (2021)
Fungi	*Aspergillus terrus*	ND	0.1	23.1	42.3	12.5	0.4	17.5	0.2	Patel et al. (2020)
	Mortierella isabellina	53.1	2.3	9.5	52.4	5.6	1.4	24.5	0.9	
	Cunninghamella elegens	31.2	2.1	17.2	38.4	6.2	0.8	20.3	0.6	
	Mortierella vinacea	43.9	2.2	13.8	51.3	3.6	1.8	23.4	0.5	
	Rhizopus oryzae	57.1	5.5	19.0	40.5	11.8	0.8	16.2	0.2	
	Trichosporon coremiiforme	37.3	ND	6.1	38.9	26.3	ND	24.5	ND	
	Mucor circinelloides	25.0	ND	22.1	ND	5.0	38.0	10.3	15.0	Alves et al. (2021)
	Claviceps purpurea	60.0	ND	8.3	19.3	2.1	ND	23.2	ND	
	Rhizopus arrhizus	57.2	12	10.2	22.1	6.2	ND	18.3	ND	
	Rhodosporidium toruloides	66.2	ND	2.1	54.6	7.7	1.1	24.3	ND	
	Rodotorula glutinis	72.4	ND	ND	ND	66.1	3.3	3.2	18.2	Mahajan et al. (2019)
	Trichosporon pullulans	65.0	1.2	24	57.1	2.2	ND	15.1	ND	
	Rhodococcus opacus	87.0	ND	ND	6 – 74	3 – 19	ND	ND	ND	Alves et al. (2021)
	Rhodotorula graminis	54.1	2.89	17.2	42.2	7.16	ND	20.5	ND	Saha and Mukhopadhyay (2021)
Yeast	*Yarrowia lipolytica*	42.0	ND	51.0	28	1.1	6.2	11.3	ND	Mahajan et al. (2019)
	Lipomyces starkeyi	63.2	ND	3.3	51	5.0	ND	4.8	34.1	
	Lipomyces lipofer	64.1	ND	3.1	38	7.2	4.1	37.2	ND	

* ND: Not defined.

3. Factors influencing microbial lipid biosynthesis

3.1. Chemical factors

The regulation of microbial lipid biosynthesis is influenced by various chemical factors, which vary depending on the type of oleaginous microbes and fermentation models used (Robles-Iglesias et al., 2023). To achieve a high lipid yield in fermentative lipid production, optimization of various chemical factors, including the carbon-to-nitrogen ratio (C/N), carbon-to-sulfur molar ratio (C/S), nitrogen trioxide/dioxide ratio (N3/N2), nitrogen-to-phosphorus ratio (N/P), phosphate concentration, pH level, and the presence of proteinogenic substances, is necessary (Mahajan et al., 2019; Uthandi et al., 2022).

The C/N ratio directly impacts the final lipid yield of oleaginous microbes on an industrial scale. For example, *Rhodotorula toruloides* can accumulate up to 60% intracellular lipids when grown on xylose with a C/N ratio of 120 (Osorio-González et al., 2023). Yeast growth on glycerol is optimal within a C/N ratio range of 60-100. Maintaining a low ratio of N3/N2 (≤ 0.03) and a C/N ratio of 700:1 results in noteworthy fat coefficient values. An industrially convenient N3/N2 ratio of 0.05-0.2 is preferred for yeast lipid overproduction (Llamas et al., 2020). Increasing glycerol concentration can positively affect the total C16 and C18 concentrations in oleaginous yeasts like *Candida freyschussii* ATCC 18737 (Guo et al., 2019). Limiting sulfate concentration can stimulate yeast (*Rhodosporidium toruloides* Y4) mediated overproduction of lipids in the fermenter while maintaining an initial C/S at 46:750 increases lipid accumulation by approximately 58% (Elfeky et al., 2019).

Oleaginous eukaryotes, such as *Chlorella kessleri* UTEX 263, can achieve up to a 10-fold increase in lipid production when grown in dark conditions with limited nitrogen and phosphorus concentrations. Interestingly, limiting iron concentration has little effect on microalgal lipid accumulation (Capusoni et al., 2017). Adjusting the concentrations of proteinogenic amino acids in the growth medium can significantly stimulate lipid productivity by up to 29–48% in oleaginous yeasts and fungi. Surprisingly, using a single selective amino acid instead of a blend can overexpress lipid accumulation by 20% in fungi. Specifically, using Trp, Leu, and Pro as substrates can show a significant increase in lipid yield from *Cryptococcus curvatus* ATCC 20509 (>0.23 g/g coefficients). To ensure maximum fungal lipid productivity, protein-mediated biorefinery systems maintain a higher pH level and a lower phosphate concentration (Dzurendova et al., 2020).

Consequently, elevated carbon concentrations (Wierzchowska et al., 2021) and restricted nitrogen, sulfur, and phosphorus levels promote the overexpression of anaerobic oleaginous bacterial lipids (Lopes et al., 2020). Meanwhile, optimizing oxygen and nitrogen concentrations enhances aerobic microbial lipid productivity. Including glycerol and specific amino acid substrates significantly boosts fungal and yeast lipid production yield (Karayannis et al., 2023).

3.2. Pretreatment

Pretreatment plays a pivotal role in bioenergy production by fractionating the rigid structure of LCB, thereby making the substrate more accessible for the enzymatic hydrolysis process (Madadi et al., 2022a). LCB pretreatment can be classified into physical, chemical, organic solvent, biological, and combined approaches (Madadi et al., 2021). The pretreatment efficiency is

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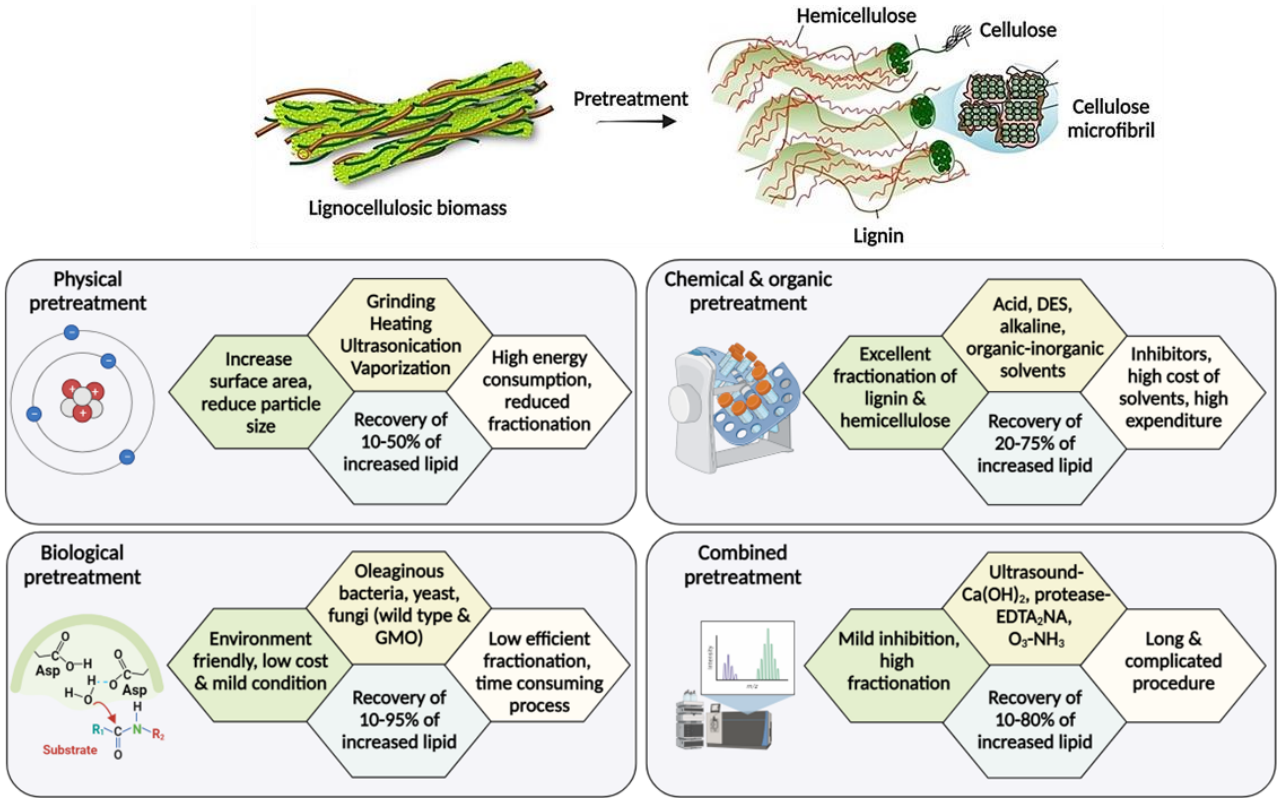

Figure 1. Pretreatment methods used for LCB fractionation in lipid production. This schematic diagram was generated and edited by BioRender.

significantly influenced by factors such as the type of LCB, temperature, pH, chemical dosage, and pressure conditions (Balan, 2019). **Figure 1** illustrates the current pretreatment techniques for LCB fractionation in microbial lipid production.

3.2.1. Physical pretreatment

The significance of physical pretreatment as a crucial step in improving the physical and physicochemical properties of the substrate has been underscored (Zheng et al., 2014). This pretreatment employs various techniques, including grinding, vaporization-induced cavitation, and ultrasonication. By effectively reducing substrate size or breaking down cellulose crystallinity, physical pretreatment enhances accessible surface area and fermentation efficiency (Yu et al., 2019). The increased volatile fatty acids (VFAs) from physical pretreatment typically range from 10% to 50%. For instance, under optimized ultrasonication conditions of 2 W/mL ultrasonic density for 15 min, a significant 45.5% increase in VFA production from food waste was observed, with dominant genera like *Parabacteroides* and *Clostridium* (Rodriguez et al., 2017).

Recently, innovative approaches have introduced radioactive components in high-throughput LCB fractionation. Isotopes, such as Co-60 and Cs-137, undergo radioactive decay, producing gamma rays capable of penetrating LCB and generating free radicals (Basak et al., 2023). Established studies have demonstrated that physical pretreatment approaches can significantly enhance VFA productivity, resulting in increased acetate (44.12 mmol/L), butyrate (9.50 mmol/L), and propionate (14.69 mmol/L) concentrations. This increase leads to an overall VFA production of up to 92.02 mmol/L, surpassing untreated LCB, especially softwood, and grasses (Rusli et al., 2019). Despite the promising results reported in the literature, physical pretreatment faces challenges, such as high energy consumption, low fractionation efficiency, and costly equipment. These factors render physical pretreatment economically unfeasible by a substantial margin, necessitating additional pretreatment steps in the process (Maurya et al., 2015).

3.2.2. Chemical and organic solvent pretreatments

Chemical pretreatment, a traditional method, is commonly conducted using both acids (*i.e.*, H_2SO_4, HNO_3, $AlCl_3$, p-Toluenesulfonic acid, and acetic acid) (Gönen et al., 2021) and alkalis (*i.e.*, NaOH, KOH, $Ca(OH)_2$, NH_4OH (Madadi et al., 2023a). Recently, organic solvent pretreatment (*i.e.*, *p*-Toluenesulfonic acid/pentanol, H_2SO_4/butanol, and chloride/ethylene glycol) has also gained prominence in LCB fractionation (Wang et al., 2022). Generally, these methods effectively fractionate by removing lignin, hemicellulose, or both, thus enhancing enzymatic hydrolysis yield (Madadi et al., 2023b). The increase in VFAs resulting from chemical and solvent methods is estimated to be between 20% and 75% (Wang et al., 2022). For instance, pretreating rice straw with 2% NaOH and 0.5% HNO_3 at 150 °C for 20 min can lead to VFA yield enhancements of 7.09 g/L and 6.0 g/L, respectively (Park et al., 2015). It has been observed that the availability of VFAs for methane generation can be reduced by other chemicals that disrupt the LCB matrix, resulting in VFA accumulation. Studies have shown that the use of sodium BES to inhibit methanogenic bacteria led to a 10% reduction in biogas production from LCB compared to the control without the compound, causing VFA accumulation (Wang et al., 2022).

Despite the high fractionation efficiency achieved with chemical and solvent methods, it has been observed that harsher pretreatment conditions can lead to the generation of unwanted inhibitory components, such as hydroxymethylfurfural, furfural, and their derivatives (*i.e.*, sodium acetate, ferulic acid, furan derivatives, absorbable organic halogen, p-coumaric acids, and phenolics) (Madadi et al., 2022b). These compounds can hinder the hydrolysis and fermentation processes, impeding efficient VFA production and necessitating the additional removal of these inhibitory pollutants (Poddar et al., 2022).

3.2.3. Biological pretreatment

This form of pretreatment is tailored for utilizing a consortium of oleaginous bacteria, fungi, and yeast. These microbes demonstrate enhanced

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results when applied synergistically on an industrial scale (Garcia-Ochoa et al., 2021). Biological pretreatment offers several advantages, including mild conditions, simple equipment, reduced downstream processing costs, decreased formation of toxic and inhibitory substances, and elimination of the need for chemical removal post-treatment (Balan, 2019; Zabed et al., 2019). The lipid yield achieved through biological pretreatment typically ranges from 10% to 80%. The excessive presence of reactive oxygen species in a fermenter significantly reduces the activity and count of oleaginous microbes (Biswas et al., 2022). Often, selective enzymes are preferred over whole microbial cells to ensure rapid catalysis, swift conversion, and reduced contamination during LCB pretreatment. The systematic application of enzyme cocktails has been proven to catalyze more efficiently than using them individually (Nakhate et al., 2021). Pretreating solid digestate, primarily composed of organic waste like fruit and vegetable residues, with *P. sajor-caju* has been reported to increase VFA yield by 1.24 and 1.17 folds compared to untreated substrate and substrate treated withautoclaving, respectively (Fang et al., 2018). The VFA yield reached 240 mg COD/g VS in just 42 days. Although biological pretreatment requires a substantial amount of time and space, its cost-effectiveness and eco-friendliness outweigh these limitations (Rouches et al., 2016).

3.2.4. Combined pretreatment

While physical pretreatment methods have proven effective in enhancing bioprocess efficiency, their energy-intensive nature is closely tied to their effectiveness. Chemical pretreatments face challenges, such as generating inhibitory compounds and secondary pollutants. Therefore, a combined pretreatment approach has been designed to overcome the shortcomings of a single method (Yu et al., 2019; Poddar et al., 2022). Several significant combined pretreatment approaches have been introduced, resulting in a high amount of cellulose and xylan following significant delignification. This issue highlights the potential of using such techniques to establish new state-of-the-art pretreatment methods. Among the most successful combined pretreatment approaches, ozone-soaking aqueous NH_3 pretreatment (Yu et al., 2014), protease-EDTA2NA pretreatment (Liu et al., 2016), HNO_2-rhamnolipid pretreatment (Wu et al., 2017), ultrasound-$Ca(OH)_2$ pretreatment (Wang et al., 2019), and temperature/pressure optimized fungal pretreatment of LCB are the most successful ones (Rusli et al., 2019). Combined pretreatment has been reported to achieve the maximum microbial lipid production (10–90%).

A comprehensive analysis of different combined pretreatment methods reveals that increased VFAs and biogas can be obtained from the ozone-soaking aqueous NH_3 pretreatment process, achieving around 89% lignin fractionation (Yu et al., 2014). Comparatively hard LCB sources can be significantly fractionated by some other proposed methods (Khan et al., 2018; Garcia-Ochoa et al., 2021; Biswas et al., 2022; Madadi et al., 2022c). The authors confirmed the high production of VFAs by successfully applying first-order and modified Gompertz models and the prevalence of VFA-producing genera *Ruminiclostridium* and *Halocella*. It was demonstrated that the potential of combined pretreatment methods to improve lignin degradability and VFA production (Rusli et al., 2019). They found that pretreating oil palm fronds with combined physical and biological methods, including enzymatic extracts from two white-rot fungi, resulted in the lowest residual lignin content and the highest VFA generation. Most of the VFAs produced under this scenario consisted of propionate and butyrate with great quality. Like biological methods, this pretreatment is also time-consuming in many cases (Al-Battashi et al., 2019).

3.3. Enzymatic hydrolysis

The process of enzymatic hydrolysis involves metabolizing fractionated carbohydrates into fermentable sugar molecules, which is a crucial step preceding the fermentation process. During fermentation, microorganisms such as oleaginous bacteria, fungi, and yeast metabolize these sugar molecules into intracellular lipids (Chen and Liu, 2017). In ethanol production, the solid content in the enzymatic hydrolysis stream plays a vital role in determining economic feasibility (Chen and Liu, 2017; Song et al., 2024a). For most types of LCB, an initial solid loading exceeding 20 wt% is necessary for efficient enzyme hydrolysis. When the solid loading in the enzymatic hydrolysis process exceeds 15 wt%, it is commonly referred to as high solids enzymatic hydrolysis in classical methods (Koppram et al., 2014).

Based on LCB types and solid loading, the enzyme concentration is estimated. For example, with a 20 wt% sugarcane bagasse loading, an enzyme loading of 0.1 g/g cellulose achieved 69.2% conversion following hydrolysis at 50 °C in previous studies (Ramos et al., 2015). Similarly, for 28 wt% corn stoves, 22 FPU/g cellulase was used to achieve 73% cellulose conversion (Hodge et al., 2008; Lu et al., 2010). Moreover, for 30 wt% olive plant, 15 FPU g/solids; 20 wt% poplar wood, 20 FPU/g cellulose; and 17 wt% wheat straw, 9.6% FPU/g solids were used to recover 50%, 83%, and 39% of sugars (Chen and Liu, 2017). Enzyme hydrolysis offers increased productivity, lower energy input, higher economic feasibility, and lower capital and operational costs (Dessie et al., 2018; Song et al., 2024b). In contrast, enzyme inhibitors, water constraints, rheological alterations, and lowered efficiencies are considered significant limitations of enzymatic hydrolysis (Chen and Liu, 2017). Generally, degraded inhibitors hinder the efficiencies of catabolic enzymes, including furan derivatives, weak acids, phenolic compounds, and unwasted lignin models (Madadi et al., 2023b). It has been reported that the unwanted presence of typical phenolic aldehydes can suppress the catabolic activity of cellulase by 0.05–8 g/L, while it is around 2–4 g/L for phenolic acids. Various surfactants and non-catalytic proteins can be used to address these issues and improve cellulase activity (Chen and Liu, 2017).

The biological pretreatment system relies on the enzymatic activity of bacteria, fungi, and Archaea to hydrolyze LCB, with lipolysis essential to this process. It utilizes ligninolytic enzymes like versatile peroxidase, lignin peroxidase, laccase, and manganese peroxidase (Paul et al., 2022). Cellulolytic enzymes, including exocellulases, endocellulases, cellobiases, and oxidative cellulases, are combined to hydrolyze cellulose (Madadi et al., 2021). Enzymatic hydrolysis processes use hydrolytic and lignolytic enzymes (Sun et al., 2023a). Enzymes, rather than microbes, are significant in individual and combined pretreatment methods (Zabed et al., 2019), where many enzymes, especially cellulases, can be recycled following hydrolysis.

4. Microbial lipid biosynthesis from lignocellulosic hydrolysate

Two distinct metabolic pathways are responsible for synthesizing and accumulating lipids. These pathways are differentiated based on the polarity of the substrate, with de novo lipogenesis using hydrophilic substrates and ex novo lipid metabolism relying on hydrophobic ones. This section thoroughly speculates the differences in de novo lipogenesis of oleaginous microbes and the advantages and limitations of using bacteria, yeast, and fungi as single-cell factories.

In de novo lipid synthesis, a complex series of three biochemical processes unfold, involving initial intracellular intermediary generation, lipogenesis, and lipolysis. The initial phase involves the conversion of available carbon sources into pyruvate within the cytosol, a universal step shared by bacteria, yeasts, and fungi (Novy et al., 2018). Yet, the disparity between eukaryotes and prokaryotes becomes apparent when delving into lipogenesis and lipolysis. In eukaryotic organisms, these processes depend on the intricate carbon flux between organelles (Dourou et al., 2018).

An organism earns the designation of “oleaginous” based on its remarkable ability to synthesize and accumulate lipids, often exceeding 20% of its cellular dry weight (Annamalai et al., 2018). Notably, under specific nutrient-restricted conditions, these organisms demonstrate a heightened production of cytosolic acetyl-CoA, the paramount precursor to lipogenesis. Notably, inhibiting the Krebs cycle’s isocitrate dehydrogenase leads to citrate accumulation under nitrogen-deficient conditions. These oleaginous organisms adeptly facilitate the carbon flow from this precursor toward the intricate enzymatic processes that catalyze lipid biosynthesis.

Remarkably, the metabolic process responsible for the hydrolytic degradation of synthesized fats into their constituent molecules, known as lipolysis, does not exhibit a corresponding increase in kinetics alongside lipogenesis in these organisms. Consequently, this metabolic imbalance culminates in the accumulation of lipids within the cellular milieu (Prasad et al., 2019). The following are some differences between the three main groups of oleaginous microorganisms at the metabolic level (**Fig. 2**).

Oleaginous microorganisms utilize abundant acetyl-CoA as a substrate for synthesizing various lipids (Qadeer et al., 2017). The biosynthesis of

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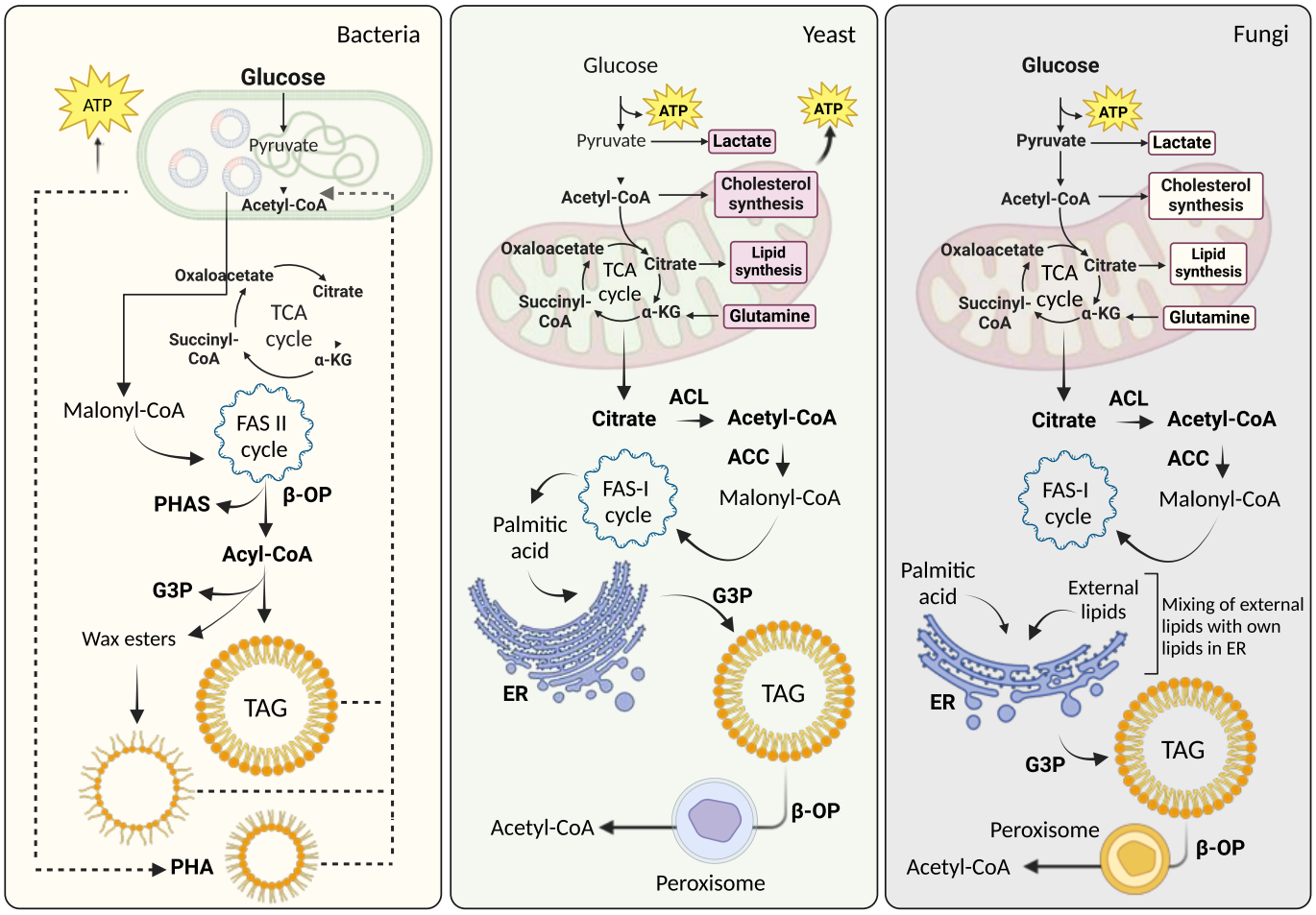

Figure 2. Metabolic deviations among the oleaginous bacteria, yeast, and fungi. β-OP: β oxidation pathway; PHA: Polyhydroxyalkanoates; PHAS: Polyhydroxyalkanoates Synthase; WS: Wax Ester Synthase; FAS: Fatty Acid Synthase; G3P: glycerol-3-phosphate; ER: Endoplasmatic Reticulum; ACL: ATP Citrate Lyase; ACC: acetyl-CoA Carboxylase. This schematic diagram was generated and edited by BioRender.

TAGs begin with the conversion of acetyl-CoA to malonyl-CoA, which is then used by individual enzymes of type 2 fatty acid synthetase to synthesize acyl-CoA through consecutive condensations. The type 2 fatty acid synthetase complex also controls the length and degree of unsaturation of the acyl group (Qadeer et al., 2017). G3P provides the glycerol backbone enzymatically esterified with acyl-CoA to generate TAGs. Wax esters can also be synthesized from acyl-CoA, which is transformed into alcohol and transesterified by wax ester synthase (Dourou et al., 2018). PHA synthase can also produce polyhydroxyalkanoates using monomers derived from acetyl-CoA (Zhuang and Qi, 2019). All these hydrophobic structures are accumulated, forming micellar structures called lipid droplets (Lupette and Maréchal, 2020). Lipolysis involves utilizing accumulated lipids as a substrate to recover acetyl-CoA, achieved through a sequence of enzymatic reactions known as the β-oxidation pathway (Pavoncello et al., 2022).

In yeast systems, citrate overproduction is facilitated by two key enzymes involved in lipogenesis: ATP citrate lyase and acetyl-CoA carboxylase (Athenaki et al., 2018). These enzymes drive carbon flux towards Malonyl-CoA, an intermediate required by the type 1 fatty acid synthetase complex to produce free FAs, specifically palmitic acid (Athenaki et al., 2018). The acyl-CoAs derived from the free FAs migrate within the endoplasmic reticulum, where elongation and unsaturation of the carbon chain occur. Like bacteria, G3P is utilized as a source of glycerol for the formation of triglycerides that accumulate in lipid droplets. These droplets are degraded by a group of Acyl-CoA synthetases that form the β oxidation pathway in the peroxisome (Pavoncello et al., 2022). It is important to note that the synthesis of sterols also primarily occurs in the endoplasmic reticulum, utilizing the acyl-CoAs pool as a substrate for this parallel metabolism to triglyceride synthesis (Girardi Piva et al., 2022).

Lipid biosynthesis in oleaginous fungi can be carried out through heterotrophic pathways (Chen et al., 2018). Fungi primarily obtain energy by breaking down complex organic molecules, such as carbohydrates and organic matter. They secrete enzymes that can break down these complex compounds into simpler molecules, which they can then absorb and use as a source of energy. This process is known as extracellular digestion. The latter serves as a precursor for forming acyl-CoA *via* type 1 fatty acid synthetase, which can undergo elongation in the ER where sterols are synthesized or react directly with G3P to TAGs. Like yeasts, oleaginous fungi catabolize lipids via the peroxisomal β oxidation pathway. To achieve profitable production of lipids, it is essential to effectively utilize pretreated waste to attain high growth rates, productivity, and yields (Cordell et al., 2023). The single-cell factories must resist possible inhibitors present in these carbon sources and contamination in industrial scales, and downstream purification must be as consistent and effective as possible to be considered a possible producing strain (Park and Ledesma-Amaro, 2023). Furthermore, a final product with sufficient experimental value is imperative for economic feasibility.

Precision fermentation techniques have advanced to develop systems that optimize the utilization of accessible carbon sources. Sustained fermentation, which maintains a stable C/N ratio, increases lipid accumulation (Blazeck et al., 2014). Furthermore, selecting specific temperature, pH, and aeration conditions enhances the production of

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valuable lipids. In the instance of oleaginous fungi, adopting a two-stage fermentation process proves advantageous. Hydrolysates are harnessed to produce biomass, followed by subsequent lipid accumulation during autotrophic fermentation under nitrogen deficiency (Xu et al., 2017).

Concluding that one microbe is inherently superior to another for lipid production oversimplifies the matter. As previously elucidated, specific products are more closely associated with particular microorganisms due to metabolic pathway disparities. These microorganisms act as intermediaries, bridging the gap between industrial requirements and the chosen substrate. Additionally, metabolic engineering can enhance the productivity of a givenmicrobial strain, even incorporating pathways that lead to the synthesis of high-value fermentative products, thereby rendering the process more economically sustainable (Tang and Xue, 2019; Qiao et al., 2021). **Table 3** comprehensively represents the diverse array of wild-type oleaginous microorganisms being considered for producing FAs from LCB sources. Altogether, oleaginous yeast and fungi have been experienced as significant lipid producers compared to the bacterial species, where lipid productivity changes based on the substrates.

Table 3.
Examples of batch fermentation using different species of bacteria, yeast, and fungi with pretreated LCB as the main carbon source.

Microorganism	Biomass	DCW* (g/L)	Lipids (wt%)	Productivity (g lipids/d)	Reference
Bacteria					
R. opacus	Sugarcane bagasse	2.7	64.47	0.35	Mahmood and Singh (2023)
R. opacus	Corn stover	2.02	12.6	0.05	Le et al. (2017)
Gordonia sp.	Orange waste	1.42	62.5	0.44	Gouda et al. (2008)
R. opacus	Paper waste	6.41	35	0.4487	Nair and Sivakumar (2022)
R. opacus	Loblolly pine and sweetgum	1.03	28.6	0.107	Wei et al. (2015)
Yeast					
C. curvatus	Wheat straw	17.2	33.5	0.82	Diamantopoulou et al. (2020)
Y. lipolytica	Sugarcane bagasse	16.39	58.7	1.37	Vasaki et al. (2022)
L. starkeyi	Sugarcane bagasse	9.6	26.1	0.84	Xavier et al. (2017)
R. toruloides	Apple pomace	11.67	21.1	0.30	Caporusso et al. (2023)
R. toruloides	Tea waste	10.75	44.61	0.95	Qi et al. (2020)
Fungi					
Aspergillus caespitosus	Sugarcane bagasse	10.45	37.27	1.21	Srinivasan et al. (2021)
Mortierella sp.	Agricultural biomass	27.01	63.4	1.8	Yao et al. (2019)
Mortierella alpina	Forest biomass	6.60	17.00	1.10	Dellero et al. (2020)
Mortierella schmuckeri	Forest biomass	11.70	70.00	1.83	
Backusella sp.	Agricultural biomass	9.67	58.08	1.32	Zhao et al. (2021)
Mortierella amoeboidea	Agricultural biomass	10.08	59.15	1.34	

* DCW: Dry cell weight; % Lipids: g of lipids/g of dry cell; Productivity: g of lipids/d of fermentation.

5. Bioprocessing strategies in high lipid yield

5.1. Microorganism-specific strategic alterations

Novel industrial fermentative lipid production strategies encompass a range of fermentation optimization and operational techniques. These approaches are designed to maximize microbial lipid yield while minimizing time, cost, and waste generation (**Table 4**). In most cases involving the use of oleaginous microbes, especially genetically modified yeast and fungi, there have been reports of these organisms biosynthesizing and accumulating higher amounts of intracellular lipids than their wild counterparts, based on various fermentation operating systems (Xue et al., 2018). Recent studies have identified *Yarrowia lipolytica*, *Lipomyces starkeyi*, and *Rhodosporidium toruloides* as the highest lipid-producing yeasts, while *Mortierella isabellina* and *Cunninghamella echinulata* are among the fungi with the highest lipid production (Samavi and Rakshit, 2022).

5.2. Microorganism-specific fermentation modeling for high lipid yield

In recent times, different fermentation methods have been introduced to ensure microbial lipid production, restoring all economic-feasibility aspects, such as separate hydrolysis and lipid production (SHLP), simultaneous saccharification and lipid production (SSLP), solid-state fermentation (SSF), and consolidated bioprocessing (CBP). Based on the operating conditions - biomass loading, pretreatment method, and types of microorganisms (bacteria, fungi, and yeast), the productivity of lipids is significantly regulated (**Fig. 3** and **Tables S1-S3**).

In SHLP systems, diversified oleaginous microbes have been applied to produce lipids where a group of bacterial (*i.e., Tepidibacillus fermentans*), yeast (*i.e., Yarrowia lipolytica, Rhodosporidium toruloides, Lipomycis kononenkoae, Lipomycis tetraporus,* and *Lipomycis starkeyi*), and fungi (*i.e., Cryptococcus curvatus, Mortierella isabelline, Rhodotorula glutinis, Rhodococcus opacus, Trichosporon cutaneum, Trichosporon mycotoxinivorans*) are significantly used (**Table S1**). Among the reported oleaginous fungi, *Rhodotorula* spp., and *Mortierella* spp. produce the highest amount of lipids in acidic conditions (**Fig. 3A**). Besides, *Lipomyces tetrasporus* was the highest lipid producer among the fermentative yeasts in the presence of acidic environments (**Table S1**). Unlike the SHLP method, only different groups of fungal species are utilized in producing lipids in the SSLP and SSF methods (**Fig. 3B**). In the SSLP system, a variety of fungi, including acidophilic and alkaline-tolerant strains, have been documented. For instance, *Cryptococcus curvatus* exhibits a preference for slightly acidic conditions, while one of its strains, *Cryptococcus curvatus* ATCC20509, has been observed to operate effectively under alkaline conditions (0.5 M NaOH) during the feed-batch mode of SSLP fermentation (refer to **Table S2**). Alike SSLP, there are some fungal strains from the same family working in both acidic and alkaline conditions have been found in the SSF method (**Fig. 3B**). In batch operating mode, Aspergillus oryzae A-4 produces lipids in the acidic condition, whereas *Aspergillus tubingensis* TSIP9 metabolizes lipids more spontaneously in the alkaline environment of the feed-batch model of SSL fermentation. *Microsphaeropsis, Sclerocystis, Phomopsis, Cephalosporium* , and *Nigrospora* can biosynthesize lipids in batch systems based on different mixed-solid systems (**Table S3**).

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Table 4.
Novel fermentation strategies for increased microbial lipid production.

Strategic Facts	Used Microorganisms	Reference
Use of the mutated form of any microbes through sequential plasma and chemical mutagenesis rather than using their wild types	*Trichosporon dermatis mutant* (L7)	Sun et al. (2021)
Activity profiling and optimization of the glycerol synthetic enzymes at reduced oxygen levels in a bioreactor	*Candida krusei*	Liu et al. (2003)
Increased accumulation of erythritol, controlling the osmotic pressure of a fed-batch fermentation	*Yarrowia lipolytica*	Yang et al. (2019)
Use of oleaginous yeast to accumulate high lipids when VFAs are the main energy source	*Pichia kudriavzevii, Kodamaea ohmeri, Lipomyces lipoferrus, Candida tropicalis, Rhodotorula babjevae*	Law et al. (2023)
Application of different salts (KH_2PO_4, Na_2SO_4, NaCl, MSG) and novel osmotic pressure controlling system for increased DHA production	*Schizochytrium* sp.	Hu et al. (2015)
Application of monosodium glutamate wastewater as the culture media for high lipid yield in a bioreactor	*Rhodotorula glutinis*	Maza et al. (2020)
Use of food waste hydrolysate for high yield and sustainable production of lutein and lipid	*Chlorella mixotrophic*	Wang et al. (2020)
Use of novel yeast for simultaneous xylose and glucose conversion mediated high-yield lipid biosynthesis	*Cystobasidium iriomotense*	Tanimura et al. (2018)
Xylose and cellobiose co-fermentation by oleaginous yeasts for lipid	*Lipomyces starkeyi*	Kalyani et al. (2017)
Yeast-mediated continuous self-provided fermentation for lipids	*Trichosporon porous Cryptococcus podzolicus*	Qian et al. (2020)
Use of controlled nitrogen and oleaginous fungus when lipid is synthesized from plant substances	*Aspergillus oryzae*	Tamano et al. (2015)
Application of ex novo and de novo techniques for converting acidic oil wastes into docosahexaenoic acid during lipids synthesis	*Aurantiochytrium limacinum*	Laddha et al. (2021)
Use of transcriptomically characterized microbes for fermentative lipid production	*Mortierella alpina*	Sun et al. (2021)
Increased levels of docosahexaenoic acid using fungi	*Aurantiochytrium limacinum*	Dellero et al. (2020)

In summary, the SHLP approach stands out as a preferred method among the extensively reviewed fermentative strategies for microbial lipid production. This method leverages the remarkable capabilities of oleaginous bacteria, fungi, and yeast, showcasing versatility across a range of conditions, including both acidic and alkaline environments, and employing batch-to-feed-batch models on various individual and mixed-solid systems. The oleaginous yeast are the most lipid producers among the reviewed oleaginous microbes concerning all the models means SHLP, SSLP, and SSF. Surprisingly, oleaginous bacteria (*i.e., Tepidibacillus fermentans)* are the least lipid producers among the fermentative microbes.

5.3. Fermentative lipid production controlling strategies

Conventionally, an industrial controlling system for fermentative lipid production considers several parameters, including substrate flow rate, water for injection, phenylacetic acid flow rate, aeration rate, discharge rate, and nitrogen. In contrast, for the novel bioreactor controlling system, automated measurement and controlling system are installed to figure out acid/based flow rate, cooling water flow rate, process analytical technology (PAT) measurement (Raman spectroscopic/Spec), penicillin concentration, viscosity, weight, dissolved oxygen, the power of the agitator system, off gas O_2, off-gas CO_2, and enzyme concentrations (Denardi-Souza et al., 2018). In the structure-based control system of fermentation, the Fieldbus control system, knowledge-based control system (Wang et al., 2020), and networked control system have been operated for fermentative lipid synthesis (Peng et al., 2017).

Common and special-type bioactive compounds, such as diacetyl and pentane-dione, can be produced through enzymatic activity control (**Fig. 4**). The process starts with saccharifying polysaccharides into simple sugars, which are then converted into pyruvate following glycolysis (Maleki and Eiteman, 2017). The pyruvate is enzymatically converted inside the plasma membrane into ethanol, acetic acid, and α-Acetolactate and α-Acetohydroxybutyrate (Joo et al., 2016; Achanta and Rae, 2017). Afterward, through passive diffusion, diacetyl and pentanedione are formed outside the plasma membrane through a non-enzymatic downstream processing. This approach allows for the production of various value-added products alongside bioethanol by strategically controlling microbial fermentations (**Fig. 4**).

6. Metabolic engineering for lipid overproduction

6.1. Genetic modifications

Metabolic engineering integrating genetic modifications of the oleaginous microbes upregulates their lipid productivity concerning factors such as microbial types, target gene engineering methods, energy sources, lipid types, and the modes of fermentation (Hernández et al., 2015). For specific genetic modifications, a group of *E. coli* has been experimented for overexpressing transgenes amongst the oleaginous bacterial strains (**Table 5**). Engineering of *E. coli* with *fadE* and *fabR* mutant for the overexpression of *fabZ, tesA, nadK,* and *pntAB* can synthesize up to 4.8 g/L of FFA in a glycerol system (Chen et al., 2015). Similarly, overexpression of the *ws1* gene from *M. hydrocarbonoclasticus* induces 7.5 g/L of total FA production in *E. coli,* taking glucose as an energy source (Rahman et al., 2019). In combined glucose and xylose substrate, 3.6 g/L free FA is found as the maximum in *E. coli* engineered with the *fadD* and *ptsG* mutants in overexpressing *fabZ* and TE isolated from *R. communis* (Chen et al., 2015). On the other hand, in the lactose medium, 12.1 g/L of TAGs have been synthesized as the highest productivity of *E. coli* in a feed-batch fermentation system engineered with the *dgkA* mutant to upregulate *atfA* and *fadD* genes (Wang et al., 2020). Interestingly, *fadD, fadR, plsB, pgpB, tesA,* and *atfA* inserted *E. coli* can produce 16.1 and 9.2 g/L of total FAs and TAGs respectively in complex medium containing multiple energy sources (Hernández et al., 2015). Maximal FA ethyl ester from *E. coli* (0.27 g/L) has been produced following the overexpression of a group of genes from diversified sources such as *atfA, fadD, tesA, alsS,* and *ilvCD* (*Bacillus subtilis)*; *aro10* and adh2 (*Saccharomyces cerevisiae*) in LB medium (Tao et al., 2015), where the lowest was in lactose (0.015 g/L).
In contrast, *R. opacus* is the highest producer of TAGs (48 g/L) in glucose but 54.2 g/L in corn stove hydrolysate, which was engineered to overexpress *xylA* and *xylB* genes isolated from *S. padanus* (Donini et al., 2021). To ratify, the range of lipid production was between 39.7 and 54.2 g/L (**Table 5**). Besides, *R. fascians* and *Rhodococcus jostii* can produce 16.2 and 50.1 g/L of lipids in fructose and glucose, respectively (Hernández et al., 2015). Moreover, the productivity of *E. coli* was significantly inferior to the other engineered microbes, though most of the reviewed experiments strongly

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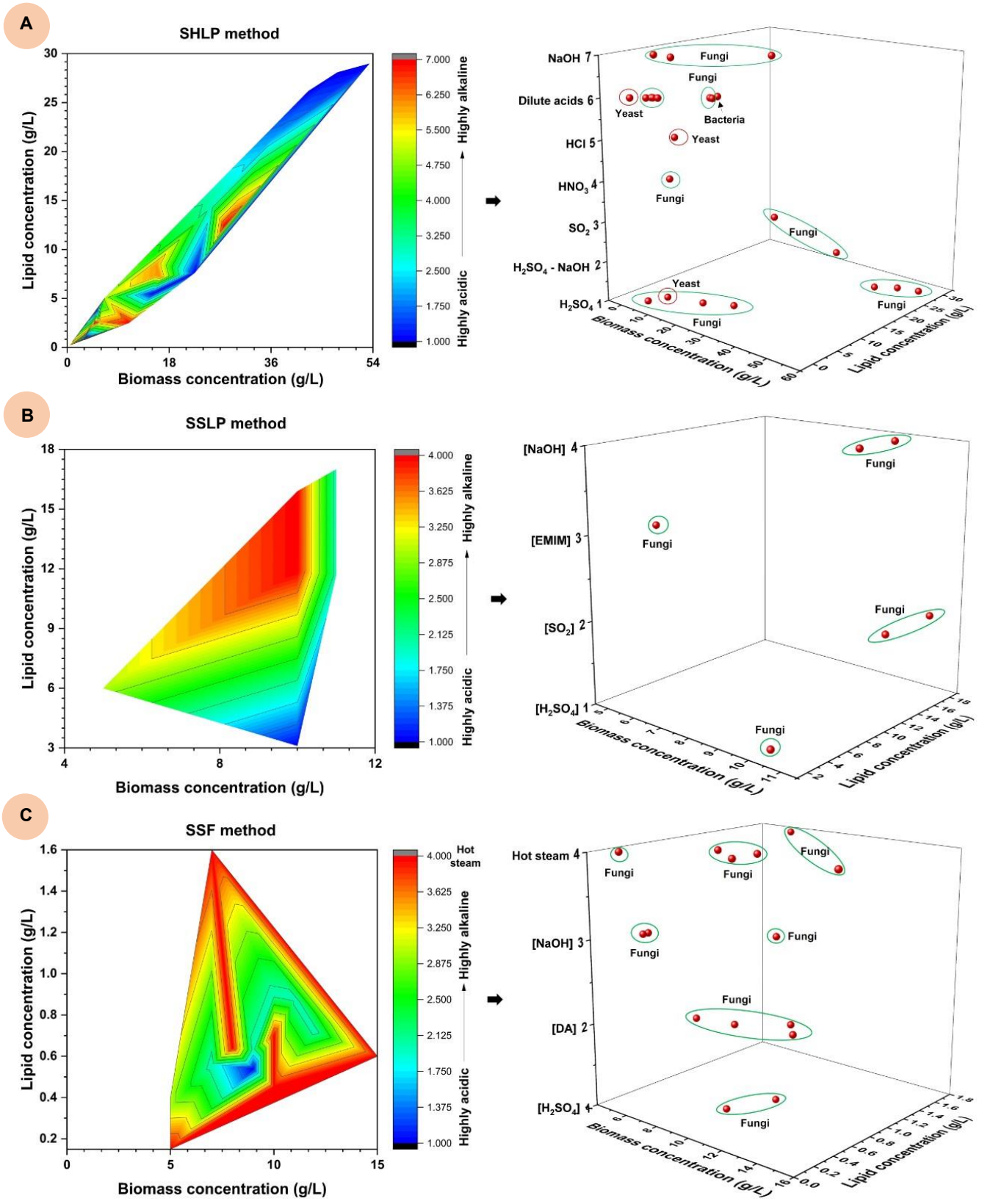

Figure 3. Profiling of lipid concentrations based on fermentation methods such as **(A)** separate hydrolysis and lipid production (SHLP); **(B)** simultaneous saccharification and lipid production (SSLP); and **(C)** solid-state fermentation (SSF), biomass loading, pretreatment methods, and types of microorganisms. The data in the contour plots are based on the literature review in **Tables S1-S3**.

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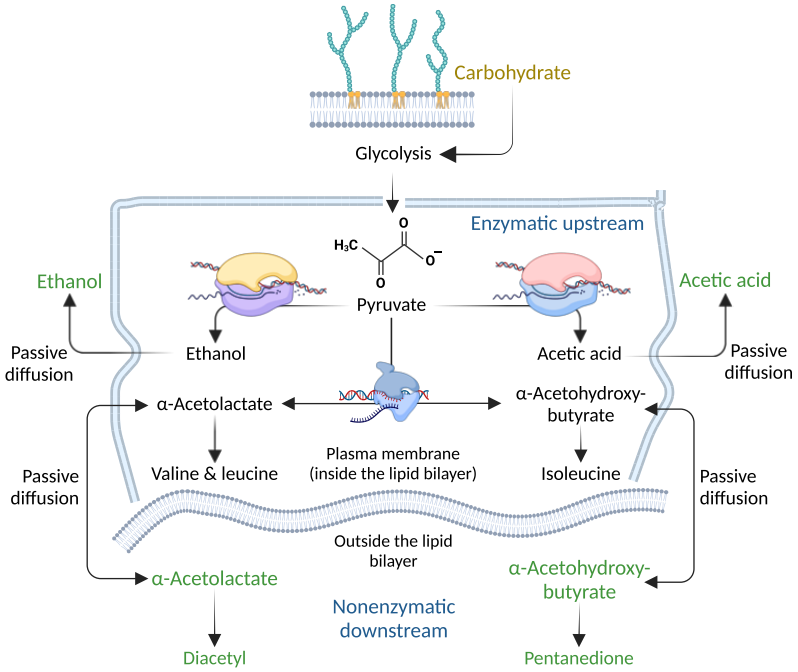

Figure 4. A novel biorefinery process combining enzymatic and non-enzymatic conversion of microbial lipids into other functional value-added products. This schematic diagram was generated and edited by BioRender.

emphasized manipulating *E. coli* productivity using genetic modification (**Table 5**).

6.2. Metabolic alterations

Metabolic alterations are regulated by the manipulation of a group of enzyme-coding genes among a group of prokaryotic and eukaryotic microorganisms (**Table 6**). In terms of percentile increased lipid production, *Arabidopsis* engineered with SLC1-1 (LPAT) gene from yeast was the highest of all (+48%) (Zhang et al., 2021), followed by *Chlamydomonas reinhardtii* (+46.4%), and *E. coli* (+46.9%) increased lipid (Fathy et al., 2021; Jothibasu et al., 2021). In contrast, upregulating lipid production by folds, oleaginous yeast engineered with a yeast-derived gene by 3–9 folds of TAG content and 200–600 folds of DGAT activity (Son et al., 2022). *E. coli* resulted in a 20-fold increase in FA synthesis following a multi-gene engineering approach where ACC, thioesterase, and D fadD (acyl-CoAsynthetase) genes were considered from different *E. coli* strains (Cai et al., 2015). ACS and ACC gene overexpression stimulate around 9-fold and 3-fold increased lipid, respectively, in *E. coli* (Vrablik et al., 2015; Sitepu et al., 2017). Surprisingly, oleaginous fungi like *A. oryzan* have no significant changes in lipid concentration following ACC expression (Cai et al., 2015). To summarize, the overall increase of *E. coli* following different gene-mediated metabolic modifications was more significant in most aspects than that of the fungal and yeast strains (**Table 6**).

6.3. CRISPR-induced engineering model for oleaginous microorganisms

Early endeavors to enhance lipid production through microorganism engineering primarily concentrated on model organisms, such as *E. coli*, which possessed well-established genetic tools for transgene overexpression, genome integration, and precise gene knockout techniques. In the case of *E. coli*, elevating lipid titers primarily involved the application of combined "push" and "pull" strategies. These strategies entailed the overexpression of pivotal genes involved in FAs synthesis, such as acetyl-CoA carboxylases previously demonstrated (Fathy et al., 2021), or enzymes in the FAs synthase elongation cycle (Marella et al., 2018), thus "pushing" carbon into FAs biosynthesis. Concurrently, downstream enzymes like acyltransferases or thioesterases were harnessed to "pull" FAs toward the production of specific compounds, including TAGs (Röttig et al., 2015; Xue et al., 2018) or FAs (Liu et al., 2019). To further enhance lipid yields, gene knockout strategies were employed to suppress genes involved in competing metabolic pathways, such as *fadD* (Azadbakht et al., 2022), responsible for long-chain FAs CoA ligase in the context of FAs oxidation.

The titers attained through these approaches in *E. coli* typically fall short of the levels achieved by oleaginous organisms. More comprehensive engineering strategies, such as reversing the beta-oxidation cycle, resulted in titers reaching 7 g of free FA/L (Lozada et al., 2020). These approaches yielded approximately 85% of the theoretical maximum (0.33 g/g glucose in *E. coli*), with 0.28 g of free FA/g glucose (Wang et al., 2015). To attain a concentration of 8.6 g free FA/L in a glucose-fed-batch fermentation, a noteworthy effort was required, involving the overexpression of fifteen FA biosynthetic genes in an *E. coli* BL21 *fadD* knockout strain, as exemplified (Song et al., 2016). Recent advancements in gene technology have broadened the horizon for genome-scale engineering, enabling the maximization of lipid synthesis. Notably, CRISPR/Cas technologies, such as CRISPR activation (CRISPRa) and CRISPR inhibition (CRISPRi), have ushered in a new era of microbial engineering by facilitating the dynamic activation or repression of multiple genes simultaneously (**Fig. 5**). Traditional CRISPR/Cas techniques have also streamlined knocking out multiple genes in a single transformation step, obviating the need for iterative recycling of selectable markers. For an in-depth exploration of these CRISPR-based technologies, comprehensive reviews can also be found in the other works (Lu et al., 2022).

A key advantage of CRISPRi is that it can be used to dynamically downregulate ordinarily essential genes for survival (and cannot be knocked out). This CRISPR was used to further tune the engineered reverse beta-oxidation cycle to favor the production of medium-chain FAs (C6, C8, C10), achieving 3.5 g MCFAs from 15 g glucose/L (Wu et al., 2017). The ability to repress or activate multiple genes in a combinatorial fashion can greatly expand the scope of metabolic engineering campaigns. In a recent study on identifying new gene knockout targets to support lipid overproduction in *E. coli*, over 100 genes were systematically repressed using CRISPRi. Several genes were identified that have an important impact on free FA accumulation but are not part of core FA metabolism pathways.

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Table 5.
Genetic modifications of different microbial strains behind fermentative production of lipids.

Bacterium	Type of Genetic Modification	Energy Sources	Lipid Types	Fermentation Mode	Lipid Yield (g/L)	Reference
E. coli BL21XL100/pMSD8/pMSD15/pXL49	*ΔfadD, accBCDA* overexpression, *'tesA* and TE' from *C. camphora*	Glycerol	Free FA	Fed-batch	2.5	Jeucken et al. (2019)
E. coli LL18	Expression of '*AcTes*A' from *A. baylyi*	Glucose	Free FA	Fed-batch	3.6	Li et al. (2018)
E. coli ML 190 pXZ18z	*'fadD* and *ptsG* mutant' for the overexpression of *fabZ* and TE from R. communis	Glucose and xylose	Free FA	ND	3.6	Chen et al. (2015)
E. coli ML 103 pXZ18	*'fadE* mutant' for the overexpression of TE from the Ricinus communis	Glucose contained LB medium	Free FA	ND	2.3	Bai et al. (2022)
E. coli BL21 *ΔfadD*/pE-*AtFatAssi2&PA-acc*	*'fadD* mutant' for the overexpression of acc, *AtFatA*, and *SSi2* from Arabidopsis thaliana	Glucose	Free FA	Fed-batch	1.3	Rahman et al. (2019)
E. coli MLK 211pXZ18z pBADNP	*'fadE and fabR* mutant' for the overexpression of *fabZ*, *tesA*, *nadK*, and *pntAB*	Crude glycerol	Free FA	ND	3.5	Chen et al. (2015)
E. coli MLK 211pXZ18z pBADNP	*'fadE* and *fabR* mutant' for the overexpression of *fabZ*, *tesA*, *nadK*, and *pntAB*	Glycerol	Free FA	ND	4.8	
E. coli BL21 (DE3) *pDucFatB/pDccFatB*	Pant TEs ucFatB (U. California)/ccFatB (C. camphorum), and *atfA*	Ethanol contained LB medium	Free FA	ND	0.5	Rahman et al. (2019)
E. coli EDE3 pJR1	*'pgpB*	LB medium	TAGs	ND	5	Xu et al. (2020)
E. coli MG1655 Δp2AAF	*dgkA* mutant' for the overexpression of *atfA* and *fadD*	Lactose	TAGs	Fed-batch	12.1	Wang et al. (2020)
E. coli MG1655 Δp2AAF	*dgkA* mutant' for the overexpression of *atfA* and *fadD*	Lactose	Total FAs	Fed-batch	8.5	
E. coli BL21 PC::ff::ppt	*fadD, fadR, plsB, pgpB, tesA* and *atfA*	Complex medium	Total FAs	ND	16.1	Hernández et al. (2015)
E. coli BL21 PC;:ff::ppt	*fadD, fadR, plsB, pgpB, tesA* and *atfA*	Complex medium	TAGs	ND	9.2	
E. coli BL21 ΔdgkA/WS1	Overexpression of *ws1* from *Marinobacter hydrocarbonoclasticus*	Glucose	Total FAs TAGs	ND ND	7.1 1.3	Rahman et al. (2019)
E. coli BL21 ΔdgkA/WS1	Overexpression of *ws1* from *Marinobacter hydrocarbonoclasticus*	LB medium containing glucose	Total FAs TAGs	ND	7.5 4.9	
Acinetobacter baylyi M4	*ΔdgkA*	Glycerol and gluconate	Total FAs	ND	8.7	
R. opacus Xsp8	Cloned from the gene library of *Streptomyces padanus*	Kraft hardwood hydrolysate	ND	ND	45.8	Donini et al. (2021)
R. opacus MITXM-61	Overexpressing *xylA* and *xylB* from *S. padanus*	Glucose	TAGs	ND	48.0	
R. opacus MITXM-61	Overexpressing *xylA* and *xylB* from *S. padanus*	Corn stove hydrolysate	TAGs	ND	54.2	
R. opacus MITAE-348	Overexpressing *araD, araA, araB* from S. cattleya	Arabinose	TAGs	ND	39.7	Hernández et al. (2015)
R. opacus MITAE-348	Overexpressing *araD, araA, araB* from *S. cattleya*	Glucose in combination with arabinose	TAGs	ND	42.1	
R. fascians F7 pTip-QC2-RO00075-pPR27/Atf2	Overexpressing PAP2 *rhO00075* and *atf2*	Fructose	FAs	ND	16.2	
Rhodococcus jostii RHA1 pTip-QC2/RO00075	Overexpressing PAP2 *rhO00075*	Glucose	FAs	ND	50.1	
E. coli MG1655 Δp2AAF	*dgkA* mutant' for the overexpression of *atfA* and *fadD*	Lactose	FA ethyl ester	ND	0.015	Wang et al. (2020)
E. coli BL21 (DE3) *pDucFatB/pDccFatB*	Pant TEs ucFatB (U. California)/ccFatB (C. camphorum), and *atfA*	Ethanol contained LB medium	FA ethyl ester	ND	0.25	Xu et al. (2020)
E. coli BL21 *pC::ff::t*	*fadD, fadR, tesA* and *atfA*-G355I	Complex medium	FA ethyl ester	ND	0.5	Hernández et al. (2015)
E. coli strain Y	*ΔfadE*, overexpressing *tesA, fadR, pdc, atfA,fadD,* and *adhB*	Glucose	FA ethyl ester	ND	1.5	Tao et al. (2015)
E. coli A2A	*ΔfadE*, overexpressing *tesA, fadR, pdc, atfA,fadD,* and *adhB*	Glucose	FA ethyl ester	ND	0.4	Kim et al. (2019b)
E. coli BL21/pDG104/pDG105	Overexpression of *atfA, fadD, tesA, alsS,* and *ilvCD* (*Bacillus subtilis*); *aro10* and adh2 (*Saccharomyces cerevisiae*)	LB medium	FA ethyl ester	ND	0.27	Tao et al. (2015)
E. coli BL21 TL101/pDG102/pMSD15	Overexpression of *tesA, atfA, fadD, ARO10,* and *ADH2*	Yeast extract with glycerol	FA ethyl ester	ND	0.21	Zeng et al. (2018)

TE: Thioesterase; ND: Not Defined.

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Table 6.
Metabolic engineering for the strain development of the high lipid yields oleaginous microbes.

Genes for Enzymes	Sources of the Genes	Recipient Species	Productivity Manipulated*	Reference
ΔAGPase	*Chlamydomonas reinhardtii* BAFJ5	*Chlamydomonas reinhardtii* BAFJ5	+46.4% lipid	Jothibasu et al. (2021)
Antisense PEPC	*Anabaena sp.*	*E. coli* DH5a	+46.9% lipid	Fathy et al. (2021)
ΔAGPase	*Chlamydomonas*	*Chlamydomonas*	10-fold ↑TAG content	Jothibasu et al. (2021)
FAT	*Jatropha curcas*	*E. coli ML103*	>2.0 g/L FA content	Tan and Lee (2017)
	Diploknema Butyracea			
	Ricinus communis			
	Umbellularia californica	*E. coli*	+ FA biosynthesis	
FAT, *fabD* (MAT)	*Streptomyces avermitilis* MA-4680	*E. coli*	+11% FA	Tan and Lee (2017)
ΔTGL3, ΔTGL4	*Y. lipolytica*	*Y. lipolytica*	+lipid	Son et al. (2022)
DGAT	*Arabidopsis*	*Yeast*	3–9 folds ↑TAG content, and 200–600 folds ↑DGAT activity	Son et al. (2022)
SLC1-1 (LPAT)	*Yeast*	*Arabidopsis*	+48% oil content	Zhang et al. (2021)
GPAT	*E. coli*	*Arabidopsis*	+29% oil content	Matsumoto and Awai (2020)
ACC1(ACC)	*Yarrowia lipolytica*	*Yarrowia lipolytica*	2-fold ↑lipid content	Pereira et al. (2022)
accA-D (ACC), tesA (thioesterase I)	*E. coli*	*E. coli*	6-fold ↑FA synthesis	Liu et al. (2014)
ACC1(ACC)	*Mucor rouxii*	*Hansenula polymorpha*	+40% FA content	Nosheen et al. (2021)
ACC	*Acinetobacter calcoaceticus*	*E. coli*	3-fold ↑lipid content	Sitepu et al. (2017)
ACC	*Aspergillus oryzan*	*Aspergillus oryzan*	No significant changes	Cai et al. (2015)
ACL	*Aspergillus oryzan*	*Aspergillus oryzan*	1.9-fold ↑TAG content	Cai et al. (2015)
ACS	*E. coli*	*E. coli*	9-fold ↑ACS activity	Vrablik et al. (2015)
malA (ME)	*Mucor circinelloides*	*Mucor circinelloides*	2.5-fold ↑lipid synthesis	Fazili et al. (2022)
Multi-gene approach to enhance lipid biosynthesis ACC, thioesterase, D fadD (acyl-CoA synthetase)	*E. coli*	*E. coli*	20-fold ↑FA content	Cai et al. (2015)
ACC1, DGAT1	*Y. lipolytica*	*Y. lipolytica*	5-fold ↑lipid synthesis	Bao et al. (2021)
ACP, KAS, FAT	*Haematococcus pluvialis*	*Haematococcus pluvialis*	+FA content	Hu et al. (2021)
GUT2 (GPDH)	*Y. lipolytica*	*Y. lipolytica*	2.9-fold ↑TAG content	Son et al. (2022)
GPD1 (GPDH)	*Y. lipolytica*	*Y. lipolytica*	1.5-fold ↑TAG content	

*+: Increased by percent; -: Decreased by percent; Δ: Knock-out; ↑: Increased by folds; >: more than.

Incorporating four new gene knockouts identified in this study resulted in a strain that can produce 30 g free FA/L in a glucose-fed batch (Fang et al., 2021).

The model non-oleaginous yeast *Saccharomyces cerevisiae* has also been engineered for hyperaccumulation of TAGs. A recent study systematically engineered a series of previously identified gene knockouts and gene overexpression into *S. cerevisiae* to produce a strain that can accumulate 65% TAGs (Arhar et al., 2021). However, a key step in this engineering campaign was to identify a wild-type strain that already accumulated over 25% TAGs, a level of lipid content that would normally be considered an indicator of an oleaginous organism, whereas the lipid content of model *S. cerevisiae* strains like BY4741 and CEN.PK ranges from ~5–10% (Arhar et al., 2021). Ongoing challenges in developing lipid-producing *E. coli* and *S. cerevisiae* for industry include their generally poor tolerance of industrially relevant LCB. *E. coli* and *S. cerevisiae* are sensitive to LCB-derived growth inhibitors like furfurals. Furthermore, the best-performing engineered strains reported to date have been grown using refined sugars and are not optimized for consuming the diversity of carbohydrates in LCB hydrolysates.

The universal applicability of CRISPR/Cas-based engineering has significantly accelerated the modification of oleaginous organisms, overcoming previous limitations in their available genetic tools. The benefits of engineering such organisms are particularly evident in cases where they already exhibit high lipid yields and possess well-established strategies for lipid accumulation during fermentation.

Rhodococcus opacus, an oleaginous prokaryote that can naturally accumulate over 35% TAGs/cellular dry weight (Castro et al., 2016), was extensively engineered in a single study to produce free FAs, FA ethyl esters, and long-chain hydrocarbons (Kim et al., 2019a). In an optimized fermentation, the wild-type strain produced 82.9 g TAGs/L in glucose fed-batch. Overexpression of lipases and lipase chaperones, combined with acyl-CoiA synthetase knockouts, resulted in free FA titers of 50.2 g/L. Alternative engineered strains were developed from this same oleaginous platform and fermentation method, producing high titers of FA ethyl esters (21.3 g/L) and long-chain hydrocarbons (C12-C27, 5.2 g/L). The oleaginous yeast *Yarrowia lipolytica* has also been subjected to metabolic engineering to further enhance its lipid yield. Based on observations of cellular metabolism in mammalian obesity, *Δ9*-stearoyl-CoA desaturase was

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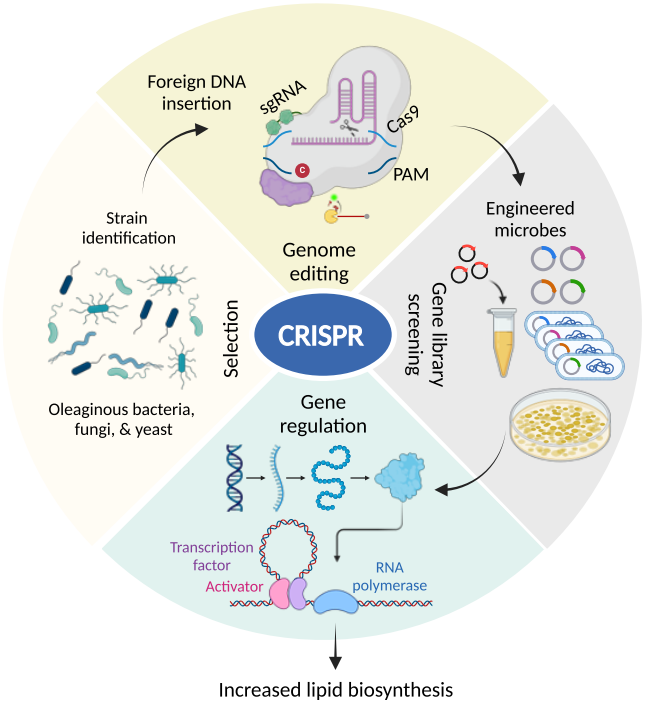

Figure 5. An overview of engineering oleaginous microbes for increased lipid biosynthesis through CRISPR-induced genome editing. This diagrammatic illustration was generated and edited by BioRender.

identified as a potential rate-limiting step in lipid accumulation in *Y. lipolytica* (Qiao et al., 2015). Overexpression of this gene in conjunction with acetyl-CoA carboxylase and diacylglycerol acyl-transferase resulted in yields of 0.23 g lipid/g glucose and high titer and productivity (55 g/L and 0.71 g/L/h) in batch fermentations with 150 g glucose/L (Qiao et al., 2015). Further engineering, including overexpression of additional diacylglycerol acyltransferases and deletion of a key regulator of lipase expression, improved lipid titer to 85 g/L with the accumulation of lipids to 77% DCW (Friedlander et al., 2016), and optimization of redox metabolism to increase the availability of NADPH and decrease oxygen demand improved productivity to 1.2 g FA methyl esters/L/h and a yield of 0.27 g/g glucose (Qiao et al., 2017).

While most intensive metabolic engineering studies in oleaginous organisms have used refined glucose as a carbon source, these organisms are generally more tolerant of industrially relevant biomass hydrolysates. *R. opacus*, in particular, is highly tolerant of lignin-derived small molecules and can assimilate many aromatic compounds from lignin hydrolysate *via* the β-ketoadipate pathway (Anthony et al., 2019). Co-cultivation of *R. opacus* strains engineered to secrete ligninolytic laccase enzymes and overexpress TAG biosynthesis genes enabled lipid production from corn stover at titers of 2.54 g/L in a fed-batch fermentation (Xie et al., 2019). *Y. lipolytica* can tolerate industrially relevant concentrations of most common growth inhibitors found in LCB hydrolysates, including furfural, hydroxymethylfurfural, formic acid, and acetic acid at least 1 g/L, though it is relatively sensitive to formic acid concentrations greater than 1 g/L (Konzock et al., 2021). Tolerance to ferulic acid has been improved to greater than 1 g/L via adaptive laboratory evolution (Wang et al., 2021), and aromatic aldehydes tolerance has been improved by increased expression of an aldehyde ketone reductase (Zhou et al., 2021).

Despite bearing genes encoding the xylose reduction-isomerase pathway, *Y. lipolytica* is generally poor at metabolizing xylose, particularly in the absence of glucose. Overexpression of the native xylose reductoisomerase pathway enables the growth of xylose, but this can be improved by the incorporation of heterologous xylose reductase and xylitol dehydrogenase genes (Ledesma-Amaro et al., 2016). Batch fermentations yielded 0.17 g FAs/g xylose, with xylose as the sole carbon source (Ledesma-Amaro et al., 2016). Adaptive laboratory evolution of an engineered lipid overproducing strain with a similar heterologous xylose reductoisomerase pathway resulted in over 50% of DCW as lipids with xylose as the sole carbon source.

6.4. Engineering methods for microbes other than CRISPR

While the CRISPR-mediated manipulation of oleaginous microbes has exhibited commendable precision and efficiency in augmenting lipid biosynthesis, alternative molecular engineering methodologies have also come to the fore in refining microbial strains. Noteworthy among these are zinc finger nucleases (ZFN) (**Fig. 6A**), transcription activator-like effector nucleases (TALEN) (**Fig. 6B**), and RNA interference (RNAi) (**Fig. 6C**), which have been extensively employed in this domain (Shanmugam et al., 2020). Gram-negative prokaryotic microorganisms are predominantly subjected to lipid overproduction *via* ZFN, whereas oleaginous yeasts such as *S. cerevisiae* and many other eukaryotes favor TALEN for their engineering endeavors (Aouida et al., 2015).

However, ZFN and TALEN encounter formidable limitations, including off-target cutting effects, error-prone digestion of DNA bases, and intricate design processes, significantly compromising their overall efficiency (Manghwar et al., 2020). In response to these challenges, RNAi has emerged as the method of choice for numerous oleaginous microbes, particularly where gene silencing approaches take precedence over genome editing (**Fig. 6C**). Notably, in the realm of oleaginous yeast (*i.e., Rhodosporidium toruloides*) and fungi (*i.e., Mortierella alpina*), RNAi technology has gained prominence as the method of preference for high lipid yield (Liu et al., 2019; Chang et al., 2023).

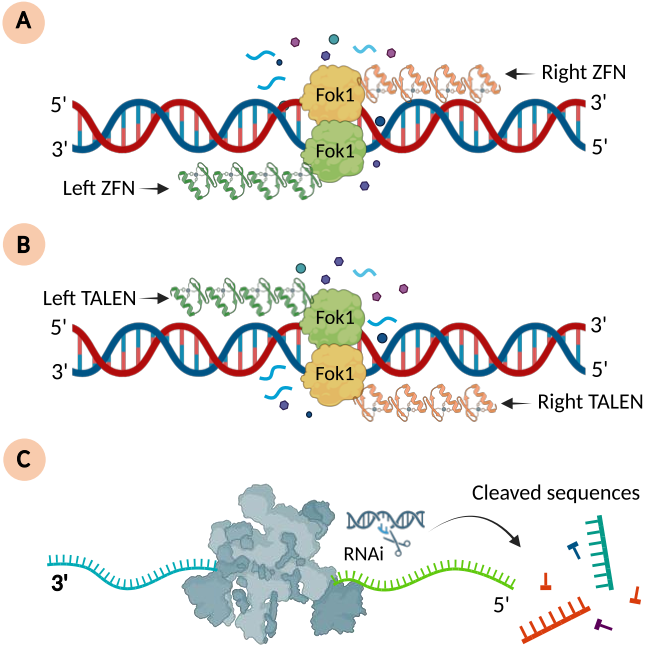

Figure 6. Illustration of different genome editing tools for oleaginous microbes. **(A)** ZFN: Zinc finger nucleases; **(B)** TALEN: Transcription activator-like effector nuclease; and **(C)** RNAi: RNA interference.

7. Trending approaches to converting microbial lipids into value-added products

The biochemical conversion of SCOs into multiple functional components can pave the way for their broad-spectrum applications into multidisciplinary aspects, such as biosurfactants and biocatalysts in chemical industries, nutritional and flavoring additives in food industries, nutraceutical supplements in pharmaceutical industries, detoxifying agents in the environment, and functional bioactive compounds in biomedical industries (**Table 7**). Biochemical approaches render excellence in sustainable downstream processing to achieve maximum distinguishable

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Table 7.
Routes of novel fermentative microbial lipid conversions into functional components.

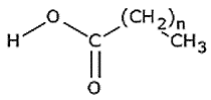
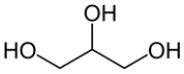
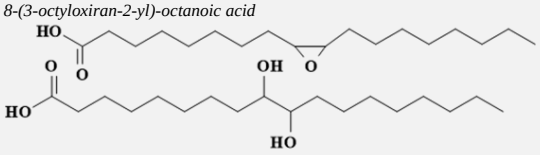
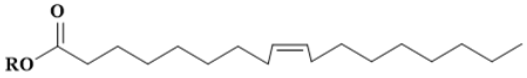
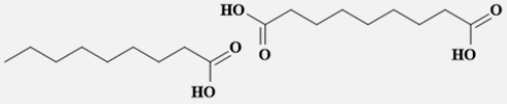
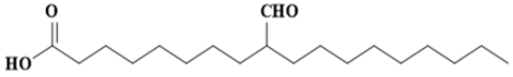
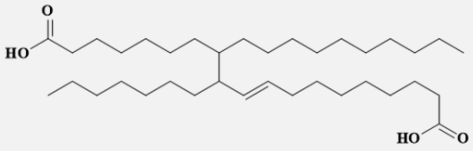
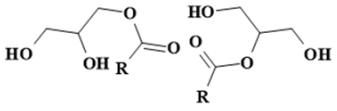
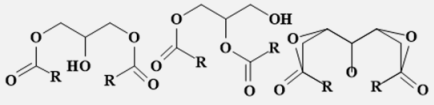
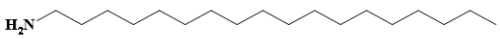
Lipid Types	Reaction Types	Chemical Compounds	Commercial Applications	Reference
TAGs	Hydrolysis	 *FA*	Pharmaceuticals, biosurfactants, cosmetics, biofuel, ethanol, biomedical sciences	Jin et al. (2015)
		 *Glycerol*	Pharmaceuticals, biosurfactants, cosmetics, biofuel, ethanol, and biomedical sciences	
FAs	Alkaline hydrolysis	*8-(3-octyloxiran-2-yl)-octanoic acid*  *9,10-dihydroxyoctadecanoic acid*	antioxidants, light stabilizers, flame retardant, plastic additives	Riecan et al. (2022)
FAs	Acid/base Alcoholic catalysis	 *Alkyl oleate*	Biodiesel	Jin et al. (2015)
FAs	Ozonolysis	 *Nonanal* and *Dicarboxylic acid*	Biopolymers, biodegradable solvents, perfumery, pharmaceuticals, bioplastics, and corrosion protector	Riecan et al. (2022)
FAs	Formylation	 *9-formyloctadecanoic acid*	Polyesters, bioplasticisers, polyacids, and polyols	Syed et al. (2022)
FAs	Dimerization	 *(E)-12-decyl-11-heptylnonadec-9-enedioic acid*	Epoxy coating, adhesives, polyurethanes, printing inks	Syed et al. (2022)
FAs	Acid/base esterification	 *α-Isomer* and *β-Isomer*	Food and surfactant	Marić et al. (2020)
FAs	Acid/base esterification	 *1,3-Isomer*, *1,2-Isomer*, and *TAGs*	Food and surfactant	Marić et al. (2020)
FAs	Amphoteric hydrolysis	 *Octadecan-1-amine*	Gasoline, floating agent, fungicide, bactericide, emulsifier, lubricating additives	Ostermann et al. (2020)

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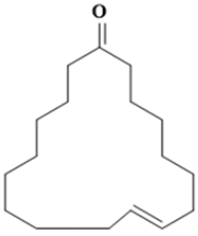
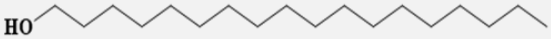
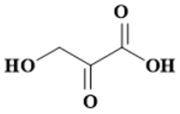
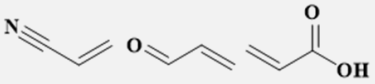
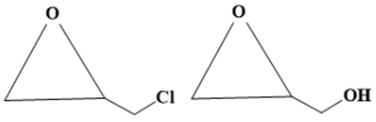
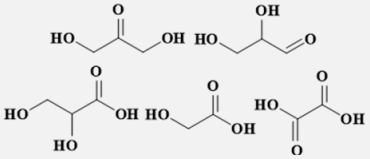
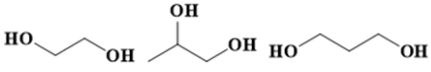
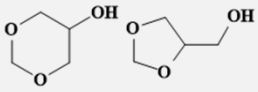
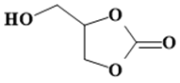
Table 7.
continued.

Lipid Types	Reaction Types	Chemical Compounds	Commercial Applications	Reference
FAs	Amphoteric hydrolysis	 *Cycloheptadec-8-enone*	Pharmaceuticals, oleochemicals, petrochemicals, polymers, pheromones, agrochemicals, fragrances	Yarazari and Jayaraj (2022)
FAs	Hydrogenation	 *Octadecan-1-ol*	Lubricants, surfactants, emulsifiers, plasticizers, oleochemicals	Jin et al. (2015)
FAs	Oxidation	 *3-hydroxy-2-oxopro-panoic acid*	Biocatalysts, intermediate in organic synthesis	Riecan et al. (2022)
FAs	Amphoteric hydrolysis	 *Acrylonitrile, Acrolein, and acrylic acid*	Carbon fiber, organic building blocks (monomers), antimicrobial agents, fumigant	Yang et al. (2019)
FAs	Amphoteric hydrolysis	*2-(chloromethyl)-oxirane*  *2,3-epoxy-1-propanol*	Building blocks at organic synthesis, biopolymers, Stabilizers, biopolymers	Ostermann et al. (2020)
FAs	Hydrolysis	*1,3-dihydroxy-acetone*  *Glyceraldehyde*	Building blocks at organic synthesis, cosmetics, pharmaceuticals, tannin agents	Riecan et al. (2022)
FAs	Hydrolysis	*1,2-ethanediol* *1,3-propanediol*  *Propene-glycol*	Monomers, additives, antifreeze, solvents	Sparsø (2014)
FAs	HD-HG^{L}	*1,3-dioxan-5-ol*  *1,3-dioxan-5-ol*	Semi-toxic solvent used in intravenous injection and other pharmaceutical aspects	Castanheiro et al. (2020)
FAs	Hydrolysis	 *4-(hydroxymethyl)-1,3-dioxolan-2-one*	Monomer, additive, solvent, chemical intermediate	Castanheiro et al. (2020)

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product recovery at minimal time, cost, and waste generation, substituting the conventionally used chemical protocols (Khan et al., 2018). The techno-economic analysis (TEA) of feasible SCO-derived product formation can ensure multidimensional industrial consolidation in sustainable biorefinery management for having variations in product types and quality (Parsons et al., 2019). Besides, SCO-based industries can obliterate selective eco-hazards perilous industries to mitigate the ever-increasing demands of the 4th Industrial Revolution (Nanda et al., 2016).

8. Life cycle and techno-economic assessments of microbial lipids from lignocellulose

Life cycle analysis (LCA) is a method used to assess the environmental impacts of a product throughout its life cycle, from raw material production to disposal (Ali et al., 2024). It is a valuable tool for evaluating the ecological benefits of bioenergy processes, particularly regarding their greenhouse gas balance. While some LCA studies have explored plant fatty oil-based biodiesel processes from LCB, there is room for more comprehensive investigations (Bradley and Maga, 2019). For instance, a comprehensive LCA was conducted to transform yeast lipids from sugarcane bagasse into biodiesel, lube base oil, and BTEX (benzene, toluene, ethylbenzene, and xylene). The study disclosed emissions associated with global warming potential (GWP), indicating values of 260.03 kg CO_{2eq} for non-polar lipid, 572.16 kg CO_{2eq} for lube base oil, 27.83 kg CO_{2eq} for biodiesel, and 85.19 kg CO_{2eq} for BTEX processes. GWP uncertainties remained consistently low, with values less than 0.3 across all processes. However, uncertainties persist in measuring ozone layer depletion, human toxicity, and marine aquatic ecotoxicity. These uncertainties underscore the imperative for process development or improvements in the life cycle inventory to attain lower impact and reduced uncertainties in these categories (Sharma et al., 2020).

A notable biorefinery practice involves synthesizing integrated ethanol and biodiesel from microbial oils (Longati et al., 2022). This innovative process utilized the oleaginous yeast *R. toruloides* to generate 2.55 m^3/h of biodiesel and 71.7 m^3/h of ethanol from four million tons of sugarcane bagasse. The approach yielded a remarkable 86.3 MW of additional bioelectricity, effectively replacing 75.6% of the in-field demands for diesel. Notably, there was a 14.5% increase in annual revenue generation compared to the previous fiscal year despite manipulating the same volume of LCB. This biorefinery process necessitates the operation of microbial oil-type bioreactors, demonstrating lower environmental impacts than conventionally designed first-generation ethanol plants (Longati et al., 2022). TEA and environmental analysis have been integrated for analyzing a microbial biorefinery. This integration was applied in the context of integrated microbial and ethanol-bioelectricity-biodiesel production from the hemicellulose fractionation of LCB, providing a comprehensive assessment of environmental and economic aspects (Longati et al., 2022).

The assessment primarily focused on the impact of selectively operating anaerobic digestion processes to curb the escalation of methanogenesis (Gálvez-Martos et al., 2021). This targeted approach led to a substantial reduction in the generation of toxic byproducts and fumigation. Notably, agro-industrial waste, comprising 65.5% of the total, exceeded alternative sources like microalgae (21.6%) in carbohydrate content. The findings indicate that VFA production from agro-industrial wastes produced higher CO_{2eq} output (~350 kg) than microalgal wastes (~180 kg). Despite the increased byproduct generation, the productivity of compost, which retains carbohydrates and VFAs collectively from agricultural residues, proved surprisingly higher than that of microalgal compost. This finding translates to approximately ~460 and ~160 kg CO_{2eq}, respectively. Consequently, the retention of additional carbons in compost forms contributes to a reduction in stratospheric ozone depletion (~0.0049 kg CFC11 eq), establishing agricultural residues as a more effective alternative compared to previously reported microalgal usage (Gálvez-Martos et al., 2021).

By maintaining oxygenates and lignin monomers below 25% in bio-oil, it is possible to achieve a six-fold increase in levoglucosan productivity while simultaneously reducing unwanted acidification by about eightfold, consequently mitigating global warming effects (Zhang et al., 2023). In the context of oleaginous fungal lipid synthesis for the formulation of ethanol and biodiesel, strategies have been employed to minimize the emission of toxic secondary metabolites into the environment. Nitrogen-limited conditions in the fermenter facilitate a full-swing de novo synthesis of TAGs from acetyl-CoA. Additionally, highly concentrated carbon within the culture medium significantly enhances the fungal lipid conversion rate, allowing for the biological recycling of additional toxic carbon atoms in a more environmentally friendly manner (Hosseinzadeh-Bandbafha et al., 2020). Furthermore, research suggests that optimizing fungal lipid production from LCB demonstrates strong economic feasibility due to minimal nitrogen and oxygen requirements under culture conditions (Hosseinzadeh-Bandbafha et al., 2020).

Considering the factors related to LCA and TEA, oleaginous yeast and fungi hold greater significance in microbial lipid synthesis from LCB compared to bacterial systems. This promising result is attributed to superior energy efficiency, cost-effectiveness, eco-friendliness, market applications, productivity, and operational safety considerations, facilitating the realization of an operationally zero-waste circular bioeconomy (Caporusso et al., 2022).

9. Social and political acceptance concerning bioenergy

The evaluation of social sustainability encompasses various indicators, including energy factors, food security, socio-economic aspects, and promoting a healthy social environment. Repurposing waste biomass from sugarcane biorefining not only enhances soil quality and enriches it with valuable nutrients but also contributes to social development in rural areas. Oligosaccharides, used as prebiotics for medicinal and nutraceutical purposes, play a crucial role in safeguarding public health (Chen et al., 2023). Many countries incentivize LCB biorefineries through policies, such as subsidies and tax-free systems, intending to boost biofuel production. For example, integrated microbial and sugarcane biorefineries, supported by policies like RenovaBio, yield cost-effective ethanol with reduced carbon emissions (Chandel et al., 2021). Another approach involves implementing combined cycle power systems in sugarcane and eucalyptus biorefineries, aiming to decrease greenhouse gas emissions and enhance the overall value chain of the biorefinery (Bressanin et al., 2020). The waste-to-energy concept is pivotal in establishing a circular bioeconomy, transforming banana waste into briquettes for heat or electricity. This approach, integrated with effective waste management policies and financial investment, fosters long-term sustainable development (Ashokkumar et al., 2022).

Moreover, numerous countries have embraced nationwide practices promoting a circular bioeconomy, aligning their policies with the increasing demand for environmentally friendly technologies. Brazil, for instance, has implemented a regulated market for decarbonization credits, enabling fuel distributors to reduce their carbon footprint either through credit purchases or the adoption of low-impact biofuels (Dhamodharan et al., 2020). In India, efforts to diversify feedstocks for ethanol production have been underway since 2018. This strategy involves allowing the use of sugarcane juice, utilizing surplus grain for ethanol production, and facilitating the establishment of supply chain systems for biodiesel production from non-food organic sources. Following its national biofuel policy, Malaysia mandates adherence to blending directives for palm oil biodiesel and petroleum diesel (5 vol% biodiesel+95 vol% diesel). A licensing and regulatory system oversees blending, storage, transportation, and export operations related to biofuels in the country (Subramaniam et al., 2021). Germany employs a blending quota system, providing tax exemptions exclusively for pure biofuels. This system incentivizes oil companies to meet quotas and allows biofuel producers to sell surplus quotas to oil firms (Ebadian et al., 2020). Support for business groups and consumers has been consistent in the USA, especially in the aftermath of novel biorefinery product recoveries. The country has also developed a roadmap for achieving a budget-friendly, low-carbon economy. This strategic plan emphasizes biomass production and utilizing LCB waste, converting it into value-added products such as bioenergy and innovative bio-based solutions (Pascoli et al., 2022).

10. Current challenges and future directions

The potential of utilizing various systematic processes, enzymatic hydrolysis, and fermentation approaches for refining LCB into biofuels and other functional products is impeded by several significant barriers. These include the altered catabolic activity of enzymes, the presence of inhibitors, operating costs, and the need for specialized expertise (Agrawal et al., 2021). Furthermore, using genetically and metabolically engineered

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microorganisms raises environmental concerns in microbial lipid synthesis, especially in accidental release into the natural environment (Rafeeq et al., 2023). Many of these oleaginous bacterial and fungal strains are potential environmental hazards and pose risks to human health due to their high pathogenicity (Al-Mamun et al., 2016; Al Azad et al., 2016). Addressing these concerns necessitates the development of an integrated biological, biochemical, and biophysical environment that considers the physicochemical aspects of LCB biorefinery.

In the foreseeable future, metagenomic techniques offer the potential to revolutionize VFA production processes by providing methods to selectively eliminate undesirable microorganisms that compete for resources, thereby reducing VFA output. This integration, alongside a thorough analysis of microbial communities—especially through complete metagenome sequencing—deepens the understanding of these ecosystems and optimizes operational aspects. This approach brings the industry closer to realizing the viability of VFA production through fermentation (Varghese et al., 2022).

Furthermore, the application of artificial intelligence is poised to usher in transformative changes in LCB biorefinery industries. An artificial intelligence model has been developed, incorporating predictors for various pretreatment and enzymatic hydrolysis parameters, such as cellulose, hemicellulose, lignin content, cellulose-lignin ratio, acid concentration, temperature, time, pretreatment severity, and enzyme concentration (Haldar et al., 2023). The introduction of such technologies is expected to become increasingly prevalent in achieving maximum sugar molecule recovery. Moreover, leveraging artificial intelligence will enhance the precision of large-scale LCB pretreatment. This improvement includes deploying both partially and fully automated sensors and robotic systems for sophisticated and critical pretreatment steps, ensuring efficiency in processes that are challenging for individual operations (Velidandi et al., 2023). The systematic integration of machine learning algorithms in maintaining an alert and optimized working environment, regulating parameters such as temperature, pH conditions, presence of unwanted compounds in the hydrolysate, and toxicity indexing of the reaction chamber represents a promising avenue for high-throughput LCB fractionation in biofuel industries (Phromphithak et al., 2021). Besides, the optimization of molecular dynamic simulation (Rahman et al., 2023) and quantum mechanics behind the DFT profiles of different pretreatment-solvent systems (Xie et al., 2024) have been emphasized in recent times in predicting the quality and types of fermentative products expected to recover from consecutive biochemical reactions.

Robust and straightforward genetic modification methods have been firmly established in industrially relevant oleaginous organisms. Over the past decade, genetic engineering efforts have predominantly concentrated on enhancing lipid yields and titers. The logical progression in developing economically viable biorefineries is the optimization of these organisms for the assimilation of ultra-low-cost LCB. This approach involves integrating high lipid production genotypes with tolerance to growth inhibitors derived from LCB and maximizing total available carbon assimilation. Additionally, there is potential for further value creation by genetically engineering organisms to produce specific high-value lipids. For instance, enhancing arachidonic acid production in the oleaginous fungus *Mortierella alpina* (Hao et al., 2015) or eicosapentaenoic acids (EPA) in the oleaginous microalgae *Nannochloropsis* (Liu et al., 2022) represents a promising avenue in this regard. EPA has been reported to be used in formulating different anti-neoplastic agents using systems and structural biochemistry (Morshed et al., 2023).

11. Conclusions

Using microbial lipid-based biorefinery for LCB in biofuel and value-added product production represents a novel trend in industrial biotechnology, offering eco-friendly alternatives to reduce dependence on fossil fuels. The core processes of LCB biorefinery, namely pretreatment, enzymatic hydrolysis, and fermentation systems, are crucially regulated. Combined pretreatment methods have shown superior effectiveness in LCB fractionation compared to other approaches. Notably, genetically and metabolically modified oleaginous microorganisms, including bacteria, fungi, and yeast, can biosynthesize significantly higher quantities of lipids than their natural, wild-type counterparts. Additionally, the efficiency of microbial lipid metabolism is heavily influenced by fermentation methods and conditions. Among various fermentation models, the order of viability for microbial lipid production is SHLP > SSLP > SSF.

The optimization of biorefinery conditions, addressing inhibitors and environmental toxicity, is poised for significant advancements in the future. Systematic integration and operational modeling of pretreatment, enzymatic hydrolysis, and fermentation, utilizing artificial intelligence, alongside advanced microbial strain-improving techniques like transcription activator-like effector nucleases, ZFN, TALEN, RNAi, and CRISPR-Cas9, are anticipated to play a pivotal role. Furthermore, integrating molecular dynamic simulation to study enzyme targets, considering their structural and functional properties in catabolizing complex carbohydrates, represents an innovative approach to exploring microbial lipid-based biorefinery processes.

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Supplementary Data

Table S1.
Differential lipid biosynthesis from SSF depends on the substrates, microorganisms, and fermentation modes over different times.

Substrate types	Microbes	Pretreatment Methods	Fermentation Process	Lipid Productivity			Reference
				BC (g/L)	LC (g/L)	LYS (g/g)	
Wheat straw and bran mixture	*Aspergillus oryzae* A-4	0.7% H_2SO_4 (121 °C for 1 h)	Batch	9	0.54	0.06	Khot (2022)
	Microsphaeropsis spp.	Exploded steam (15% water, 1.5 MPa, 10 min)	Batch	15	0.6	0.04	Šantek et al. (2021)
	Sclerocystis spp.			10	0.7	0.07	
	Phomopsis spp.			8	0.64	0.08	
	Cephalosporium spp.			10	0.45	0.02 – 0.03	
	Nigrospora spp.			5	0.15	0.03 – 0.04	
Palm empty fruit branch and kern cake	*Aspergillus tubingensis* TSIP9	10% NaOH (100 °C for 15 min)	Batch	5	0.4	0.08	Chattopadhyay et al. (2021)
			Feed batch	5	0.35	0.7	Fang et al. (2016); Šantek et al. (2021)
			Repeated batch	10	0.9	0.09	Fang et al. (2016)
Palm processed fiber and palm empty fruit branch	*Aspergillus tubingensis* TSIP9	0.5% H_2SO_4 (121 °C for 1 h)	Batch	10	0.9	0.09	Cheirsilp et al. (2022)
Rice straw and wheat bran mixture	*Alternaria* spp.	DA	Batch	12	0.72	0.06	Bao et al. (2021)
				7	0.56	0.08	
	Colletotrichum spp.			9	0.63	0.07	
				11	0.88	0.08	

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Table S2.
SHLP fermentation system for microbial lipid biosynthesis.

Substrate Types	Microbial Types	Names	Pretreatment Methods	Fermentation Process	Lipid Productivity			Reference
					BC (g/L)	LC (g/L)	LYS (g/g)	
Corn stoves	Fungi	*Cryptococcus curvatus*	Ionic liquid (140 ºC for 1 h)	Batch	16.5	7.2	0.14	Šantek et al. (2021)
			NaOH (0.5 M; 80 ºC for 75 min)	Batch	27.7	12.4	0.15	
		Mortierella isabellina	Hot steam (200 ºC for 7 min)	Batch	36.1	18.7	0.05	Fang et al. (2016)
			NaOH (1%, 121 ºC for 2 h)	Batch	10.9	2.5	0.02	
		Rhodosporidium toruloides	Combined NaOH (0.4%) and H_2SO_4 (0.8%)	Batch	36.2	21.4	0.2	Zhao et al. (2022)
			H_2SO_4 (1%) 140–180 ºC for 5–10 min	Batch	22.5	7.6	0.15	Dai et al. (2019)
		Trichosporon cutaneum	H_2SO_4 (1%) 190 ºC for 3 min	Batch	3.2	1.0	0.05	Zhao et al. (2022)
			NaOH (variable concentration, time, and temperature)	Batch	11	2.5	0.21	Chattopadhyay et al. (2021)
Corn cobs	Fungi	*Rhodotorula glutinis*	H_2SO_4 (0.5%) and H_3PO_4 (1.5%) mixed	Batch	15.1	5.5	0.13	Fang et al. (2016)
		Trichosporon cutaneum	ND	Batch	38.4	12.3	0.13	Zhao et al. (2022)
Sweet sorghum bagasse	Fungi	*Cryptococcus curvatus*	Microwave radiation at 100 ºC (~4 min)	Batch	15.5	7.3	0.11	Fei et al. (2016)
Switchgrass	Fungi	*Rhodosporidium toruloides*	H_2SO_4 (0.9%)	Batch	42.6	26.2	0.16	Slininger et al. (2016)
Jerusalem artichoke	Fungi	*Cryptococcus* spp.	HNO_3 (0.57%) for 49 min	Batch	6.1	4.3	0.05	Šantek et al. (2021)
Plant residues	Fungi	*Mortierella isabellina*	SO_2 (140 ºC for 1 h)	Batch	25.5	14.4	0.18	Harde et al. (2016)
Wheat straw	Fungi	*Cryptococcus* spp.	DA	Batch	5.8	2.6	0.05	Liu et al. (2020)
	Fungi	*Mordellistena isabellina*	DA	Batch	4.3	2.2	0.04	Harde et al. (2016)
	Fungi	*Rhodotorula* spp.	DA	Batch	3.5	1.6	0.03	Liu et al. (2020)
Rice straw	Fungi	*Trichosporon mycotoxinivorans*	DA	Batch	11.5	4.8	0.11	Sagia et al. (2020)
Kraft hardwood pulp	Fungi	*Rhodococcus opacus*	ND	Batch	11.0	4.1	0.2	Sagia et al. (2020)
Sweet sorghum bagasse	Yeast	*Lipomyces starkeyi*	ND	Batch	6.4	2.3	0.08	Rolz et al. (2019)
Switchgrass	Yeast	*Lipomyces tetrasporus*	H_2SO_4 (0.9%)	Batch	53.4	29.0	0.16	Slininger et al. (2016)
	Yeast	*Lipomyces kononenkoae*	H_2SO_4 (0.9%)	Batch	47.7	28.1	0.15	
Wheat straw	Yeast	*Lipomyces starkeyi*	DA	Batch	4.6	2.3	0.04	Liu et al. (2020)
	Yeast	*Yarrowia lipolytica*	DA	Batch	0.4	0.21	0.03	
Defatted rice bean	Yeast	*Yarrowia lipolytica*	H_2SO_4 mediated	Batch	5.2	3.8	0.65	Lazar et al. (2018)
Sugarcane bagasse	Yeast	*Yarrowia lipolytica*	HCl mediated	Batch	6.7	5.1	0.33	Lazar et al. (2018)
Sugarcane bagasse	Bacteria	*Tepidibacillus fermentans*	DA	Feed batch	15.8	8.6	0.14	Sagia et al. (2020)

BC: Biomass concentration; LC: Lipid concentration; LYS: Lipid yield / g of pretreated LCB; DA: Dilute acid; ND: Not Defined

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Table S3.
Lipid biosynthesis using the SSLP fermentation system.

Substrate Types	Microbes	Pretreatment Method	Fermentation Process	Biological Conversion and Productivity Profiling			Reference
				BC (g/L)	LC (g/L)	LYS (g/g)	
Corn stove	*Cryptococcus curvatus* ATCC 20509	0.5 M NaOH (80 ºC for 75 min	Batch	11	11.9	0.17	Liu et al. (2020)
			Feed batch	10	15.9	0.2	Dai et al. (2019)
	Cryptococcus curvatus	1-ethyl-3-methylimidazolium acetate (140 °C for 1 h)	Batch	5	6	0.1	Chaiyaso et al. (2018)
Douglas fir forest residue	*Mortierella isabellina* NRRL 1757	11 g/L of SO_2 (140 °C for 60 min)	Batch	VA	17	0.2	Harde et al. (2016)
		11 g/L of SO_2 (140 °C for 2 h)	Batch	11	11.7	0.2	
Corn stove	*Trichosporon cutaneum*	H_2SO_4-presoaking (190 °C, 3 min)	Batch	10	3.1	1.0	Wang et al. (2020)

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