

Review

Harnessing the Power of Mutagenesis and Adaptive Laboratory Evolution for High Lipid Production by Oleaginous Microalgae and Yeasts

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Abstract: Oleaginous microalgae and yeasts represent promising candidates for large-scale production of lipids, which can be utilized for production of drop-in biofuels, nutraceuticals, pigments, and cosmetics. However, low lipid productivity and costly downstream processing continue to hamper the commercial deployment of oleaginous microorganisms. Strain improvement can play an essential role in the development of such industrial microorganisms by increasing lipid production and hence reducing production costs. The main means of strain improvement are random mutagenesis, adaptive laboratory evolution (ALE), and rational genetic engineering. Among these, random mutagenesis and ALE are straight forward, low-cost, and do not require thorough knowledge of the microorganism's genetic composition. This paper reviews available mutagenesis and ALE techniques and screening methods to effectively select for oleaginous microalgae and yeasts with enhanced lipid yield and understand the alterations caused to metabolic pathways, which could subsequently serve as the basis for further targeted genetic engineering.

Keywords: oleaginous; microalgae; yeast; lipid; mutagenesis; adaptive laboratory evolution

1. Introduction

Microorganisms with the inherent ability to synthesize and accumulate lipids to over 20% of their dry cell weight (DCW) are termed oleaginous. These microbial lipids are also called single-cell oils (SCO) as they resemble lipids and oils extracted from plants and animals [1,2]. The extent of lipid accumulation by oleaginous microorganisms depends on their genetic profile and varies considerably among species and even among strains of the same species [3]. A crucial factor affecting lipid accumulation is nutrient limitation, particularly nitrogen deprivation in the presence of excess carbon, which is directed towards lipid synthesis [4]. The lipids are mostly composed of triacylglycerols (TAGs) and, to a lesser extent, of stearyl esters [5,6]. TAGs comprise three fatty acid chains with a glycerol backbone and can be utilized for the production of biodiesel (fatty acid methyl esters) and other fuels, high-value nutraceuticals (omega-3 and omega-6 fatty acids), natural pigments, and cosmetics. In the context of escalating greenhouse gas (GHGs) emissions and climate change, the development of cost-competitive and eco-friendly biofuels and bioproducts is essential. Biodiesel is a prime example of TAG-based biofuels that require low feedstock and production costs to become cost-competitive with fossil fuels [7]. Currently, plant-derived oils, such as palm, corn, and soybean, are being utilized for biodiesel production, but competition with food crops for land and nutrients (nitrogen and phosphorous) raises serious sustainability concerns about food vs. fuel [8]. Additionally, the upsurge in market demand for essential lipids, such as the omega-3 fatty acids; α linolenic acid

(C18:3n3), eicosapentaenoic acid (C20:5n3), and docosahexaenoic acid (C22:6n3), and the omega-6 fatty acids; γ -linolenic acid (C18:3n6) and linoleic acid (C18:2n6), necessitates the development of alternative lipid sources [9]. Furthermore, palmitic acid (C16:0) is utilized as an emollient and diluent by the cosmetics industry [10].

In this regard, oleaginous microalgae, yeasts, and fungi, are on the forefront of lipid biotechnology research. Microalgae are unicellular photosynthetic microorganisms that convert sunlight and CO₂ into biomass. The key advantages of microalgae as sources of lipids include fast growth rate, CO₂ fixation, ability to grow in waste, brackish, or saline water, ability to tolerate a range of abiotic conditions, such as temperature, salinity, and pH, and ability to also consume organic carbon, thus being able to grow on abundant and low-cost carbon, nutrient, and water sources [11–13]. To date, several oleaginous microalgae have been reported to accumulate up to ~70% lipid yield (% of DCW) in their biomass under stress conditions [14], while they produce a variety of lipids that vary among species and strains and depend on cultivation conditions [15]. In parallel with microalgae, oleaginous yeasts are non-pathogenic asexual unicellular microorganisms that have also emerged as excellent sources of lipids [8,9]. In particular, yeasts belonging to the genera *Yarrowia*, *Candida*, *Rhodotorula*, *Rhodospiridium*, *Cryptococcus*, *Trichosporon*, and *Lipomyces* can accumulate up to ~80% lipid yield in their biomass under stress conditions, while being cultivated on a wide range of carbon sources [16–18]. Lipids synthesized by fungi, particularly by Zygomycetes species like *Mortierella* sp. and *Mucor* sp., have been used for production of mainly polyunsaturated fatty acids (PUFA), such as eicosapentaenoic acid, docosahexaenoic acid, γ -linolenic acid, and α -linolenic acid [3,19]. Indeed, classical mutagenesis using methylnitrosoguanidine (MNNG) has been reported to increase rare PUFA production in fungi [20–23]. For instance, mutants of *Mortierella alpina* IS-4 defective in Δ^5 desaturase (Mut 44) showed increased γ -linolenic acid content (33.9%, *w/w*) as compared to wild type (7.8%; *w/w*) at 12 °C [21]. Furthermore, the mutant with lower n-3 desaturation activity (Y11) showed increased α -linolenic acid content (4.97 mg/mL) but similar eicosapentaenoic acid as the wild type (3.74 mg/mL) [23]. However, the present review will focus on the most widely reported lipid producers, namely oleaginous microalgae and yeasts.

Despite the plethora of research data on oleaginous microalgae and yeasts, large-scale production is still not economically viable. The major drawback is that the energy needed for lipid production is currently higher than the energy output [24]. The main factors that have impeded industrial production are low lipid productivity (production rate), high cost of agitation and aeration during cultivation, and high cost of downstream processing (mainly harvesting and drying) to extract and purify lipids from biomass [25]. As a result, the oil content of oleaginous microorganisms should reportedly be at least 40% and should be accompanied by robust and fast growth for economic feasibility of large-scale production [4]. In order to bridge the gap between lab-scale and commercial production, we need to develop more productive strains. This can be readily achieved through strain improvement with random mutagenesis, adaptive laboratory evolution (ALE), or targeted genetic engineering. Given that the latter has already been covered by recent reviews [16,26–28], the present review focuses on the simple and low-cost strategies of random mutagenesis and ALE for strain improvement and on the screening strategies that are subsequently employed to identify high lipid yield strains among the mutated or evolved oleaginous microalgae and yeasts.

2. Methodology for Strain Improvement

For an oleaginous microorganism (yeast or microalgae) to be promising at industrial scale for lipid production, it needs to possess certain key characteristics: (1) Fast growth rate; (2) robustness; (3) high lipid content; (4) ability to survive under varying environmental conditions; (5) ability to grow in low cost media, such as lignocellulosic hydrolysates, wastewater, and brackish or saline water; (6) good cell flocculation/settlement properties; (7) ease of downstream processing and lipid extraction; and (8) ease of genetic manipulations. Most strains isolated from natural environments cannot meet these industrial standards, necessitating the efforts for strain improvement. Strain improvement strategies aim at the

development of robust strains that possess most of the desired key characteristics outlined earlier [29]. Three available techniques for strain improvement are classical or random mutagenesis, adaptive laboratory evolution (ALE), and genetic engineering [30]. Among them, mutagenesis and ALE offer the advantages of being comparatively straight forward and inexpensive, requiring little knowledge of the microorganism's genetic composition, biochemical pathways, and genetic regulation, and needing few technical manipulations and tools [12,13]. Furthermore, strains generated via mutagenesis and ALE do not come under the strict regulatory restrictions of genetically engineered microorganisms, thus requiring no or less legal and regulatory permitting before deployment at commercial scale [31].

2.1. Use of Random Mutagenesis

Random mutagenesis in yeasts and microalgae can be carried out by using physical or chemical mutagens or insertional mutagenesis, which cause alterations to the organism's DNA, followed by selection of mutants with the desired metabolic properties. The physical mutagens include exposure of microbial cells to radiation (ultraviolet, ion beams, nuclear, X- and γ -rays) or atmospheric and room temperature plasma (ARTP) (Figure 1). Ultraviolet (UV) radiation at 250–290 nm induces either formation of thymine dimers that cause transition of guanine (G) and cytosine (C) to adenine (A) and thymine (T) or deletion of A-T base pairs in the DNA [32]. The heavy-ion beam utilizes heavier charged particles, such as $^{12}\text{C}^{6+}$, which cause higher mutation rates and a wider spectrum of mutations due to their high linear energy transfer (LET) as compared to X and γ -rays [33]. Energy deposition by heavy-ion beams causes double strand breaks in the DNA, base substitutions, and small deletions/insertions [34]. Another technique is the use of the low energy nitrogen (N^+) ions, which damage only the microbial cell surface, and then the ion implantation generates free radicals inside the cell causing DNA damage and a high mutation rate [35,36]. In addition, gamma radiation from radioactive cobalt ^{60}Co possesses strong penetration power and results in reactive oxygen species (ROS) generation, such as free radicals (O_2^- and $\text{OH}\cdot$) and non-radicals (H_2O_2 and $^1\text{O}_2$) from water radiolysis, which subsequently cause cellular DNA damage [37].

ARTP is an emerging microbial mutation breeding technique produced by ionized pure helium discharge plasma in a high frequency electric field [38]. The radio frequency (RF) is generated uniformly under atmospheric pressure (~760 Torr) at room temperature (~300 K) without any vacuum and is applied to the microbial cell surface through a nozzle [13,21]. Under certain conditions of RF power input, helium rate, plasma distance, and treatment time, ARTP causes sublethal damage to the DNA, enzyme activities, and the metabolism of microbial cells [39,40]. Compared to the other physical mutagenesis techniques, ARTP is advantageous, since it has low operating cost and greater operational flexibility, does not require an expensive and complicated vacuum system, and operates at room temperature, thus limiting thermal damage to the oleaginous microorganisms [40].

Chemical mutagenesis involves the use of chemicals that react with DNA and cause errors in base pairing, deamination of purines and transitions, transversions, and frameshift mutations [29]. The chemical mutagens can be classified into four broad categories, as summarized in Figure 1: (1) Intercalating agents that insert themselves in DNA resulting in genetic frameshift mutations (acridine orange, ethidium bromide, proflavine, and acriflavine); (2) base analogs that have similar chemical structure to nucleic acids causing base transitions (5-bromouracil, 5-bromodeoxyuridine, and 2-amino purine); (3) non alkylating reagents that cause crosslinking between DNA and proteins, deletions, and deamination (formaldehyde, hydroxylamine, methoxamine, nitrous acid, bisulphite and hydrazine); and (4) alkylating agents (alkyl sulphates and N-nitroso compounds) [41]. It should be noted that alkylating agents are the most commonly used mutagens for enhancing the lipid yield of oleaginous microorganisms, including ethyl methane sulphonate (EMS), methyl methane sulfonate (MMS), diethyl sulphate (DES), and N-methyl-N-nitro-N-nitrosoguanidine (NTG) [29]. The alkylating agents add an alkyl group to the hydrogen-bonded oxygen at N^7 and N^3 positions of G and A base pairs, respectively [41]. This leads to a N-glycosidic bond that undergoes hydrolysis resulting in depurination and pairing errors in DNA [29]. For instance, EMS selectively removes C and G base pairs resulting in

C/G to A/T transitions, while NTG induces a wide spectrum of mutations by targeting the replication fork [42,43].

Another mutagenesis technique is insertional mutagenesis, which has also been utilized to augment the lipid yield in oleaginous microalgae [44,45]. Insertional mutagenesis involves the addition of exogenous DNA into genomic DNA via non-homologous recombination using a plasmid with an antibiotic resistance marker [44]. The insertion results in gene disruption, mostly null mutation, that alters metabolic activity potentially leading to high lipid accumulation [45].

2.2. Use of Adaptive Lab Evolution

Unlike mutagenesis, adaptive lab evolution (ALE), also called evolutionary engineering, exploits the flexibility of microorganisms to adapt to adverse environmental conditions for the purpose of strain improvement [46]. During ALE, the selected microorganism is continuously cultivated under defined stress conditions (such as high temperature, salinity, nutrient deprivation) for prolonged periods of time (weeks to years) to generate better or improved phenotypes (Figure 1) [47]. Such adaptation results in accumulation of several mutations in the DNA, including single nucleotide polymorphisms (SNPs) and small-scale insertions and deletions, leading to an evolved strain with improved characteristics, such as high growth rate, high lipid content, tolerance to toxic compounds, or capability to survive under extreme environment conditions [48]. Although ALE results in the generation of several phenotypes during the evolution process, only the dominant phenotypes manage to survive, eventually leading to a heterogenous population [48]. One key variable in ALE is the amount of environmental stress imposed on the microorganism: If the stress is very low, the efficiency of ALE will be low and it will take a significant amount of time for evolution to occur, whereas if the stress is high, the microbial cells may not survive [49]. Compared to targeted genetic engineering, ALE causes alterations in multiple genes at the same time resulting in enhanced fitness of the evolved microorganism. The two most commonly used techniques for ALE are batch and continuous cultivation [48]. In batch cultivation, the adaptation is performed in shake flasks or deep-well plates with small culture volumes, and at regular intervals the culture is serially transferred to new shake flasks or wells containing fresh media [48]. The advantages of batch cultivation include inexpensive lab equipment and low cost of chemicals, ease of conducting multiple experiments in parallel, and well-controlled abiotic conditions, such as temperature and mixing [48,50]. However, batch cultivation suffers from variable population density and fluctuating pH, dissolved oxygen, and nutrient availability that affect the growth rate of the microorganism [48]. On the other hand, continuous adaptation is performed in bioreactors operated under tightly regulated pH, dissolved oxygen, and nutrient availability that result in a rather steady growth rate and population density [48]. Its main drawback is the higher cost of equipments and the difficulty in running multiple adaptations at the same time [51]. Indeed, the choice of method depends primarily on whether nutrient limitation is important. Furthermore, the critical factors that affect the end ALE population structure are passage size, rate of mutation, and frequency of beneficial mutations [51]. Among these factors, passage size, which is the number of cells inoculated for gradual adaptation, can be controlled and optimized experimentally, hence controlling the rate of evolution and stacking beneficial mutations in the adapted microorganism [50]. For instance, if the passage size is very small, the rate of mutation decreases, whereas if the passage size is very large, it increases resource utilization with continuous loss of beneficial mutations over time. Ideally, the passage size is between 13.5% and 20%, but this number can vary depending on the microorganism to be adapted and decreases with every round of adaptation [50].

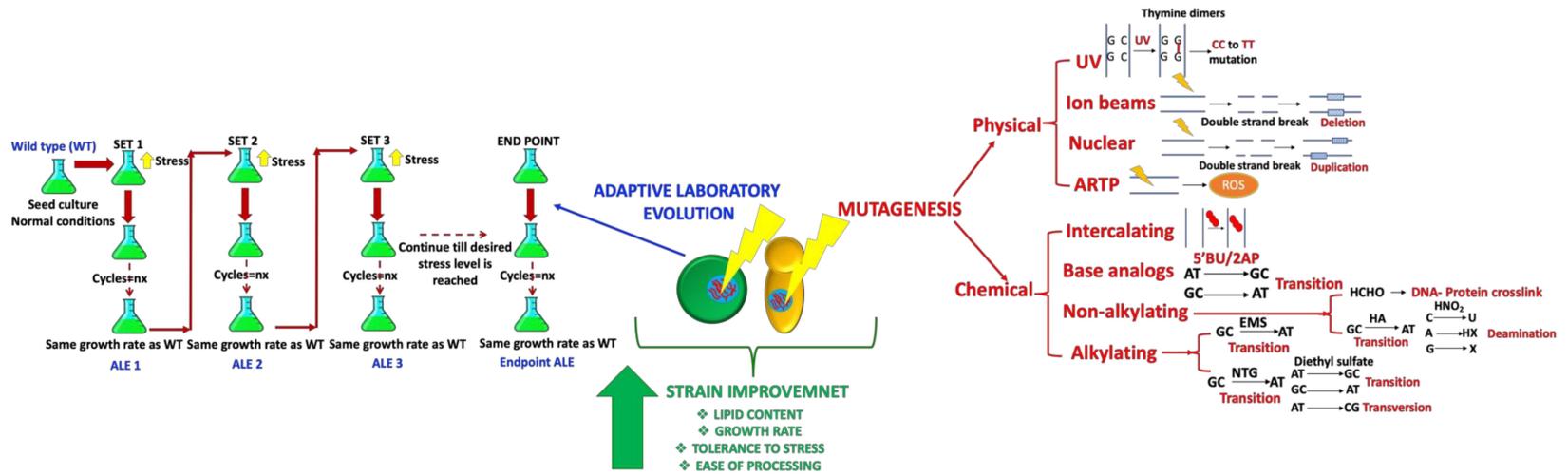


Figure 1. Overview of mutagenesis and adaptive laboratory evolution (ALE) strategies for strain improvement of oleaginous yeasts and microalgae.

2.3. Screening Methods for Selecting Promising Mutants

Random mutagenesis results in generation of hundreds of mutated colonies, making the screening and selection of high lipid producing cells time consuming and challenging. The conventional screening methods employ manual selection by cultivating each mutant colony in shake flasks and then analyzing it for lipid content, which is cumbersome and inefficient [52]. The utilization of large-scale screening methods, including fluorescence-activated cell sorting (FACS), percoll density gradient centrifugation, and chemical inhibitors of lipid synthesis makes the selection process more selective and faster. FACS appears to be the most efficient method to isolate individual cells with the desired properties from a mutant library. High lipid accumulating cells are sorted by staining all the mutated cells with lipophilic dyes, such as Nile red (9-diethylamino-5H-benzo [α] phenoxazine-5-one) or BODIPY 505/515 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene), which bind to neutral lipids emitting yellow ($\lambda = 488/510$ nm) or green ($\lambda = 488-530/575-580$ nm) fluorescence, respectively [53]. The mutated cells, sorted at a rate of 10,000 cells/sec, showing maximum fluorescence, as a result of high lipid content, are sorted out by the FACS machine [54]. Percoll density centrifugation selectively sorts the mutant cells based on their relative densities [55]. The low-density cells are enriched during the procedure, as they are the ones with high lipid content, since lipids have lower density (0.9 g/cm^3) than other cellular biomolecules, such as carbohydrates, proteins, and nucleotides [56].

Lipid biosynthesis inhibitors offer an indirect way to select for mutant cells that show higher tolerance to the chemical. The cells capable of tolerating high concentrations of the lipid inhibitor either enhance the metabolic synthesis or activity of the inhibited enzyme to alleviate the effect of the chemical or activate alternate pathways to bypass the inhibition of lipid accumulation, thereby increasing overall lipid synthesis. The triacylglycerol (TAG) synthesis in both yeasts and microalgae begins with the formation of acetyl-CoA from pyruvate catalyzed by the pyruvate dehydrogenase complex (PDH), which is followed by carboxylation to malonyl-CoA by acetyl-CoA carboxylase (ACCase) (Figure 2). ACCase exists in two isoforms, cytosolic homomeric encoded as a single polypeptide (ACC1) and plastidal heteromeric composed of multiple subunits (ACC2) [8]. Among the isoforms, only the homomeric isoform (ACC1) is present in the cytosol of yeasts and microalgae and is inhibited by herbicides belonging to the aryloxyphenoxypropionate (quizalofop, haloxyfop-R, diclofop-p, and fluazifop-p), cyclohexanedione (sethoxydin), and phenylpyrazolin (pinoxaden) classes [57–59]. These herbicides inhibit the carboxyltransferase (CT) domain of the ACCase enzyme, thereby disrupting the lipid synthesis pathway [59].

The next step of lipid synthesis is the transfer of malonyl-CoA to acyl carrier protein (ACP) catalyzed by the fatty acid synthase (FAS) complex (Figure 2). In yeasts, the type I FAS complex is present, composed of six copies of independent functional domains (α_6) [60]. The α subunit comprises ACP, -keto ACP reductase (KAR), β_6 -keto ACP synthase (KAS), and phosphopantetheinyl transferase (PPT), while the subunit consists of acetyltransferase (AT), enoyl-ACP reductase (ER), 6-hydroxyl acyl ACP dehydratase (HD), and malonyl transacylase (MPT) [60]. On the other hand, in microalgae, the type II FAS complex is present comprising of ACP, KAS, KAR, HD, and ER monofunctional proteins encoded by the *acpP*, *Fab B/Fab F*, *Fab G*, *Fab A/Fab Z*, and *Fab I* genes, respectively [41,42]. The formation of malonyl-ACP is catalyzed by malonyl-CoA acyltransferase followed by its conversion to 3-ketoacyl ACP, then to 3-hydroxylacyl ACP, to trans-enoyl ACP, and finally to acyl-ACP using the KAS, KAR, HD, and ER enzymes, respectively (Figure 2). Several chemicals can inhibit this lipid biosynthesis pathway and hence are useful for mutant screening. Cerulenin, an antibiotic isolated from the fungus *Cephalosporin caerulens*, is a non-competitive inhibitor of KAS (in yeasts) and of Fab B and Fab F (in microalgae), interfering with both de-novo fatty acid synthesis and chain elongation starting with palmitoyl-CoA as depicted in Figure 2 [61–63]. Triclosan and isoniazid are broad spectrum antimicrobial agents, which selectively inhibit the ER enzyme, thus hindering the final step of the fatty acid synthesis (Figure 2).

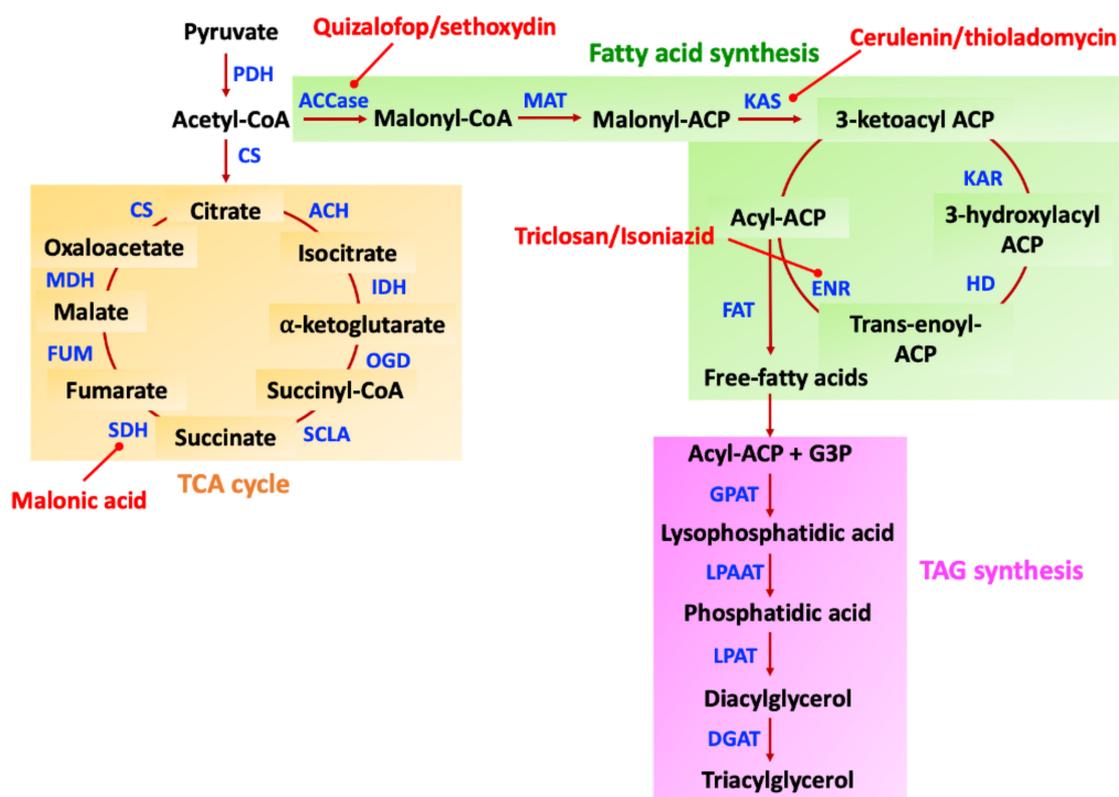


Figure 2. Schematic of the lipid biosynthesis pathway in microalgae and yeasts highlighting the action of chemical mutagens on metabolic enzymes.

Apart from fatty acid inhibitors, other chemical compounds, such as seasmol, iodoacetic acid, malonic acid, and erythromycin, have also been utilized to select for strains with high lipid productivity from generated mutant libraries. Seasmol is a natural non-oil phenolic component of sesame oil that inhibits the malic enzyme and scavenges ROS in oleaginous microorganisms [64]. The malic enzyme (ME) catalyzes the reversible oxidative carboxylation of malate to pyruvate with concomitant reduction of NADP^+ to NADPH , thereby supplying reducing power for fatty acid biosynthesis [65]. It has been reported that seasmol enhances the growth of the microalga *Cryptocodinium cohnii* and increases its docosahexaenoic acid (DHA, 22:6n-3) synthesis [64]. Iodoacetic acid, on the other hand, interacts with phosphoglyceraldehyde dehydrogenase inhibiting the Embden–Meyerhof pathway (glycolysis), which catabolizes glucose to generate energy equivalents, including ATP and NADH, and precursor molecules for other metabolic pathways, including pyruvate and 3-phosphoglycerate [66]. This in turn activates a parallel pathway, hexose monophosphate pathway (HMP) or pentose phosphate pathway (PPP), which produces NADPH and ribose-5-phosphate [67]. Iodoacetic acid together with malonic acid, which is the inhibitor of succinate dehydrogenase, result in the accumulation of citric acid and acetyl-coA, thus increasing the lipid yield in oleaginous microorganisms, as depicted in Figure 2 [67]. Finally, erythromycin is a well-known inhibitor of protein synthesis in prokaryotes, but can also inhibit synthesis of membrane proteins of thylakoids in algae, such as photosystem (PS) II reaction center and cytochrome (Cyt) b_6 -f-complex [68]. Microalgae mutants resistant to erythromycin treatment (35 $\mu\text{g}/\text{mL}$) were reported in conjunction with enhanced lipid yield [69].

3. Strain Improvement Results

3.1. Use of Random Mutagenesis

To date, various microalgae and yeasts have been randomly mutated using both physical and chemical mutagenesis to enhance their lipid yield and growth rate and increase their tolerance to toxic compounds or environmental stress, such as salinity, temperature, and alkalinity. The sections below provide a snapshot of random mutagenesis applications using physical and chemical methods to obtain high lipid accumulating mutants of microalgae and yeasts.

3.1.1. Microalga Mutants

Among physical mutagens, UV exposure has been extensively utilized for generating high lipid accumulating mutants of several oleaginous microalgae (Table 1). For instance, UV mutants of *Chlorella vulgaris* exhibited a ~6% increase in total lipid yield along with enhanced DCW [43]. The authors analyzed the UV mutant using transcriptomics and identified 51 differentially expressed genes accounting for 2.5% of the genome. The major genetic alterations included overexpression of nitrate reductase (NR) and siroheme synthase (Sis) in the mutant as compared to the wild type (WT). An upregulation in NR responsible for reducing NO_3^- to NO_2^- results in increased nitrate assimilation contributing to the high biomass production [8]. Moreover, Sis is a cofactor of NR and also responsible for tetrapyrrole biosynthesis (hemes, chlorophyll, and cobalamins), overall increasing the photosynthetic capability and thus the biomass of the mutant [43]. Furthermore, the mutant strain showed better antioxidant potential with upregulation of superoxide dismutase (SOD), which catalyzes the partitioning of superoxide (O_2^-) to H_2O_2 and O_2 . The mutant strain also showed increased transcript abundance of PPP (glucose-6-phosphate isomerase and glucose-6-phosphate dehydrogenase), thereby enhancing the production of NADPH, which is the reducing power for lipid synthesis. The synthesis of the main precursor for lipid synthesis acetyl-CoA also increased in the mutant strain as compared to the wild type, as transcript abundance of the gene encoding family 2-aldehyde dehydrogenase B4 (oxides aldehydes carboxylic acids) was higher [70]. Similar increases in biomass and lipid yield in UV mutants have been reported for *Nannochloropsis oculata* and *Chlorella* sp. FC2 IITG (Table 1).

Table 1. Application of physical and chemical mutagenesis on microalga strains to enhance dry cell weight (DCW) and lipid yield in the mutated cells (M) over the wild type cells (WT).

Microalga	Mutagen	Survival Rate (%)	Screening Method	Mode of Cultivation	Cultivation Time (days)	DCW(g/L)		Lipid Yield (%)		Reference
						WT	M	WT	M	
<i>Aurantiochytrium</i> sp.	Heavy ion (120 Gy)	50	Triclosan and Isoniazid	5 L fed batch culture	4	32.1	34.2	41.38	46.82	[34]
<i>Nannochloropsis oceanica</i>	Heavy ion (160 Gy)	n.r.			18	6	9.36	40.57	43.48	[71]
	NTG	10			7	0.19	0.18	24.14	31.23	[72]
<i>Desmodesmus</i> sp.	ARTP	4		Flask	31	0.59	0.54	26.64	61.75	[73]
<i>Chlorella pyrenoidosa</i>		1			7	0.4	0.52	6.12	5.38	[38]
	⁶⁰ Coγ (500Gy)	n.r.	n.r.				0.58	1.12	n.r.	n.r.
						0.46	1.05	n.r.	n.r.	
<i>Chlorella vulgaris</i>	UV + EMS (25 mM for 60 min)			25 L Flat panel bioreactors, airlift	10	0.96	1.3	25	41	[70]
	UV (254 nm)	2.5		Flask	14	0.62	0.68	15.35	21.94	[43]
	EMS (25 mM for 60 min)	10	0.64				15.35	22.46		
	EMS (0.24 mM for 120 mins)	0.56	Decreased pigments	2 L Flat panel PBR	13	2.01	2.9	n.r.	n.r.	[75]
<i>Schizochytrium</i> sp.	NTG (2 mg/mL for 30 min) +UV (30 W for 3 min)	2.5	Iodoacetate and Malonic acid		3	45.44	45.24	29.51	39.41	[67]
<i>Chlamydomonas</i> sp. JSC4	Heavy ion (100 Gy)	n.r.	Salinity (7%)		9	6.09	4.08	18.3	34.7	[76]
<i>Chlamydomonas reinhardtii</i> acc-124	EMS (40 μL/mL for 120 min)	n.r.	n.r.		7	0.859	0.933	8	12	[77]
<i>Chlamydomonas reinhardtii</i>			FACS			5.5	6.5	9.09	8	[78]
<i>Scenedesmus</i> sp.	N ⁺ ion beam (1.8 × 10 ⁵ ions/cm)	5.20	n.r.	Flask	7	0.88	0.89	46.92	47.77	[35]
	⁶⁰ Coγ (500Gy)	2			7	2.31	2.66	16.8	28.9	[79]
	UV (254 nm for 60 s)	56	Highly concentrated cellulosic ethanol wastewater		27	n.g.	1.07	n.g.	21.4	[80]
	UV (3.4 W/m ² for 10 min)	n.r.	n.r.		12	1	2.56	40	60	[81]
<i>Scenedesmus obliquus</i>	UV (30 min)	5–10	Starchless		12	4	12.6	4.8	11.5	[82]
<i>Desmodesmus</i> sp.	EMS (0.8 M) for 60 min	9	High light tolerance			0.61	0.73	35.66	46.01	[83]
	Heavy ion (¹² C ⁶⁺ , 120 Gy)	20	n.r.		8	4.95	5.23	48	37.78	[84]

Table 1. Cont.

Microalga	Mutagen	Survival Rate (%)	Screening Method	Mode of Cultivation	Cultivation Time (days)	DCW(g/L)		Lipid Yield (%)		Reference
						WT	M	WT	M	
<i>Nannochloropsis salina</i>	EMS (0.24 mol/L for 30 min)	3	FACS		22	n.r.	n.r.	17.5	34.1 (FAME content)	[85]
	EMS (0.24 mol/L for 30 min) + UV (45 s)				27	n.r.	n.r.	17.5	78.7 (FAME content)	
<i>Nannochloropsis</i> sp.	InDels	n.r.		Flask	12	2.2	3	24.9	32.8	[86]
	EMS (1 M)	8			18	0.20	0.22	34	50.8	[30]
	EMS (0.5 M)	n.r.	n.r.	11	0.79	1.08	8.32	11.30	[87]	
	UV (354 nm for 120 min)		Cerulenin and Quizalofop	8	1.03	1.40	49	57.05	[88]	
<i>Nannochloropsis gaditana</i>	EMS (70 mM for 60 min) + InDels	10	-	Decreased pigments	7	2.59	3.29	n.r.	n.r.	[89]
<i>Chlorella</i> sp.	EMS (100 mM for 60 min)	n.r.	Thermo tolerance	40 L PBR outdoor	8	0.90	1.8	16.2	12.0	[90]
<i>Chlorella pyrenoidosa</i>	EMS (2% for 60 min)	10		Outdoor (10 L bottles)	5	n.g.	1.97	n.g.	44.5	[91]
<i>Chlorella</i> sp.	NTG (5 µg/mL for 60 min)	23	Alkali tolerance	PBR (1 L)	7	0.06	0.35	n.r.	n.r.	[42]
	EMS (100 mM for 30 min)	n.r.	Quizalofop		6	0.30	0.64	10.07	12.44	[92]
<i>Chlorella</i> sp. FC2 IITG	UV-C (30 W for 8 min)	2	n.r.	Flask	10	0.5	0.67	56	68	[32]
<i>Chlorella sorokiniana</i>	EMS (0.5%, w/v for 4 h)	n.r.	Starchless		4	5.5	6.1	n.r.	11.2	[93]
	UV	10	Reduced antenna size	7-L outdoor hanging bags	10	2.2	2.8	n.r.	n.r.	[94]
<i>Chlorella minutissima</i>	EMS (2 M for 30 min)	5	n.r.	Flask	10	1.5	2.4	27	42	[95]
<i>Tetraselmis</i> sp.	EMS (50 µ mol/mL 30 min)	1.9			21	0.27	0.58	32	40	[96]
<i>Brotriyococcus braunii</i>	UV (15 W/cm ²) for 6–21 min	10			24	1.04	1.98	21	34	[97]

Note: n.g. = no growth; n.r. = not reported.

Apart from augmenting the lipid content, UV mutagenesis has been used to increase the tolerance of *Scenedesmus* sp. to highly concentrated cellulosic ethanol wastewater (Table 1). A total of three UV mutant strains were identified (MU1, MU2, and MU15), which showed high growth (~0.73–1 g/L), lipid yield (18–22%), and chemical removal ability in wastewater, while the wild type was unable to adapt [80].

Physical mutagenesis has been utilized to generate high DHA (docosahexaenoic acid) production, salt resistance, and CO₂ tolerance in various microalgal strains [16,52,53]. DHA is a PUFA that is an essential component of the cellular membrane, particularly for the visual and nervous system in newborn infants [64]. It is also a health supplement and essential for normal functioning and disease prevention (hypertension, cancer, diabetes) in humans. Microalgae including *Cryptocodinium cohnii*, *Schizochytrium* sp., and *Aurantiochytrium* sp. are heterotrophic dinoflagellates utilized for industrial-scale DHA production [98,99]. Heavy-ion beam mutagenesis combined with Triclosan (100 µm) or Isoniazid (50 µm) screening were used to generate high DHA accumulating *Aurantiochytrium* sp. mutants [34]. The authors reported the mutants could grow at low temperatures (4 °C) without the PUFA content changing significantly as compared to the WT (~41% of DCW). The mutant's resistance to triclosan (T-99) or isoniazid (I-33) resulted in high PUFA synthesis accounting for 44% and 47% of DCW, respectively [34]. The reason for the high PUFA production in the mutant strains could be direction of lipid precursors (acetyl-CoA and NADPH) towards the polyketide synthase-like polyunsaturated fatty acid synthase pathway (PKS) involved in DHA synthesis, since triclosan and isoniazid selectively block the FAS complex [34]. Kato et al. [76] developed salt-resistant strains of *Chlamydomonas* sp. JSC4 (KH1 lines) that were able to grow in 7% salinity. The salt-resistant strains showed increased growth under saline conditions (3% and 7% NaCl), however the lipid yield decreased by ~10% under 3% salinity [76]. The authors reported that the wild type switched from starch to lipid synthesis, when cultivated in 3% salinity, as an upregulation in the level of starch phosphorylase (SP), which catalyzes degradation of starch, was observed. However, no such change in SP activity was recorded in the mutant strain indicating delayed starch degradation and hence a lower lipid content.

Microalgae are considered a potential large-scale sink for CO₂ sequestration from flue gases, which contain 12–15% CO₂ [56,57]. However, most of the microalgal strains can only survive up to 5% CO₂, hence development of high tolerance CO₂ strains is crucial [100–102]. Nuclear radiation (⁶⁰Co) was utilized to enhance the CO₂ tolerance of *C. pyrenoidosa* and *C. vulgaris* [74]. The mutated strains of both species were able to grow in 6% CO₂ and demonstrated an increase in biomass compared to the WT strains (Table 1). The authors further enhanced the CO₂ tolerance of the *C. pyrenoidosa* mutant to 15% by ALE. The evolved strain, when grown at 15% CO₂, reached a concentration of 2.41 g/L, which was 51% higher than the mutated strain with a CO₂ fixation rate of 32% [74].

In addition to physical mutagenesis techniques, chemical mutagens, particularly EMS and NTG, have been successfully employed for enhancing the lipid production of several microalgal strains (Table 1). Depending on the species, the optimum EMS or NTG concentration that is essential for inducing positive mutants varies. The EMS mutants of various microalgae, including *Chlorella minutissima*, *Nannochloropsis* sp., and *N. salina*, showed a 15–17% increase in total lipid yield along with a significant enhancement in DCW as compared to the WT [12,60,61]. Furthermore, combined mutagenesis or successive rounds of mutagenesis have also been utilized, wherein a microalga is mutated using two different mutagens (physical + chemical) in an effort to increase the number of positive mutants. For instance, *C. vulgaris* mutated using UV radiation (2.9×10^{-2} W/m² for 10 min) and then treated with EMS (25 mM for 60 min) resulted in 1.5-fold and 2.3-fold higher lipid productivity, as compared to the UV mutant and the WT, respectively [70]. Similarly, *N. salina* EMS mutants treated with UV reached 78.7% lipid content, although cell growth dropped by 56% [85].

EMS has also been utilized to generate thermotolerant, high light intensity tolerant, and reduced pigment/truncated antenna size mutants (Table 1). All of these characteristics of algae are essential for sustainable outdoor cultivation particularly in closed photobioreactors and in hot climates [98]. Although tropical and semitropical climates are better suited for large-scale microalgal cultivation due

to longer/stronger light exposure and warm temperatures year-round compared to temperate climates, high temperatures and excessive light intensities inhibit microalgal growth [103], necessitating the development of thermotolerant and high light intensity tolerant strains. To this end, two thermotolerant EMS mutants of *Chlorella* sp. and *C. pyrenoidosa* were able to grow at ~42 °C without any effect on growth [63,64]. Moreover, when the *Chlorella* sp. mutant was cultivated outdoors in a 40-liter PBR for eight days at 40 °C, the mutant showed 43% higher DCW with a ~4% decrease in lipid yield as compared to the WT [90]. In terms of tolerance to high light intensities, microalgae generally have large-size light harvesting antennas bonded to hundreds of chlorophyll molecules to capture light in their photosynthetic apparatus [89]. Such large antenna size can result in excessive light absorption, which will cause oxidative damage and photoinhibition, and thus preclude the algae from large scale production [104]. The excessive light absorbed by the microalgal cells is either dissipated as heat (non-photochemical quenching) or re-emitted as fluorescence [105]. Truncated chlorophyll antenna sizes could potentially alleviate this damage by reducing the amount of photons captured by the cells, while diminishing shading among cells and ensuring a more uniform illumination of the culture [106]. Shin et al. [75] developed a *C. vulgaris* mutant strain (E5) with truncated antenna size and reduced photosynthetic pigments. The mutant strain was able to grow under high light intensity (200 μ mol photons/m²/s¹) with high biomass productivity as compared to the WT (Table 1). The authors analyzed the light harvesting complex (LHC) proteins of the mutant and reported a 22.8% decrease in Lhcb 1 and Lhcb 4 proteins and a ~70% decline in Lhcb 2 and Lhcb 3 proteins as compared to the WT, clearly indicating Photosystem II (PSII) downregulation [75]. Interestingly, high light tolerant EMS mutants of *Desmodesmus* sp. (G3) showed upregulation of LHC I, LHC II, and Photosystem I (PSI) genes namely *psa D*, *psa F*, *psa K*, *psa L*, and *psa N*, with downregulation of only the *psa A* gene [70,71]. The reduction of *psa A* gene expression in the mutant could be a cellular mechanism to reduce light energy flux from saturated LHC antennas, thereby shielding the PS1 [107]. The mutant also accumulated more lipids (41%) compared to the WT (35%), which was attributed to the recycling of membrane lipids to TAGs by the mutant, since a decline in the glycerophospholipids, Fab F, Fab H, Fab G and Fab I and an increase in ACCase and SP were recorded [74].

Another potential means of decreasing the cost of carbon capture is by generating alkali-halophilic microalgae, which can tolerate high bicarbonate resulting in (1) more inorganic carbon in the media, which increases lipid content; (2) stronger pH buffering capacity; and (3) enhanced generation of carbonate, thereby absorbing more CO₂ [108]. Two natural microalgae, *Neochloris oleoabundans* and *D. tertiolecta*, have been shown to tolerate 320 mM NaHCO₃, with the former one showing a maximum DCW of 1.56 g/L and 23.4% lipid content. Further increasing the tolerance of such microalgae using mutagenesis or ALE technique could enhance the industrial prospects for large scale cultivation.

3.1.2. Yeast Mutants

Initial attempts to attenuate the lipid content of yeast using mutagenesis were made in the 1990s to produce cocoa butter equivalents from *Apiotrichum curvatum* (renamed *Cryptococcus curvatus*) [109–111]. Cocoa butter, an essential ingredient of chocolate and a staple in various skincare and cosmetic products, is extracted from the mature beans of *Theobroma cacao* [111]. Its unique properties are due to the presence of high stearic acid (C18:0; 32–37%), palmitic acid (C16:0; 23–30%), and oleic acid (C18:1; 30–37%) [110]. The fatty acid profile of *Cryptococcus curvatus* primarily comprises 44–49% C18:1 and 12–15% C18:0 [110]. In order to use these yeast lipids as a cocoa butter equivalent, the content of C18:0 was increased using UV, EMS, and MNNG mutagenesis for generating unsaturated fatty acid (Ufa) autotrophs [109–111]. Using MNNG, six Ufa mutants were obtained and showed stability for 50 generations. They had a defective Δ^9 desaturase (responsible for introducing a double bond in palmitic and stearic acid), thus requiring C18:1 for growth [110]. Among the mutants, one showed the highest saturated fatty acid (SFA) content of 75%, when cultivated in media containing 0.2 g/L of C18:1 [110]. Moreover, Ufa auxotroph mutants were generated using EMS with a 65.2% SFA content in the presence of 0.2 g/L of C18:1 [111]. However, the need to add C18:1 renders industrial production

expensive, so the Ufa mutants were subjected to UV and MNNG mutagenesis to identify revertants that had partially deactivated Δ^9 desaturase enzyme [109]. Among the 279 revertants generated, one showed 80.2% SFA content in comparison to the wild type's 47.2% SFA, when cultivated in whey permeate hydrolysate [109]. The increasing demand for cocoa butter and the loss of crop produce to harmful insects and viruses are pushing prices up and could potentially make cocoa butter equivalents from yeast a viable option in the future [5].

In the last decade, random mutagenesis of oleaginous yeasts has been mainly carried out using UV and EMS to improve lipid productivity (Table 2). Yeasts have the inherent capacity to utilize pentose sugars, such as xylose and arabinose, for growth and lipid synthesis. The stoichiometry of carbon source utilization by oleaginous yeasts generates 1.1 and 1.2 moles of acetyl-CoA from 100 g of glucose (and similar sugars like lactose and fructose) and 100 g of xylose, respectively [5]. Thus, the maximum theoretical yield of lipids is 32 g and 34 g per 100 g of glucose and xylose utilized, respectively. However, the reported conversion is reported to be only 20–22 g of oil [4]. Given that the sugars used for cultivating yeast represent a major part of the fermentation cost, there is interest in identifying inexpensive and renewable sugar resources, such as lignocellulosic biomass from agricultural and industrial residues and wastes, to both reduce costs and enhance sustainability [5]. Cellulosic biomass hydrolysate constitutes an abundant and low-cost carbon source for large-scale yeast cultivation, but the necessary thermochemical pretreatment of biomass releases toxic compounds, such as acetic acid, furfural, and hydroxymethyl furfural (HMF), which are toxic to the yeast and inhibit its growth [112–114]. Thus, before utilizing biomass hydrolysate, additional costly processing is needed to minimize/eliminate the toxic compounds and adjust the pH to levels appropriate for oleaginous yeast cultivation and lipid production. Another important feature is the simultaneous utilization of glucose and xylose present in the hydrolysate by yeast. Generally, the preferable carbon source of yeast is glucose, which is consumed before xylose, so there is strong interest in developing mutants that can co-ferment multiple sugars [55]. Oleaginous yeast mutants have been generated to utilize non-detoxified hydrolysate obtained from sugarcane bagasse hydrolysate (SBH) [73,74]. The ARTP mutants of *Rhodospiridium toruloides* were able to grow in SBH with high biomass and lipid accumulation, while the WT failed to grow (Table 2). Moreover, the UV mutants of *R. toruloides* showed reduced glucose and xylose consumption rate as compared to WT, but a high lipid content, which was due to the upregulation of lipid biosynthesis genes, including ATP citrate lyase [55]. A similar increase in lipid biosynthesis genes was reported in a *R. toruloides* UV mutant resistant to ethanol-H₂O₂ or LiCl [115].

Overall, when compared to microalgae mutants, yeast mutants generated by UV and EMS have a 40–50% higher lipid content, as seen in Table 2. For instance, the maximum increase in lipid yield (by 56%) was recorded in UV mutants of the yeast *R. toruloides* followed by 48% and 44% higher lipid yield in heavy ion mutants of *R. glutinis* and *R. mucilaginosa*.

3.2. Use of Adaptive Laboratory Evolution

Adaptive laboratory evolution (ALE), also called whole genome directed evolution, has been mainly exploited to enhance the tolerance of oleaginous yeasts and microalgae towards environmental stressors, such as nutrients, salinity, temperature (high/low), and toxic compounds, with the objective of reaching high lipid production (Table 3). The section below provides an overview of ALE studies carried out to date on various oleaginous microalgae and yeasts.

Table 2. Application of physical and chemical mutagenesis on yeast strains to enhance dry cell weight (DCW) and lipid yield in the mutated cells (M) over the wild type cells (WT).

Yeast	Mutagen	Survival Rate (%)	Screening Method	Cultivation Time (days)	DCW (g/L)		Lipid Yield (g/L)		Reference
					WT	M	WT	M	
<i>Cryptococcus curvatus</i>	UV (2 W/m ²) for 200 s	10	Ceruleinin	4	n.r.	n.r.	0.501	0.529	[116]
					n.r.	n.r.	0.816	1.86	
					12	11	0.94	1.25	
<i>Rhodospidium toruloides</i>	UV (15 W/m ²) for 8 min	5	Ethanol-H ₂ O ₂ and LiCl	7	n.r.	n.r.	1.5	2.24	[115]
	ARTP	1–5	n.r.	8	n.g.	7.2	n.r.	4.2	[113]
	ARTP + NTG (0.5 mg/L) for 45 min			6	8.9	7.2	2.7	2.99	[40]
	<i>Rhodotorula glutinis</i>	Heavy ion (40 Gy, 55 Gy)	8–19	Ceruleinin		2.03	2.21	0.34	0.65
<i>Rhodotorula mucilaginosa</i>		11.58			2.08	2.72	0.292	0.52	
<i>Yarrowia lipolytica</i>	EMS (75 mM) for 60 min	11.22			1.92	2.44	0.36	0.52	[119]
<i>Trichosporon asatii</i>		7.7	n.r.	4	1	1.16	0.148	0.236	
<i>Debaryomyces hansenii</i>	EMS (75 mM) for 45 min	3.16			0.64	0.76	0.072	0.076	
<i>Candida tenuis</i>	EMS (75 mM) for 30 min	6.11			0.68	0.84	0.088	0.096	
<i>Lipomyces starkeyi</i>	UV (15 W/m ²) for 40 min	5	Ceruleinin	7	12.31	13.74	4.41	5.44	[63]
	EMS (340 µL) for 30 min	6	Percoll density gradient		11	14	4.3	6.3	[55]

Note: n.g. = no growth; n.r.= not reported.

3.2.1. Microalga Evolved Strains

Among the diverse microalgae species, *Chlamydomonas reinhardtii* has been extensively studied as a model system for algal physiology, photosynthesis, circadian rhythm metabolism, and genetic toolbox development [120]. An evolved strain of *C. reinhardtii* was reported exhibiting faster growth rate, cell size, loss of mobility flagella, and negative phototaxis as compared to the WT [121]. The authors reported high biomass production (1.74 g/L) and lipid yield (8.18%), when cultivated under nitrogen-deprived conditions as compared to the ancestral strain (1.45 g/L and 7.58%, respectively) (Table 3). Moreover, whole genome sequencing of the evolved strain showed 44 alterations in coding sequences (CDS) that affected 33 genes [121]. The altered genes were mainly involved in cell division, cycle regulation, and nucleotide synthesis and binding. In another study, low-starch or starchless mutants of *C. reinhardtii* (BAFJ6 and I7) were evolved over a period of 84 days, which resulted in increased biomass and lipid production in evolved strain as compared to the WT (Table 3). BAFJ6 was defective in isoamylase (de-branching enzyme), while I7 had a defective large subunit of ADP-glucose pyrophosphorylase (AGPase) [76,78]. Starch synthesis in microalgae is initiated with the AGPase catalyzing the reaction of glucose-1-phosphate and ATP to form ADP-glucose and pyrophosphate (PP_i) [120]. Together with the branching enzyme, starch synthase elongates the glucan chains to form starch granules. The possible reason for high biomass in the evolved strains as compared to the WT was attributed to the low NPQ (non-photochemical quenching), suggesting more light was utilized for photosynthesis and less dissipated as heat [122].

As discussed earlier, successful microalgae commercialization for lipid production will be facilitated by developing strains capable of surviving under high salinity, CO₂, and temperature. The ALE technique has been successfully utilized to generate microalgal strains capable of growing in saline waters (30 g/L NaCl), high CO₂ (30%), and flue gases (Table 3). Li et al. [123] reported gradual evolution of *Chlorella* sp. (ALE 10 and ALE 20) to 10% and 20% CO₂ with two-fold higher DCW and 5–6% increase in lipid yield as compared to the parent strain (Table 3). Moreover, when the adapted strains were grown in 30% CO₂, ALE 10 produced more biomass as compared to ALE 20, indicating a difference in their evolution [123]. The ALE 10 strain was further adapted for high salt tolerance (30 g/L NaCl), which resulted in 26% decrease in DCW with a similar lipid yield (~18%) [47]. The transcriptomics analysis of the salt-adapted microalga (ALE S30) showed downregulation of fatty acid biosynthesis, photosynthesis, and oxidative phosphorylation, while the antioxidant defense mechanism was upregulated [47]. Under salinity stress, to quench the oxidative stress, microalgae upregulate the biosynthesis of antioxidant enzymes, including SOD, catalase, and ascorbate peroxide, which neutralize the ROS [124]. Interestingly, the adapted strain showed activation of all three CO₂ concentration mechanisms (CCM), namely the Calvin–Benson cycle (C3), the C4-dicarboxylic acid cycle (C4), and the crassulacean acid metabolism (CAM), which aided the microalga in sustaining the required photosynthesis activity under salinity [47]. The same research group also evolved *Chlorella* sp. to tolerate and degrade 500 mg/L of phenol (L5) with a ~1.3- and 2.5-fold increase in DCW, when cultivated in 0 g/L and 500 g/L phenol, respectively, as compared to the WT [125]. Transcriptomics analysis of the phenol-adapted strain showed upregulation of PS I and PS II genes, antioxidant enzymes (SOD, APX, and CAT), carotenoid biosynthesis, and fatty acid synthesis, which explained the tolerance of the evolved strain towards phenol along with high growth and lipid yield [126]. *Chlorella* sp. has also been evolved to a simulated flue gas environment comprising of 10% CO₂, 200 ppm NO_x, and 10% SO_x [49]. The evolved strain was able to efficiently grow in simulated flue gas exhibiting high DCW, while the parental strain showed no growth (Table 3). The evolved strain showed upregulation of photosynthesis, oxidative phosphorylation, nitrate reductase, and intracellular sulphur transport indicating successful adaptation of the microalga towards high NO_x and SO_x concentrations [49].

Table 3. Application of adaptive laboratory evolution (ALE) to oleaginous microalgae and yeasts via various stress conditions to enhance dry cell weight (DCW) and lipid yield.

Microorganism	Stress Condition	Generations	Time for ALE (Days)	Cultivation Time (Days)	DCW (g/L)	Lipid Yield (%)	Reference
Microalga							
<i>Aurantiochytrium</i> sp.	Sugarcane bagasse hydrolysate	10	n.r.	5	25	30	[127]
<i>Cryptocodinium cohnii</i>	Glucose tolerant	260	650	4	n.r.	35	[99]
	Sethoxydoxin + Sesamol	100	300	5	6	60	[128]
<i>Chlorella</i> sp.	Flue gas	110	138	7	3.4	n.r.	[49]
	CO ₂ tolerance	31	97	11	3.68	20	[129]
	Phenol degradation	31	95	8	3.40	26	[125]
	Salinity	46	138	7	2.7	18.14	[47]
<i>Schizochytrium</i> sp.		n.r.	150		52.3	22.7	[130]
	Salinity + low temperature	40	n.r.	3	47.23	37.60	[131]
	Glucose tolerant	40	n.r.		62.15	49.78	[132]
<i>Chlamydomonas reinhardtii</i>	n.r.	28	84	9	0.48	40	[122]
	High salt	1255	510	n.r.	n.r.	n.r.	[133]
	Non-intentional	n.r.	4 years	8	1.71	8.18	[121]
<i>Chlorella vulgaris</i>	LED-Red light (660 nm)	38	114	3	5.2	n.r.	[134]
Yeast							
<i>Yarrowia lipolytica</i>	Ionic liquids (18%; v/v)	200	n.r.	4	n.r.	n.r.	[86]
	Nitrogen and Magnesium limited	77	n.r.	7	8.8	44	[135]
<i>Rhodospiridium toruloides</i>	Non-detoxified hydrolysate (75%)	8	4 months	4	6.6	55	[136]
<i>Metshnikowia pulcherrima</i>	Inhibitors	22	n.r.	7	14.5	33.3	[137]

Note: n.r.= not reported.

The DHA-producing microalgae *Aurantiochytrium* sp., *Cryptothecodinium cohnii*, and *Schizochytrium* sp. have also been evolved to tolerate high levels of glucose, salinity, and biomass hydrolysate, and low temperatures (Table 3). *Aurantiochytrium* sp. was evolved to adapt to 100% sugarcane bagasse hydrolysate (SBH), exhibiting a 2-fold increase in biomass and lipid content, as compared to the parental strain, when cultivated in 50% hydrolysate [127]. Transcriptomics analysis of the evolved strain revealed upregulation of key TCA cycle genes, including malate dehydrogenase, succinyl dehydrogenase, and succinyl-CoA synthetase, which provide reducing power in the form of ATP, NADH, and FADH₂ [127]. An increase in the reducing power aids the microalga to alleviate the toxic effects of the inhibitory compounds present in the SBH. Moreover, increases in the expression of amino acid synthesis and fatty acid synthesis genes were also reported [127]. Upregulation in the fatty acid synthesis helps in maintaining the membrane integrity and protecting the cell from the inhibitory compounds [127]. Along the same lines, *C. cohnii* adapted to high glucose (54 g/L) that is toxic to the parental strain and showed increased levels of malonic acid, succinic acid, glutamic acid, and glycerol, which indicate enhanced reducing power and lipid biosynthesis [99]. Diao et al. [128] sequentially adapted *C. cohnii* first to sethoxydoxin (10 µm to 60 µm) causing an increase in the ACCase expression and in turn in lipid biosynthesis, and then to sesamol (0.5 mM to 2 mM), which led to an increase in cell growth. The authors reported a 12.5% increase in DCW and a 63% increase in lipids in the adapted strain, as compared to the parental strain (Table 3). Furthermore, the evolved strain was able to degrade starch at a faster rate resulting in more carbon flux towards lipid synthesis, as an increase in the expression of α -amylase and α -glucan water dikinase were recorded [127].

3.2.2. Yeast Evolved Strains

Although there are numerous studies on application of ALE to enhance specific traits in yeasts, particularly *Saccharomyces cerevisiae*, including ethanol tolerance, furfural and acetic acid tolerance, thermotolerance, and saline stress, only three studies have been reported to date specifically for oleaginous yeasts (Table 3). *Yarrowia lipolytica* is a potential host for industrial applications thanks to its ability to metabolize a wide range of sugars, such as glycerol, n-alkanes, and agro-industrial wastes [138]. Walker et al. [82] improved the resistance of *Y. lipolytica* to ionic liquids (18%), which are toxic to the yeast even at low concentrations. Ionic liquids are green solvents, which can effectively pretreat the lignocellulosic biomass replacing acids. The evolved strain was initially adapted to 1-ethyl-3-methylimidazolium acetate, but it also showed cross tolerance towards other ionic solvents, including ethyl-3-methylimidazolium chloride, 1-ethyl-3-methylimidazolium bromide, 1-allyl-3-methylimidazolium chloride, 1-butyl-3-methylimidazolium chloride, and 1-butyl-3-methylimidazolium bromide. The authors reported changes in the cell membrane and cell wall, as an increase in glycerophospholipids and shorter chain fatty acids with high degree of unsaturation and sterols were observed in the evolved strain to combat the toxicity of the ionic liquids. The glycerophospholipids interact with the cations/anions of such liquids, while the shorter chain fatty acids and sterols maintain membrane integrity and fluidity during stress [86]. The whole genome sequencing of the evolved strains is publicly available [139].

Rhodospiridium toruloides was adapted to non-detoxified wheat straw hydrolysate (75%), which contained high amounts of inhibitory compounds, including acetic acid and furfural [136]. The adapted strain showed high DCW and lipid content, when cultivated in 75% hydrolysate (Table 3). The strain was further adapted to grow in xylose, as sole carbon source, consuming 92% of the provided xylose in 96 h as compared to 58% consumption by the WT [136]. The authors also genetically modified the evolved strains with homologous overexpression of DGAT1 and stearoyl-CoA desaturase (SCD1). The engineered strain resulted in 29% increase in biomass production with 5 g/L of total lipid yield [136].

In a recent study, *Metschnikowia pulcherrima*, which can be cultivated under non-sterile conditions and can metabolize various types of sugars, was adapted to tolerate 1.2 g/L of formic acid and a cocktail of inhibitors (0.7 g/L of furfural + acetic acid and 0.35 g/L of formic acid and HMF) [137]. The cocktail-adapted strains outperformed the formic acid evolved strains with one strain showing

maximum DCW of 8.5 g/L and lipid accumulation of 34.7%, twice that of the WT [137]. The evolved strain was then cultivated in a 2-L bioreactor under nonsterile conditions resulting in 33.3% lipid content within seven days [137].

In another study, *S. cerevisiae* was adapted to the drug myriocin, which selectively inhibits serine palmitoyl transferase that is the first enzyme involved in sphingolipid biosynthesis [140]. The adapted strain was able to grow at 40° C as compared to WT (30° C) with a substantial increase in sphingolipid content [140]. Furthermore, the evolved strain showed a decrease in acyl desaturation and fatty acids chain length indicating an adaptation to high temperature by modulating its membrane fluidity and permeability. This approach could also be utilized to generate thermotolerant mutants of other oleaginous yeasts.

4. Conclusions

Large-scale production of microbial lipids, as sources of biofuels, nutraceuticals, pigments, cosmetics, and other high-value compounds, requires the development of fast-growing high lipid yield strains. Oleaginous alga and yeast improvement using random mutagenesis and ALE followed by high-throughput screening methods, such as FACS, can make a significant contribution towards commercialization, particularly since such mutants and evolved strains are subject to less stringent environmental permitting than genetically engineered strains [31]. Random mutagenesis can be optimized in terms of type of mutagen, dosage, and exposure time to enhance the appearance of the desired features in the treated oleaginous yeast or microalga. The oleaginous microorganism can also be evolved in the laboratory via ALE under controlled conditions to increase its tolerance towards a specific stressor or to enhance a particular trait. Moreover, ALE-developed strains tend to show cross tolerance towards other stress conditions, offering an advantage over engineered strains. The utilization of high-throughput omics technologies, such as genomics, transcriptomics, proteomics, and metabolomics, aids in quickly and comprehensively identifying the resulting mutations/alterations in the modified strains, thus helping elucidate shifts in metabolic mechanisms and identify the key genes that can be subsequently targeted with genetic engineering to further enhance the modified microorganisms. An alternative strategy for screening mutants is to identify alterations in their protein synthesis machinery, such as overexpression of lipid droplet protein 1 that protects TAG from lipase degradation in yeasts [141]. Defects in the lipid droplet protein in *Schizosaccharomyces pombe* resulted in abnormally large lipid droplet accumulation that can be used as selection marker [142], whereas deletion of one copy of ribosomal protein uL6 in *S. cerevisiae* resulted in increased life span of the cells and high metabolic rate, which can be used to identify and select robust mutants [143]. Moreover, downregulation of proteins, such as thioredoxins, can serve as a key marker for increased stress tolerance to oxidative damage and oil content [144,145]. Similar protein targets for screening high lipid accumulating mutants should be explored in microalgae as well. Future studies should aim at characterizing the modified yeasts and microalgae strains using a systems biology approach and conducting pilot- and large-scale cultivations to pave the path towards industrial deployment of promising oleaginous species.

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List of Abbreviations

ACCase	Acetyl-CoA Carboxylase
ACH	Aconitase
ACP	Acyl Carrier Protein
AGPase	ADP-Glucose Pyrophosphorylase
ALE	Adaptive Laboratory Evolution
APX	Ascorbate Peroxidase
ARTP	Atmospheric and Room Temperature Plasma
AT	Acetyltransferase
ATP	Adenosine Triphosphate
CAM	Crassulacean Acid Metabolism
CAT	Catalase
CCM	Carbon Concentration Mechanism
CDS	Coding Sequences
CS	Citrate Synthase
DAG	Diacylglycerol
DCW	Dry Cell Weight
DES	Diethyl Sulphate
DGAT	Diacylglycerol Acyltransferase
DHA	Docosahexaenoic Acid
DNA	Deoxyribonucleic Acid
EMS	Ethyl Methane Sulfonate
ER	Enoyl ACP Reductase
FACS	Fluorescence-Activated Cell Sorting
FADH ₂	Flavin Adenine Dinucleotide
FAMES	Fatty Acid Methyl Esters
FAS	Fatty Acid Synthase
FAT	Fatty acyl-ACP Thioesterase
FUM	Fumarase
G-3-P	Glycerol-3-Phosphate
GADPH	Glyceraldehyde-3-Phosphate Dehydrogenase
GHG	Green House Gases
GPAT	Glycerol Phosphate Acyl Transferase
HD	3-Hydroxyacyl ACP Dehydrase
HMF	5-Hydroxy Methyl Furfural
HMP	Hexose Monophosphate Pathway
IDH	Isocitrate Dehydrogenase
KAR	3-Ketoacyl ACP Reductase
KAS	β- Keto ACP Synthase
LHC	Light Harvesting Complex
LPAAT	Lyso-Phosphatidic Acid Acyltransferase
LPAT	Lyso-Phosphatidylcholine Acyltransferase
MDH	Malate Dehydrogenase
ME	Malic Enzyme
MGDG	Mono Galactosyl Diacyl Glycerol
MMS	Methyl Methane Sulfonate
MNNG	Methyl Nitro Nitroso Guanidine
MPT	Malonyl Transacylase
NADH	Nicotinamide Adenine Dinucleotide

NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NCBI	National Centre for Biotechnology Information
NPQ	Non-Photochemical Quenching
NR	Nitrate Reductase
NTG	N-Methyl-N-Nitro-N-Nitrosoguanidine
OGD	Oxoglutarate Dehydrogenase
PBR	Photo Bioreactor
PDAT	Phospholipid Diacylglycerol Acyl Transferase
PDC	Pyruvate Dehydrogenase Complex
PKS	Polyunsaturated Fatty Acid Synthase Pathway
PP _i	Pyrophosphate
PPP	Pentose Phosphate Pathway
PPT	Phosphopantetheinyl Transferase
PSI	Photosystem I
PSII	Photosystem II
PUFA	Polyunsaturated Fatty acid
RF	Radio Frequency
ROS	Reactive Oxygen Species
RuBisCo	Ribulose-1,5-Bisphosphate Carboxylase
SAD	Stearoyl-ACP Desaturase
SBH	Sugarcane Bagasse Hydrolysate
SCD1	Stearoyl-CoA Desaturase
SDH	Succinyl-CoA Dehydrogenase
Sis	Siroheme Synthase
SNPs	Single Nucleotide Polymorphisms
SOD	Super Oxide Dismutase
SP	Starch Phosphorylase
TAG	Triacylglycerol
TCA	Tricarboxylic Acid
UV	Ultraviolet
WT	Wild Type

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