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Review Article

Production of chemicals from microalgae lipids – status and perspectives

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The engineering of algae strains, cultivation, and further processing steps in microalgae production are considered in view of the utilization of microalgae lipids for chemicals. Insights from biofuel production trials on the one hand and existing processes for very high-value pharmaceuticals on the other hand are instructive to this end. A recent example of the production of a surfactant from chemical intermediates gained from algae oil and further opportunities are discussed.

Keywords: Carbonylation / Chemical functionalization / Cultivation / Downstream processing / Industrial production / Metathesis / Microalgae

1 Introduction

Beyond being an alternative to crude oil as a source of chemicals and fuels, renewable resources are also unique feedstocks in that they contain particular molecular structures and functional groups. These can provide access to novel intermediates and products with different or even superior traits compared to existing technology. This consideration applies to various different classes of compounds, for example sugars [1], terpenes [2], or lipids [3]. This contribution focusses on the latter.

As a source of lipids, microalgae provide attractive perspectives: Autotrophic algae cultivation does not compete with food production since they can be grown on non-arable land in fresh water, brackish water, or in seawater. Due to the high division rates of a number of species, they may double their biomass typically within 24 h [4]. The growth of algae is much less dependent on season than for higher plants. Additionally, several strains can easily be engineered genetically, for example, *Phaeodactylum tricornutum* [5, 6], *Chlamydomonas reinhardtii* [7], or *Thalassiosira pseudonana* [8, 9], providing the opportunity to design advantageous traits [10–12]. Furthermore, microalgae produce fatty acids with unusual chain lengths and a high degree of unsaturation, which are not found in significant amounts in nature elsewhere. Prominent examples are the polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA, 20:5) or docosahexaenoic acid (DHA, 22:6), ω-3 fatty acids, that are commercially produced as human health supplements [13].

Lipids constitute a major portion of microalgae [14] and serve different functions: As membrane lipids they enclose biological compartments like cells and organelles, while as storage lipids they serve as a cellular high-density energy storage. Such lipids in algae accumulate during the stationary phase of growth [15] or under stress conditions [13]. The vision of generating fuels on a large scale from algal lipids has fascinated science and politics since the 1950s [16, 17]. A prime motivation has been a reduced dependency of the global economy on crude oil deposits, which are unequally distributed around the globe and finite on the long term. Later, also concerns about greenhouse gas emissions associated with the consumption of fossil fuels came up.

The long-term continued research on biofuels from algae has resulted in production trials of several thousand tons.

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Abbreviations: 16:1, methyl palmitoleate; **18:1,** methyl oleate; **ACP,** acyl carrier protein; **CAPEX,** capital expenses; **CoA,** Coenzyme A; **CRISPR,** clustered regularly interspaced short palindromic repeats; **DGDG,** digalactosyl diacylglycerol; **DHA,** docosahexaenoic acid or 22:6; **dtbpx,** 1,2-bis[di-tert-butylphosphino-methyl]benzene; **dw,** dry weight; **EPA,** eicosapentaenoic acid or 20:5; **GMO,** genetically modified organism; **GMP,** good manufacturing practice; **MeOH,** methanol; **MGDG,** monogalactosyl diacylglycerol; **OPEX,** expenses; **PBR,** photobioreactor; **PC,** phosphatidylcholine; **PCE,** photosynthetic efficiency; **PDC,** pyruvate dehydrogenase complex; **PDK,** pyruvate dehydrogenase kinase; **PUFA,** polyunsaturated fatty acid; **sp.,** species, especially when the genus is known, but the species is uncertain or unspecified; **SQDG,** sulfoquinovosyl diacylglycerol; **TCA,** tricarboxylic acid cycle; **TALEN,** transcription activator-like effector nucleases

Such large-scale cultivations of microalgae can be carried out in an autotrophic, mixotrophic, or heterotrophic fashion. However, continuous commercial operations for fuel production are currently not foreseeable [18, 19]. Identified limitations are the efficiency of algal growth and the energy-consuming downstream processes to isolate the algae and extract the desired components [20]. Thus, current approaches for the valorization of algae focus on very high-value products, which exploits the unique ability of microalgae to produce particular complex molecules, like, for example, the pigment astaxanthin by the green alga *Haematococcus pluvialis*. One obstacle here can be the rather low amount of such secondary metabolites in the production strains (e.g., 4 wt% astaxanthin in *H. pluvialis* [21]).

In summary, fuel production from microalgae oils aims at generating hydrocarbons, which resemble diesel and kerosene from crude oil. In terms of synthesis schemes and molecular complexity and functionality, this is a downgrading. On the other hand, the purification of existing, very high-value secondary metabolites is laborious, and expensive. An ambitious option from this perspective would be the production of higher value chemicals from algae oil, also taking advantage of the unique lipid compositions. Indeed, schemes to this end are emerging from ongoing research [22–24] and even in commercial applications.

2 Lipids in microalgae

Several microalgae strains naturally show a very high productivity of lipids. From extensive screening of the diverse algal groups [25, 26], the species listed in Table 1 have been identified to have very high lipid content and/or productivity. This reported data was obtained from different strains of the same species, grown at different cultivation conditions and most likely harvested at different points in the cell cycle, which strongly influences the lipid content/productivity and also the lipid composition. For example, the reported lipid productivity of *Nannochloropsis* sp. varies from 7.27 $mg\,L^{-1}\,day^{-1}$ [27] to 37.6–90 $mg\,L^{-1}\,day^{-1}$ [28].

Nevertheless, some trends can be derived from these values. There is no general correlation between a high lipid content and a high lipid productivity (Fig. 1) [29], resulting from the often inverse correlation between the lipid production and the biomass production.

The median lipid content varies in different algal classes. Cyanobacteria usually have the lowest cellular lipid content due to their high protein demand for the phycobilisomes. In contrast, heterokonts and haptophytes exhibit the highest relative lipid content, as revealed in a recent analysis which was based on more than hundred published studies about the macromolecular composition of microalgae [30].

Microalgae possess different lipid classes: Neutral triacylglycerides (TAG) are important storage products, which are deposited in lipid droplets and which constitute the

main amount of lipid in older, stationary cells (Fig. 2 – yellow droplet).

Polar lipids such as monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), or phosphatidylcholine (PC) are essential building blocks of the membranes, especially of the thylakoid membrane (Figs. 2 and 3) [31, 32].

Depending on the intended application, the choice of algae not only depends on the lipid productivity but also on the robustness of the strains in cultivation, on the amenability to genetic modification, advantageous harvest traits, like floating of senescent cells [33], or the fatty acid composition.

Microalgae are the primary producers of essential [34] fatty acids like the *ω3* fatty acids docosahexaenoic (22:6), eicosapentaenoic (20:5), or linolenic acid (18:3) or the *ω6* fatty acids arachidonic (20:4) or linoleic acid (18:2). Additionally, they produce fatty acids with unusual chain lengths, like palmitoleic acid (16:1), not found in nature elsewhere in significant amounts (Fig. 4).

Both, neutral and polar lipids, contain different fatty acids in the same species, with usually a higher degree of saturation in the TAG fraction [35]. Hence, not only the lipid composition depends on the age of the culture, but also the fatty acid composition is significantly altered. The older the culture, the higher is the degree of saturation.

The lipid productivity and fatty acid composition can be further influenced by the optimization of culture conditions (chapter 3). *Chlorella protothecoides*, for example, usually has a relatively low lipid content (14% [29]) and lipid productivity ($3\,mg\,L^{-1}\,day^{-1}$ [36]), however, in an optimized fed-batch culture in a 5-L bioreactor, it can be tuned to a lipid content of 50% and a lipid productivity of $3670\,mg\,L^{-1}\,day^{-1}$ [37]. A higher amount of PUFAs can be achieved, for example, at higher light intensities, preferably cold light [38, 39], and lower growing temperatures [40].

Another very promising strategy to increase lipid productivity is the exposure to stress. Also, the combination of different stress conditions may strongly affect the lipid and fatty acid composition (e.g., [41, 42]). Importantly, stressful conditions that induce high cellular lipid content at the same time often decrease growth efficiency and thus, in total, reduce the gain of total lipids per time. This is especially true for nutrient starvation experiments, which can result in cells with an enormous amount of lipids per cell dry weight (e.g., over 60% in green algae and in *Nannochloropsis* [29, 43, 44]), but usually induce a complete

Table 1. Promising algae species with a high lipid content or productivity

Organism	Algae species		Lipid content [% dw]	Lipid productivity [$mg\,L^{-1}\,day^{-1}$]
Prokaryotes	Cyanobacteria	*Synechococcus* sp.	11	75
Eukaryotes with primary plastids[a]	Green algae	*Dunaliella tertiolecta*	71	10
		Tetraselmis suecica	23	36
		Chlorella sorokiniana	22	45
		Chlorella protothecoides	58	3670
		Ettlia oleoabundans	42	148
		Ankistrodesmus falcatus	32	110
		Nannochloris sp.	56	77
		Neochloris oleabundans	65	134
		Scenedesmus sp.	21	54
		Chlamydomonas sp. JSC4	43	312
		Desmodesmus sp. F2	50	113
Eukaryotes with secondary plastids[a] [6]	Red algae	*Porphyridium cruentum*	19	35
	Diatoms	*Phaeodactylum tricornutum*	57	45
		Amphora sp.	51	160
		Thalassiosira weissflogii	30	5
		Cylindrotheca fusiformis	22	5
	Eustigmatophyceae	*Nannochloropis* sp.	53	90
	Haptophyceae	*Pavlova lutheri*	36	75
		Pavlova salina	31	49
		Isochrysis galbana	40	38
	Xanthophyceae	*Monodus subterraneus*	21	31

The values are compiled from different studies under different culture conditions and harvesting points and represent maximum values reported [26–32]. Hence, they are not directly comparable, but serve as indicators for high lipid producing algae species.
[a]Primary plastids derive from endosymbiotic cyanobacteria and have only two membranes. Secondary plastids evolved from secondary endosymbiosis, where a eukaryote engulfs a eukaryote with a primary plastid, resulting in complex plastids with more than two membranes.

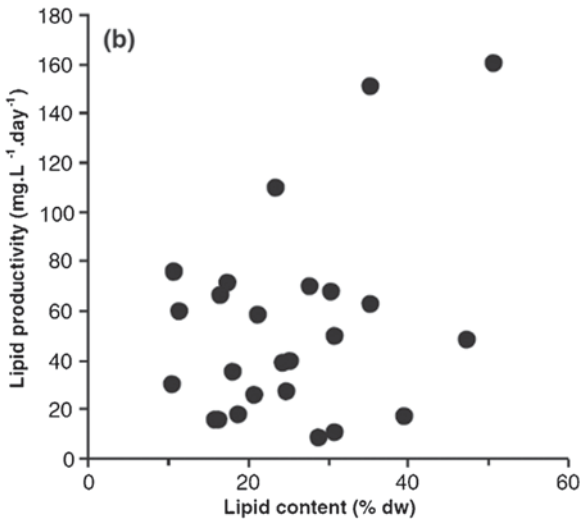

Figure 1. Correlation of lipid productivity (average of lit and calc.) with lipid content under nutrient-replete conditions for 55 algae species. Reproduced with permission.[26] Copyright 2007, Springer.

stop of cell division and thus growth. In the following, we will concentrate on different abiotic factors, which have been shown to influence the lipid content/productivity or fatty acid composition in microalgae.

3 Improving the cellular lipid productivity by adjusting cultivation conditions

There are numerous options to influence the lipid productivity as well as the lipid composition of microalgae by the cultivation conditions. Every species may react differently to a changed cultivation stimulus; hence, for a species of interest, the optimal cultivation conditions for high lipid productivity have to be determined individually.

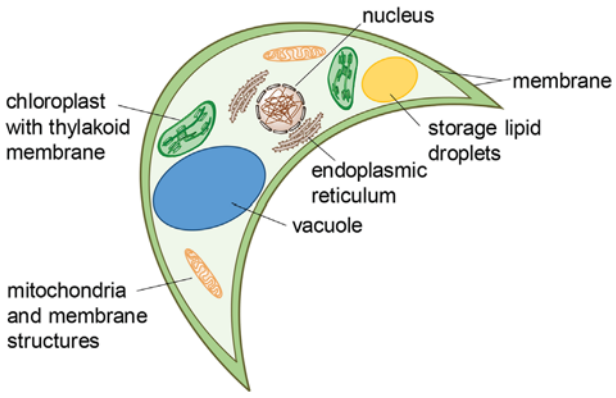

Figure 2. Schematic image of a green microalgal cell. The algae oil is stored in lipid droplets (yellow) and is also found in all cellular membranes, including those of the cell and the cell organelles, especially in mitochondria (orange) and chloroplasts (green).

3.1 Nutrients

Both, nitrogen and phosphate limitation have the highest influence on microalgal lipid composition because of their important structural role for chlorophylls, proteins, and ribonucleic acids (nitrogen) and due to their involvement in energy metabolism, signaling, and structural parts of ribonucleic acids (phosphorus).

Nitrogen starvation changes the cellular carbon flux from protein to lipid synthesis, since the reducing equivalents (NADPH/NADH) and carbon skeletons obtained by photosynthesis cannot be consumed for protein synthesis. At the same time, biomass production is slowed down [45]. Numerous studies show that nitrogen deficiency increases the total cellular lipid content in most of the investigated species such as in diatoms [46–48], green algae [8, 49–52], haptophytes [53], and the eustigmatophyte *Nannochloropsis* [43, 50, 54]. There are also a number of exceptions. For example the diatom *T. pseudonana*, the green algae *D. salina* [55], as well as most of cyanobacteria, do not respond to nitrogen-limitation with an increase of lipids [29].

Nitrogen limitation also changes the fatty acid composition in favor of short saturated or monounsaturated fatty acids, for example, in the diatom *P. tricornutum* [56], the green alga *C. reinhardtii* [52], the eustigmatophyte *Nannochloropsis gaditana* [54], the chrysophyte *Chromulina ochromonoides* [57], and the haptophyte *I. galbana* [58]. Contrasting reports exist about N-starved cells of *D. tertiolecta* where the degree of saturation increased in response to N-limitation [57] or remained unchanged compared to the N-replete culture medium [49].

Since starvation inhibits algal growth, also the influence of different nitrogen sources was investigated regarding their effect on the cellular lipid content and the composition of the fatty acids. Higher lipid productivities were achieved with nitrate compared to urea as a nitrogen source in the green alga *Desmodesmus* sp. F2 and *Neochloris oleoabundans* [51, 59], while in the diatom *P. tricornutum* the opposite effect was observed [60]. Urea as nitrogen source changed the fatty acid composition toward PUFAs in the haptophyte *I. galbana* [53] and in diatoms [61, 62] compared to NH_4^+or nitrate. In *Nannochloropsis salina*, the lipid content was unaffected by the nitrogen source [44].

Besides nitrogen, phosphorus limitation has also been reported to increase the lipid yield in haptophytes [63, 64], diatoms [56, 63–65], green algae [49, 66], and eustigmatophytes [64, 67]. Phosphorus limitation additionally changes the cellular fatty acid composition. While in the diatom *P. tricornutum*, similarly as under nitrogen starvation,

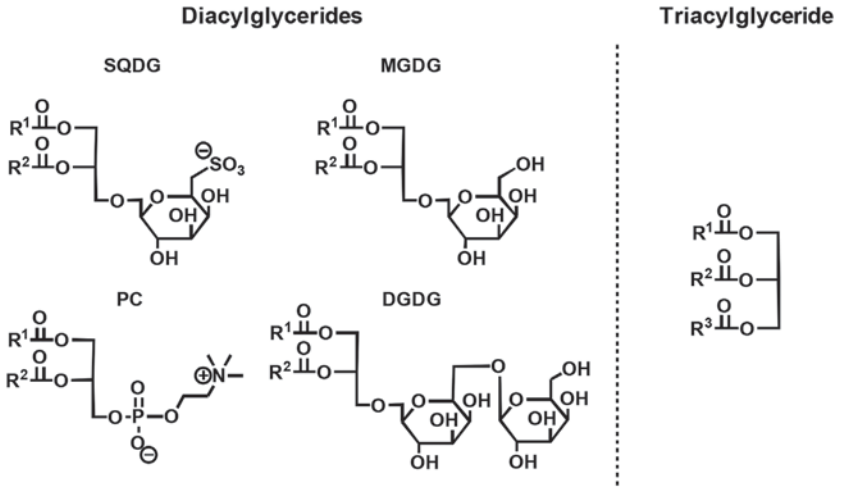

Figure 3. Polar diacylglycerides found in membranes and the neutral triacylglycerides (TAG) present in storage lipid. SQDG, sulfoquinovosyl diacylglycerol; MGDG, monogalactosyl diacylglycerol; PC, phosphatidylcholine; DGDG, digalalctosyl diacylglycerol.

more short saturated or monounsaturated fatty acids were produced [56], the eustigmatophyte *Monodus subterraneus* shows increased amounts of some saturated fatty acids but also of some PUFAs [67].

There are further nutrients that may also alter the lipid content of microalgae. Silicon limitation leads to strongly increased lipid amounts in diatoms [29, 65, 68], which was also found for the green alga *D. tertiolecta* under cobalt deprivation [49]. Also, the supplementation of several cations can increase the lipid content. In green algae, iron supplementation may increase the lipid amount [69, 70], especially when nitrogen availability is limited [42]. In *Chlorella minutissima*, when grown under mixotrophic conditions in wastewater, the heavy metal ions copper and cadmium increase lipid productivity [71], as does magnesium in *C. vulgaris* [72].

It is also worth mentioning that the application of biotic factors and specific bioactive triggers, such as cAMP, epigallocatechin gallate (a catechin), or quinacrine (an NF-κB inhibitor and p53 activator) could improve the lipid productivity remarkably. In *Nannochloropsis*, *Nannochloris*, and *P. tricornutum* the obtained lipid content in these microalgae was as high as in nitrogen starvation experiments but did not reduce growth as does nitrogen starvation [73].

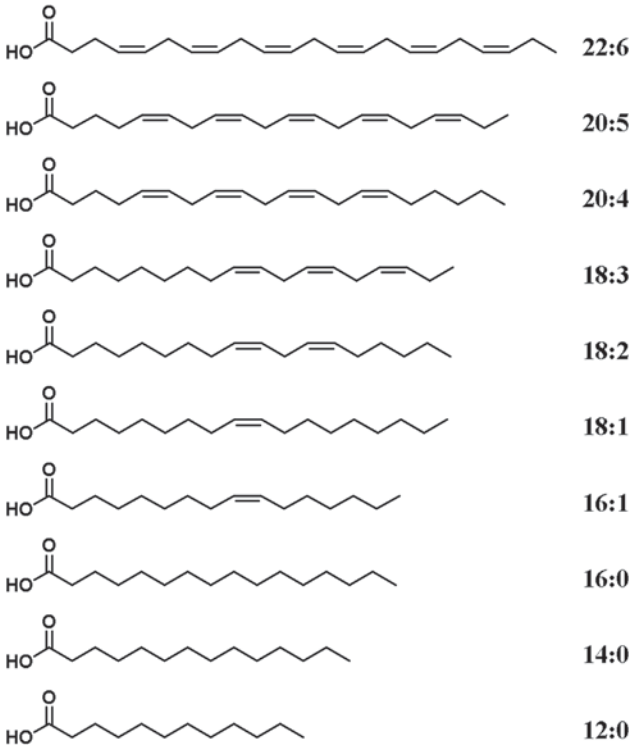

Figure 4. Examples of fatty acids produced by microalgae.

3.2 Light

Regarding autotrophic or mixotrophic processes, light, being the driving force for photosynthesis, has a strong impact on the lipid content and fatty acid composition of cells. Moreover, light quality and light quantity influence the metabolism via direct and indirect signaling processes affecting gene expression [74]. Usually, a higher light intensity leads to a higher cellular lipid content, because lipid synthesis is a possibility to get rid of excessive reducing equivalents without the need for nitrogen. This effect becomes especially important when a major nutrient is becoming limited, leading to reduced cell divisions, while on the same time photosynthesis continues [75]. The higher lipid amount mostly relates to a higher TAG content, while polar lipids rather decrease under high light conditions. Polar lipids are mostly found in the lipid rich thylakoid membranes, which are reduced under increased light intensities to protect the photosystems [35]. Moreover, the light intensity may also strongly increase the ratio of negatively charged lipids versus galactolipids in diatoms [76].

An increase of lipids during high light illumination has been shown for green algae [77–79], *Nannochloropsis* [41, 43, 80, 81], and diatoms [46, 82, 83]. No increase though has been observed for the green alga *Scenedesmus* [84] and for the haptophyte *Pavlova lutheri* [85]. Interestingly, while continuous light strongly increased growth in *Botryococcus braunii*, lipid productivity was enhanced only in regular light dark cycles [78]. Applying fluctuating light with high maximum intensities may also improve lipid productivity for certain species [86], however, apparently not for *Nannochloropsis sp* [87].

Higher light intensity additionally leads to a higher ratio of saturated and monounsaturated fatty acids versus PUFAs in eustigmatophytes [41, 80, 88], haptophytes [88, 89], and diatoms [83], while to a lesser extent in green algae [59, 90].

UV radiation can also be applied to change the lipid content of algal cells. UV-A radiation increased the saturated fatty acid content and decreased the PUFA amount in *Nannochloropsis* [91], while in diatoms the PUFA content was increased with UV-A and UV-B radiation [61]. UV-C radiation increases lipid production in the green alga *Tetraselmis* sp. M8 and the relative amount of unsaturated fatty acids [92], indicating different effects of UV-illumination on different algal taxa.

The influence of various light qualities in the VIS range on microalgal lipid content is difficult to assess, because, in order to draw valid conclusions, the light intensity at different wavelengths has to be correctly adjusted to promote the same photosynthesis rate. In a study that fulfilled these requirements, no differences in the lipid content of *P. tricornutum* cells grown at either blue or red light conditions could be detected [38]. In the green alga *Tetraselmis* sp. and in the eustigmatophyte *Nannochloropsis* sp., blue light induced a higher lipid content per volume cell culture compared to red light [93]. This higher lipid content correlated with a higher growth rate. However, both light qualities had been applied with the same intensity. Considering that blue light is more efficiently harvested in the antennae due to the high amount of carotenoids, it remains unclear whether there is a real lipid stimulating effect of blue light, or whether this was actually only a high light stimulating effect. In line with this, blue light stimulated growth and total lipid content in the haptophyte *I. galbana*, but the amount of lipid per cell was not increased and also the ratio of saturated, monounsaturated, and polyunsaturated fatty acids exhibited only minor changes [94].

3.3 Inorganic carbon and pH

Besides the availability of reducing equivalents, efficient lipid synthesis requires the availability of sufficient amounts of carbon. This carbon can be applied either directly via CO_2 aeration or by addition of bicarbonate. Bicarbonate is available in excess in aqueous environments and can be used by many algae.

Higher CO_2 concentrations (in the range of 1–5%) [62, 95–98] or bicarbonate addition [99, 100] can increase lipid content and lipid productivity in green algae, eustigmatophytes, and diatoms. In combination with other stresses, notably nitrogen-limitation, this results in an even larger shift of the additional carbon equivalents toward lipid synthesis. Regarding fatty acid saturation, CO_2 aeration, as well as bicarbonate addition, typically leads to a higher degree of saturation in green algae, haptophytes, and diatoms [62, 85, 100, 101]. In some cases, also a higher degree of unsaturation was achieved, demonstrating how species may react differentially to a change of abiotic factors [96, 97]. Another recent observation was that bicarbonate supplementation retards growth in certain species for unknown reasons [99].

One needs to take into account that addition of CO_2 or bicarbonate may alter the pH of the culture medium, which may lead to unwanted side effects. The pH can affect algal growth, carbon supply, and also the number of competitors in mixed cultures [102]. Usually, the pH fluctuates during day and night in closed cultures, as HCO_3^-/CO_2 uptake and consumption during the day lead to an alkalization of the medium, while the pH drops during the night due to CO_2 production via respiration [99, 103]. The influence of the pH on lipids is discussed controversially. In *N. salina*, only a minor effect of different pH values on lipid content and fatty acid composition was observed [104], while in green algae and diatoms more alkaline pH values increased the lipid content per cell, especially in combination with nitrogen depletion [62, 84, 104, 105]. An alkaline pH can also increase the degree of fatty acid saturation [62, 84].

3.4 Temperature and salinity

Temperature has a strong influence on lipid productivity. In green algae, haptophytes, chrysophytes, eustigmatophytes, and diatoms, temperatures lower than optimal for growth stimulated lipid accumulation [50, 57, 106]. In certain species, even increasing cultivation temperature resulted in elevated lipid productivities [50, 62, 107], in *Scenedesmus obtusus* only in combination with nitrogen deficiency [108]. Usually, there is a negative correlation between growth and lipid content depending on the temperature [57]. Interestingly, this correlation is not found in the green alga *Chlorella vulgaris* where relatively lower temperatures did not slow down growth, but resulted in a pronounced lipid accumulation, indicating a promising strategy for improving lipid production [50]. The temperature also has a strong effect on the fatty acid profile. Typically most algal groups increase the degree of fatty acid unsaturation at low temperatures [50, 57, 84, 106, 109] in order to maintain membrane fluidity, but there are contradictory reports about the fatty acid profile under different temperatures for the diatom *P. tricornutum* [62, 110].

Moderate higher salinities than normally prevailing in the habitat of the investigated species can increase lipid content in

green algae, haptophytes, and eustigmatophytes [41, 111–115] and also seem to favor a higher degree of fatty acid saturation [62, 114–116]. Diatoms apparently do not respond with an increase in lipid content to higher salt conditions [62, 115]. Changes in salinity in combination with other stress factors such as nutrient deprivation or light intensity have a species-specific effect on the lipid productivity [41, 111]. The higher lipid content recorded under higher salinity is often accompanied by a slower growth rate, but careful adjustment of the salt concentration can increase lipid productivity to some extent, for example, in *D. tertiolecta* or *Chlorococcum* sp. [112, 113]. In *Tetraselmis* sp., lower salinities even increase lipid productivity, as growth is favored while cellular lipid content remains unchanged [117].

In summary, many abiotic factors can change the lipid content, fatty acid composition, and the lipid productivity of microalgae. Probably the largest potential for increasing lipid productivity inheres in a combined approach of applying different abiotic (stress) conditions for cultivation and genetic manipulation of specific metabolic pathways in the target species (chapter 4). Importantly, the right balance between imposing modest stress to the cells (in order to direct photosynthetic electrons toward lipid production) but at the same time promoting growth has to be finely adjusted. Additionally, the degree of saturation has to be considered, because the olefinic functionalities are most valuable for chemical functionalization.

In this respect, large system biology approaches including transcriptomics, proteomics, and metabolomics, in combination with sophisticated physiological measurements, as used in refs. [81, 118, 119], could be helpful in order to understand the cellular mechanisms involved in lipid accumulation. This knowledge could then be exploited for further improving the lipid productivity in appropriate species by tuning their metabolism toward high lipid productivity with the aid of reverse genetics approaches and the cultivation under specific abiotic factors in suitable ranges.

4 Improving the cellular lipid productivity by genetic engineering

The lipid productivity of the cells can be further improved by the genetic optimization of the strains. As more and more data on genome sequences and on regulatory properties in microalgae become available, the possibilities increase to develop and use genetically modified strains that have enhanced levels of the respective natural product, like for instance lipids [120, 121].

4.1 Promising targets for genetic modification

Promising targets for genetic engineering of algae are higher lipid productivity, an increased resistance against herbicides and antibiotics [11, 12, 122], the secretion of the desired product to the culture broth [10], or thinner cell walls to facilitate product purification. The first algae genomes were sequenced in the last 10 years [5–9]. Consequently, current knowledge about the algal metabolism is still sparse [123]. However, many more recent findings can be transferred from higher plants and fungi to microalgae [121, 123, 124]. Until now, most studies and reviews [121, 125–127] aimed at an increased lipid content identifying six promising approaches (Fig. 5):

1. Overexpression of the starting material,
2. Overexpression of fatty acid synthesis,
3. Overexpression of TAG assembly,
4. Modification of fatty acid composition,
5. Knock-down of catabolism, and
6. Knock-down of competitive pathways.

A first approach targeted the starting material for fatty acid synthesis – acetyl-CoA (coenzyme A) (Fig. 5 – 1). In *P. tricornutum* silencing of the pyruvate dehydrogenase kinase (PDK) resulted in an increased lipid content, because a knocked out PDK cannot inactivate the pyruvate dehydrogenase complex (PDC), which generates acetyl-CoA from pyruvate [129]. A second approach is the overexpression of genes controlling the fatty acid biosynthesis (Fig. 5 – 2), which was, however, not as successful as expected.

Acetyl-CoA carboxylase catalyzes the conversion of acetyl-CoA to malonyl-CoA. Despite a 2- to 3-fold increase in acetyl-CoA carboxylase activity, no increase in lipid production was observed [129]. Gong et al. were, however, successful with the overexpression of a thioesterase in *P. tricornutum* thereby enhancing the total fatty acid content by 72% [130]. The thioesterase is the last enzyme in the fatty acid biosynthesis and cleaves the resulting fatty acid from the acyl carrier protein (ACP).

The overexpression of genes accompanied with TAG assembly (Fig. 5 – 3) seems to be more promising, like in the case of the glycerol-3-phosphate acyltransferase. Overexpression in *C. reinhardtii* and *P. tricornutum* leads to a 1.5- and a 2-fold increase of TAG, respectively, and a higher proportion of unsaturated fatty acids [131, 132]. *C. reinhardtii,* genetically engineered with the diacylglycerol acyltransferase 2 from *Brassica napus*, showed a threefold increase in TAGs, together with 12% more PUFAs and a shift from 7% saturated fatty acids toward unsaturated fatty acids [133].

It would also be valuable to tune the fatty acid composition (Fig. 5 – 4), by engineering the lipid modification. The chain length is mainly influenced via specific thioesterases. An overexpression of the 12:0- or 14:0-thioesterase increased the content of 12:0 and 14:0, respectively [134, 135], which might be interesting for the surfactant industry (compare chapter 8). Additional targets are PUFAs which are used for human health supplements or infant formula (chapter 7) [136–139]. PUFAs are synthesized by elongases extending the chain lengths and

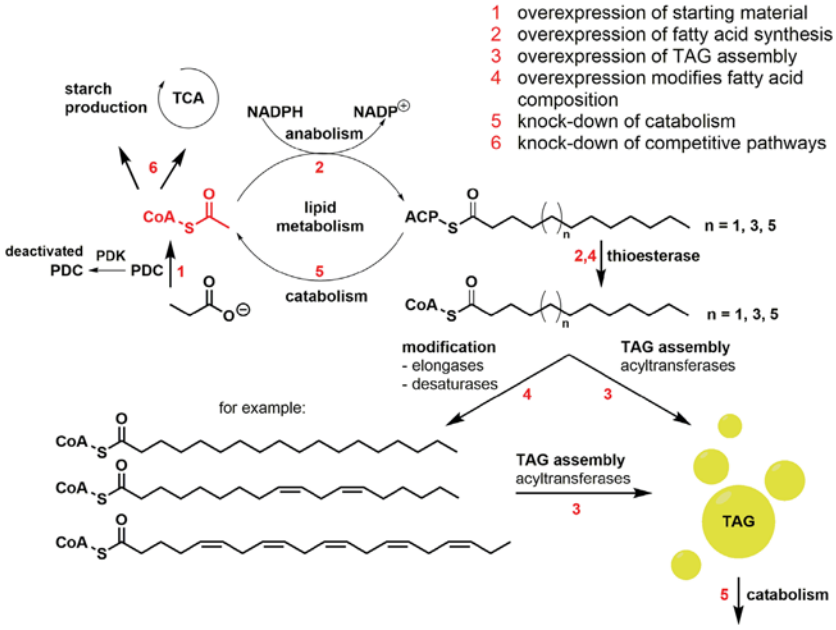

Figure 5. Schematic overview over points of attack in the lipid metabolism for the increase of the lipid content or tuning of the fatty acid composition in algae. ACP, acyl carrier protein; CoA, coenzyme A; PDC, pyruvate dehydrogenase complex; PDK, pyruvate dehydrogenase kinase; TAG, triacylglycerides; TCA, tricarboxylic acid cycle.

desaturases, inserting additional double bonds. Hamilton et al. engineered *P. tricornutum* with the Δ5-elongase from another alga resulting in an eightfold increase in docosahexaenoic acid in storage lipids [140]. Peng et al. and Zäuner et al. overexpressed desaturases in *P. tricornutum* and *C. reinhardtii,* respectively, observing an increased lipid content and a higher degree of unsaturation [141, 142]. A comprehensive review of the different genetic possibilities to increase the EPA and DHA content in microalgae was published by Mühlroth et al. They identified 106 genes in *P. tricornutum* operating in the fatty acid metabolism and in the tricarboxylic acid cycle (TCA) [123]. PUFA synthesis is very energy intensive due to the activity of desaturases and elongases. Consequently, the bottlenecks in PUFA production are enzymes producing precursors and cofactors. Their availability is important for an increased lipid production making them attractive targets for genetic engineering. One example is NADPH, which is synthesized, for example, by the malic enzyme, controlling the lipid accumulation in *N. salina* or *P. tricornutum* [143, 144].

Knocking down the lipid catabolism also results in an increased lipid content (Fig. 5 – 5). A knock-down of a multifunctional lipase/phospholipase/acyltransferase in *T. pseudonana* increased the lipid content in the exponential phase 2- to 3-fold and in the stationary phase 3- to 4-fold [145]. Barka et al. knocked down the TAG-lipase in *P. tricornutum,* which suppressed the TAG degradation, resulting in an increased TAG content [146]. Furthermore, the knock down of the enzymes of the β-oxidation of fatty acids prevents the loss of storage lipids but is also accompanied by a decreased doubling time [121].

The last possibility is a knock down/out of competing metabolic pathways (Fig. 5 – 6) for other high-energy storage products like starch or pathways using the same starting compound (acetyl-CoA) [121]. Here, the photosynthetically assimilated carbon is redirected from starch to lipid synthesis. A defective ADP-glucose pyrophosphorylase, the first enzyme of starch production, in *C. reinhardtii* resulted in an eightfold increase in neutral lipids under stress conditions [147].

Doshi and coworkers pursued a very different approach using an *Escherichia coli* test system. They introduced a ubiquitous membrane-embedded transporter resulting in a lipid efflux into the culture broth. They assume that the transporter is compatible with bacteria, cyanobacteria, yeast, diatoms and other microalgae, which would simplify the extraction procedure of lipids remarkably [10].

The introduction of unique microalgae genes into higher plants or fungi is also pursued toward different targets, like the identification of unknown genes [148–150] or the production of special microalgae compounds, like PUFAs in host organisms [151, 152]. As an example for the latter, Xia et al. heterologously expressed the Δ9-elongase gene of *Isochrysis galbana* in the plant *Arabidopsis*, resulting in 64% more elongated fatty acid products (18:2 → 20:2 and 18:3 → 20:3) [152].

4.2 Tools for the genetic modification of microalgae

4.2.1 Unspecific mutagenization

The apparently easiest way to create suitable production strains is random mutagenesis, including treatment with UV light or via chemical modification, followed by a subsequent characterization and screening/selection of strains that

produce more of a specific substance than the wild-type strain. Using this method, the nature of the mutation may remain unclear, which, however, may be unproblematic as long as the respective strain clearly does not show any new disadvantageous properties. As most mutations here will rather lead to inactivation of genes, this method is limited to those algal species that have at least one stable haploid stage and/or that offer crossbreeding opportunities, which is required for the creation of homozygous mutants. Successful strain optimization by random mutagenesis has been performed in the colorless dinoflagellate *Crypthecodinium*, strongly increasing the share of docosahexaenoic acid among the total lipids (see chapter 7) [153].

4.2.2 Specific genetic transformation

The second option for genetic modification of algae includes targeted approaches, like the introduction/overexpression of new genes, or the knock-down or knock-out of specific genes [154]. Significant progress in the development of species specific gene transfer [26] systems for eukaryotic algae has been achieved only within the last 25 years. These techniques allow the modification of algae, either in order to obtain strains, which produce certain compounds of commercial interest, or to gain information about cellular, physiological or biochemical mechanisms by switching off, downregulating or overexpressing existing or foreign genes, respectively [155]. Up to now, successful genetic manipulations of more than 40 different eukaryotic microalgae species have been reported, including the green algae *C. reinhardtii* [156], *Dunaliella salina* [157], *Chlorella vulgaris* [158], and *H. pluvialis* [159], as well as the diatoms *P. tricornutum* [160], *T. pseudonana* [9], *Cylindrotheca fusiformis* [161], and the eustigmatophyte *Nannochloropsis* sp. [162].

Most transformation methods rely on the fact that, as soon as DNA can be successfully imported into cells, there is a fair chance for integration of the DNA fragments into the nuclear genomes. For the prominent green alga *C. reinhardtii*, the largest spectrum of DNA delivery techniques was developed (Fig. 6 – 1–4) [163]:

1. Biolistics: Bombarding the cells with DNA-coated tungsten or gold microparticles [164, 165],
2. Vortexing the cells in the presence of DNA and silicon carbide whiskers [166], glass beads [167], or amino clay nanoparticles [168],
3. Electroporation: Applying a strong electrical field to the cells,
4. *Agrobacterium tumefaciens*: Utilization of bacteria that actively inject DNA into the cells [169].

In the case of diatoms, biolistics had long been the method of choice [170], while recently successful genetic transformations based on electroporation [171] as well as the utilization of transfecting *E. coli* have been reported [172].

For efficient expression of nuclear transgenes, strong promoters are required. Although there are some general promoters available from plants or fungi [169], mostly endogenous promoters of the respective target organism are used to ensure high rates of expression (see [120] for an extensive list of nuclear control elements used in algae). For nuclear expression, these promoters often include those of the small Rubisco subunit [173], or the light harvesting apparatus [170], but also others [120]. Different types of selectable markers and reporter genes can be used for the essential screening process to detect genetically modified clones among the wild-type cells that usually are the majority. Most of these selection markers confer resistance to antibiotics or herbicides, while there are also systems available that allow the rescue of auxotrophic or non-photosynthetic mutants [120].

In addition to the overexpression of proteins, silencing (knock-down), or disruption (knock-out) of key genes have been demonstrated to be a valuable tool to modify cell lines [174]. Silencing approaches take advantage of the RNAi mechanism in eukaryotic cells which results in a degradation of mRNA and in a decreased amount of the corresponding gene product [175, 176].

In order to knock-out specific genes, homologous recombination (the replacement of a region of the genome by a modified version), originally developed for prokaryotes like cyanobacteria, often fails for eukaryotic genomes. Therefore, different approaches of genome editing have been developed based on nucleases that bind specifically to distinct DNA sequences, allowing the targeting of single genes within a full genome. Meganucleases [177], zinc finger

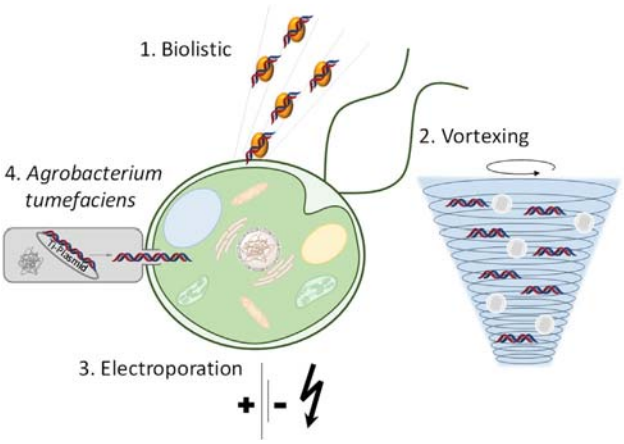

Figure 6. DNA delivery techniques for *C. reinhardtii*. (1) Biolistic: bombarding cells with DNA-coated gold particles; (2) Vortexing the cells with for example glass beads and DNA; (3) Electroporation: an electrical field causes porosity of the cell membranes; (4) The bacterium *Agrobacterium tumefaciens* has the natural ability to inject a plasmid (Ti-plasmid) into the cell, which can be genetically modified.

nucleases [178], TALENs (Transcription Activator-like Effector Nucleases) [5, 179], or CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) [180] recently became popular approaches. By cleaving the DNA strands, cellular repair mechanisms are induced that may result in insertions, deletions, or mutations within the target gene. These genome editing approaches are extremely helpful for targeting all copies of a gene in a genome, for instance in the diplontic diatoms, where no crossing options were available so far. There is a number of examples where the silencing of individual genes resulted in an increased lipid content of the respective cells [181]. An apparent limitation of genetically modified algae for production is their status as Genetically Modified Organisms (GMOs), which requires tight control of the strains in the lab or in bioreactors only. Although the question, whether CRISPR/Cas9-modified organisms are GMOs is still under debate, this requires a strict control to exclude any release of the GMOs into the environment [182].

5 Technical production of algae

Technical algae-to-product processes can be divided into four steps: Cultivation, Harvest, Downstream Processing, and Purification, which contribute very differently to the overall effort and cost of product formation.

5.1 Cultivation

Cultivation is the most time-intensive step. It can be conducted in continuous or batch operations. Capital and operating costs vary significantly for different cultivation methods. Microalgae are very flexible organisms. They grow autotrophically with sunlight as energy source and CO_2 as carbon source in open ponds [183] or photobioreactors (PBRs) [19], mixotrophically with different energy, and carbon sources or heterotrophically with an added carbon source in dark fermenters [184]. All these systems are applied commercially or have been studied in large public or private sponsored research programs [18, 19, 185]. The final system of choice will depend on the algae used and the targeted product and scale (Fig. 7- Cultivation).

In autotrophic cultivation systems, light, and the availability of nutrients determine the growth of microalgae. In dense algae cultures, the penetration depth of light is strongly reduced, and therefore large surface areas with only a shallow depth are required (this applies to both open raceway ponds or photobioreactors) leading to a very low volume per area ratio [186]. To gain higher cell concentration, heterotrophic, or mixotrophic cultivation of suitable algae species can be performed, sometimes resulting in higher lipid yields, as in the case of green algae, *Nannochloropsis*, *Spirulina* and diatoms [186–188, 190].

As a specific example, the lipid content in a heterotrophically grown *C. protothecoides* could be increased fourfold compared to autotrophic growth. However, Liang et al. found the opposite in *C. prothecoides* [189, 191]. In contrast, Cheirsilp et al. and Heredia-Arroyo et al. found no significant difference between the lipid contents of *C. prothecoides* cultivated with various methods [187, 192]. Interestingly, under mixotrophic cultivation conditions, abiotic stress effects can be different from autotrophic growth conditions as tested for *Nannochloropsis* sp. and *Chlorella* sp.: the stimulating effect of higher light intensity on the cellular lipid content in autotrophic growth (see chapter 3.2) is reversed in mixotrophic growth. A stepwise increase of light intensity and a stepwise addition of glucose resulted in the highest lipid content and lipid productivity in *Chlorella* sp. and *Nannochloropsis* sp. [187].

Moreover, nutrient limitation (especially nitrogen, see chapter 3.1) can strongly increase the lipid amount per cell also in heterotrophic and mixotrophic growth. Under these conditions, the application of an organic carbon source can even further promote lipid synthesis [193]. Different carbon sources are used by mixotrophic or heterotrophic algae with glucose as the most prominent substrate, which is, however, expensive [186, 194]. More economical feedstocks are provided by waste products like molasses or agricultural waste. Regarding the fatty acid profile, heterotrophic, and mixotrophic growth conditions result in a lower degree of unsaturation [186, 188, 195], with exceptions in diatoms producing more PUFA [188, 196–198].

Furthermore, not all algal strains are capable of heterotrophic growth [184]. It also has to be considered, that heterotrophic growth does not use the full potential of microalgae and is not independent of arable land [199, 200]. Autotrophically grown microalgae convert five times as much solar energy into biomass than higher plants which provide the sugar for heterotrophic growth (albeit providing microalgae with CO_2 feed is associated with more technical effort compared to fixation from air by higher plants) [201].

Comparing the different cultivation systems for autotrophic growth, open ponds are currently less expensive than PBRs with regard to capital and operational costs (Table 2). However, they feature a higher risk of contamination and a decreased photosynthetic efficiency because of low mixing rates and therefore poor light conditions. Additionally, this system is not suitable for GMP (Good Manufacturing Practice) since reproducibility cannot be guaranteed [199]. It is also important to note, that cultivation of GMOs in open ponds, can be problematic, and restricted by local legislation [182, 208–210]. The PBR, however, is suitable for GMP and GMO cultivation, has a higher biomass production per area than the open pond and a better PCE with around 5%. Disadvantageously, capital, and operational costs are comparatively high (chapter 5.4).

5.2 Harvest

Harvesting can be a major technical and economic bottleneck for the development of algae mass cultures and can account for up to 20–33% of total production costs [33]. There are several reasons for such a high influence on the total costs. The cell concentration in autotrophic algae culture is very low, typically 0.1–8 g dry weight L^{-1} of the culture broth [211]. Consequently, large amounts of water have to be removed and at best recycled, which adds to cost. Furthermore, the cells are in general very small (5–50 μm in diameter) and have only a marginal density difference to the culture medium (~1020 kg m^{-3}), which complicates their separation [212]. This problem was already identified in the 1980's: Gudin and Thepenier suggested naturally flocculating strains or the precise evaluation of the "harvest traits" during the cell cycle –, for example, senescent *Botrycoccus braunii* float while young cells stay in suspension [33]. However, no significant progress was made in the development of less expensive methods for harvesting. Gerardo et al. in 2015 summarized the developments in harvesting techniques and concluded that cell harvest still has the most influential effect on the economy of autotrophic microalgal production [213]. There are several approaches to facilitate this step and reduce costs. Dassey et al. evaluated the economics of different centrifugation strategies with a continuous centrifuge. A high flow rate decreases the efficiency (only 28% capture rate), but this can be compensated by several passes or the use of the biomass as inoculum for the next growth cycle, resulting in an overall decrease in harvesting costs by 82%. For a relatively high lipid content of 60% dry weight and a culture density of 500 mg/L, they calculated a prize of \$0.864 L^{-1} oil, instead of \$4.52 L^{-1} oil stated by the Department of Energy. However, such high cell densities can only be achieved with heterotrophic growth [212].

The harvest is mostly performed in a two-step approach. The culture is enriched in a bulk harvest step to around 2–5% of the original culture broth volume via flocculation, flotation or gravity sedimentation [200]. In the second step, centrifugation or filtration further increases the dry solids content to 15 or 20%, respectively (Fig. 7 – Harvest). This is generally the more energy-intensive step. Centrifugation and filtration impose significant capital and operational costs, but result in higher quality biomass, since no addition of chemicals is required. Accordingly, this approach is more appropriate for higher value products like, for example, human health supplements. Harvesting via flocculation or flotation by addition of flocculation/flotation agents is cheaper and suitable for chemical or biofuel production from algae. Since in heterotrophic or mixotrophic cultures cell densities are 100–1000 times higher, the costs for harvest are reduced in these systems, since often only one step is necessary (compare chapter 7 and 8).

5.3 Downstream processing and purification

Downstream processing greatly varies for the different targeted algae products. The subject has been reviewed comprehensively by Brannan et al. [200]. There are several steps, that all processes have in common, and these will be discussed in the following.

After harvest, the algal slurry with 15–20% dry solid content is perishable and needs to be processed rapidly. Depending on the targeted products, a drying or dehydration step is necessary, which may increase the stability of the product [200]. Biomass can be dried via sun drying, drum drying [214], spray drying [215], freeze drying [216], or refractance window dehydration [217]. Sun drying is the cheapest method, but requires large surface areas and is

Table 2. Comparison of heterotrophically and autotrophically grown algae

	Heterotrophic growth	Autotrophic growth	
	Fermenter	Open pond	PBR
Productivity (biomass)	10–50 g L^{-1} day^{-1} [200]	10–25 g m^{-2} day^{-1} [200, 202]	47–98 g m^{-2} day^{-1} [200, 203]
Biomass concentration	50–100 g dry weight L^{-1} [186, 204]	0.1–0.5 g dry weight L^{-1} [201, 204–206]	2-8 g dry weight L^{-1} [201, 205, 206]
Carbon source	Sugar cane, molasses	CO_2, loss high	CO_2, loss low
Risk of contamination	Low	High	Low
Algae strains	Restricted to strains capable of using organic carbon sources	Limited to robust algae strains insensitive to contamination	All algae can be cultivated
Photosynthetic efficiency (PCE)	No photosynthesis during algae cultivation	PCE ~ 1%	PCE ~ 5%
GMP-suitability	Suitable	Not suitable	Suitable
GMOs-suitability	Suitable	Limited suitability [207]	Suitable

time-consuming [214]. Spray drying and freeze drying are equally costly and are viable for higher value products only.

Since the desired algal products are mostly located inside the cells, a disruption step commonly increases the extraction efficiency [218, 219]. Physical (bead mill, ultrasonication, and expeller press), chemical (acidic or alkaline), and biological (lysing enzymes) methods are available to disrupt cells, which all contribute only marginally to the total cost (e.g., cell disruption, an extractor centrifuge, and the stripper contribute together with 4% to the total capital costs according to [220]).

In a next step, the products are extracted with an organic solvent. As an alternative, extraction with supercritical CO_2 is also developed [221–225]. Supercritical CO_2 extraction has several advantages: The extraction efficiency is high since the fluid density and consequently the solvent quality can be controlled by changes of pressure or temperature, it allows for low-temperature processing, is non-toxic, non-flammable, and the supercritical solvent can easily be removed [224, 226, 227]. On an industrial scale, supercritical solvent extraction is currently applied for the extraction of astaxanthin from microalgae [228, 229] or the decaffeination of coffee [230]. Points of consideration are the high expenses for high-pressure equipment and also operating costs for compression. Thus, standard organic solvent extraction is usually less costly and sufficient for chemicals production from microalgae.

Further subsequent steps strongly depend on the desired goods. Therefore, they are discussed for discrete examples of products produced. Figure 7 shows the general workflow from the microalgae culture to the product, which is detailed to the case studies in chapters 6, 7, and 8.

5.4 Techno-economic analysis

A number of techno-economic analyses calculating the costs for each step for autotrophic growth are available [206, 220, 231–233].

Davis et al. calculated the capital (CAPEX) and operational (OPEX) expenses for PBR and open ponds showing that economics are driven more by CAPEX than by OPEX (Table 3).

Installation of PBRs is in total more expensive. However, they require only 30% of the water needed for open pond cultivation. Thus, PBRs would be especially interesting in water-limited areas. It is important that the CAPEX for open pond systems are fairly evenly allocated across the number of systems, while for the PBR the lions share of the capital costs is the PBRs themselves with ~80% (Fig. 8).

From this analysis, minimum selling prices for crude TAGs or hydrocarbon fuels ("Diesel," also cf. section 6) given in Fig. 9 are calculated for this theoretically projected large facility size (38.000 m^3 of crude algae oil per year). In this scenario, capital costs (depreciation) contribute significantly to the overall cost of production, particularly for the case of PBRs.

This theoretical study suggests that even on this relatively large scale of production algae-based fuel cannot compete with petroleum-based fuels in the foreseeable future. It can also be noted, that the suggested costs are not immediately prohibitive for a production of chemical intermediates per se.

Sensitivity analysis showed that the economics are most sensitive towards the lipid content and growth rate, which can be most effectively increased via genetic engineering [206].

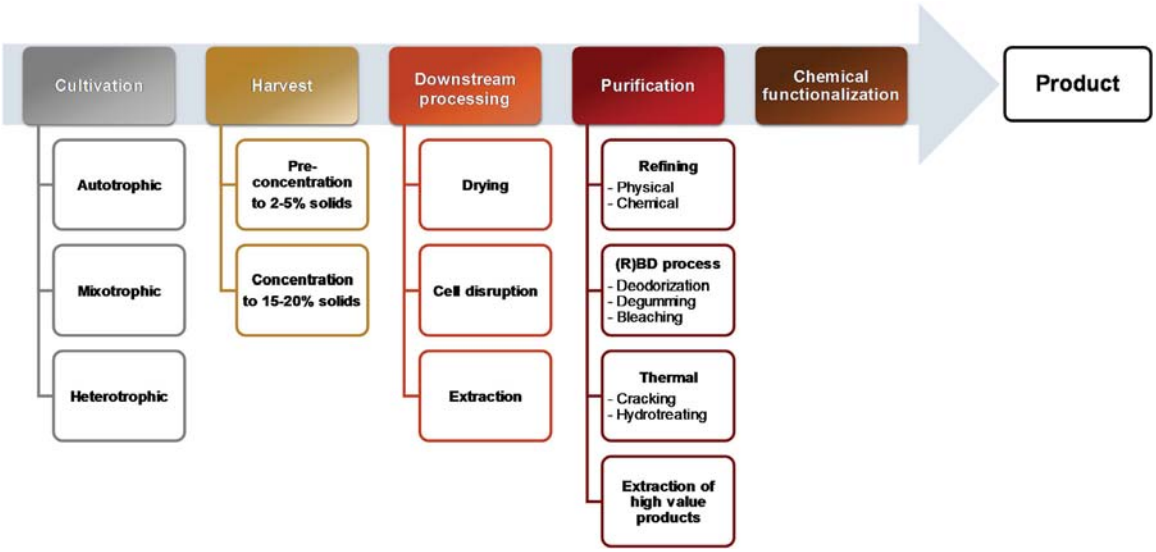

Figure 7. General scheme and methods of the production of chemicals from microalgae from cultivation downstream to the product.

We are not aware of a published detailed, immediate comparison between economics of autotrophic versus heterotrophic production on different scales. Considered by itself, heterotrophic production in principle can rely on industrially feasible established fermentation reactor and workup technologies.

6 Insights from biofuels for chemicals production

Early on in the rise of petroleum as a transportation fuel on a large scale and of the petrochemical industry, algae have been suggested as an alternative in view of finite fossil fuel resources [16]. To cover the current (2016) fuel consumption (finished motor gasoline) of the United States with soybean or rapeseed as a lipid source, an area of land that nominally corresponds to 130 and 50%, respectively, of the area of the US is required.[1] For comparison, microalgae with 30% lipid content could do with 1% theoretically (for a critical assessment cf. [20]). Since the 1970s, sizeable research and development programs have addressed the production of microalgae and their use as a source of fuel [18, 19, 185, 234, 235]. These programs discovered many of the high-lipid microalgae strains and developed systems for algae cultivation. Namely, the Aquatic Species Program (ASP – 1978–1996), funded by the US Department of Energy, started the first high-lipid-content microalgae collection for biodiesel production. The collection is still available for researchers at the University of Hawaii. They firstly investigated microalgal genetics (chapter 4) and the influence of cultivation conditions on algal lipid production (chapter 3) [236]. The Japanese research program "Biological CO_2 Fixation and Utilization" addressed photobioreactor design for microalgae cultivation in the 1990's [19].

A large body of literature addresses the optimization of microalgae cultivation, harvest, or downstream processing [201, 202, 213, 237, 238]. Some selected more recent examples and aspects are instructive here. Sapphire Energy, Inc. has developed an end-to-end process to produce algae-based fuel. For this purpose, algal strains are genetically optimized. For instance, a *C. reinhardtii* strain with an optimized thioesterase gene, integrated into the chloroplast genome, is used [239]. The biofuel ultimately produced differs from conventional common Biodiesel in that it does not consist of fatty acid methyl esters, but hydrocarbons.

Figure 10 schematically shows the Sapphire approach to this so-called "Green Crude." The microalga *C. reinhardtii* is

Table 3. Capital and operational costs of open pond and PBR according to Davis et al. for a facility with an output of 38.000 m^3 of crude algae oil $year^{-1}$ [206]

	Open pond	PBR
Productivity	25 $g\,m^{-2}\ day^{-1}$	1250 $g\,m^{-3}\,day^{-1}$
OPEX	\$37	\$55
CAPEX	\$390	\$990

Microalgae with 25% lipid yield (dry weight), 38.000 m^3/year algae oil, 330 operating days.

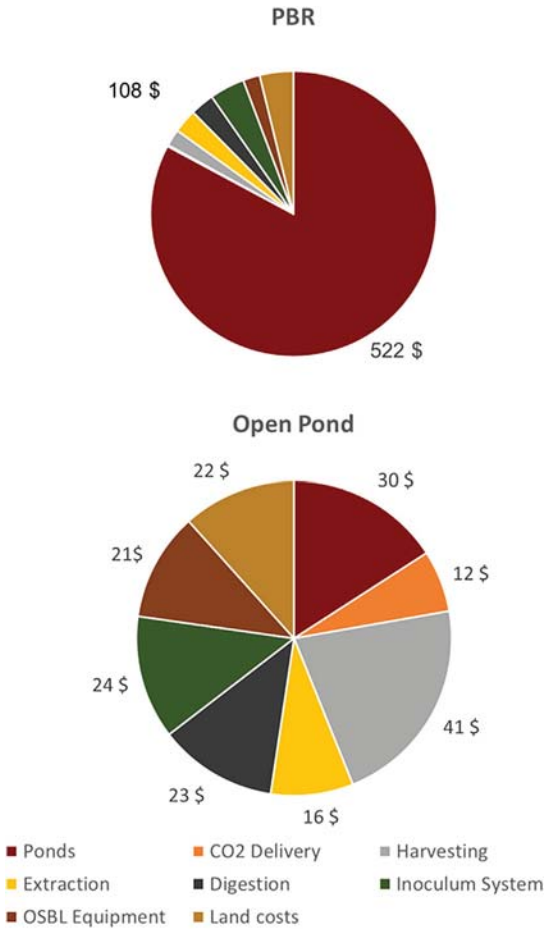

Figure 8. Capital cost composition for open ponds (bottom) and PBRs (top) in million \$. OSBL, Outside battery limits, which are costs for equipment, which is not directly associated with elements between the input and output of the plant. Adapted with permission.[187] Copyright 2012, Elsevier.

[1] $\%\ \text{US area} = \frac{\text{US fuel consumption}[L]\cdot 100}{\text{Oil yield}\left[\frac{L}{\text{ha}}\right]\cdot\text{US area [ha]}}$

US fuel consumption (finished motor gasoline) [L]: [265]. (2016) *US Energy Information Administration Independent Statistics and Analysis*, https://www.eia.gov/tools/faqs/faq.cfm?id=23&t=10 (accessed July 2016).
The oil yield [L/ha]: [201]. Chisti, Y. (2007), Biodiesel from microalgae. *Biotechnol Adv*. 25 (3), 294–306.
The US area [ha]: Blank, R.M., Mesenbourg, T.L., Commerce, U.-D.o., Bureau, U.S.C., *United States Summary: 2010, Population and Housing Unit Counts*, 2012, Table 18.

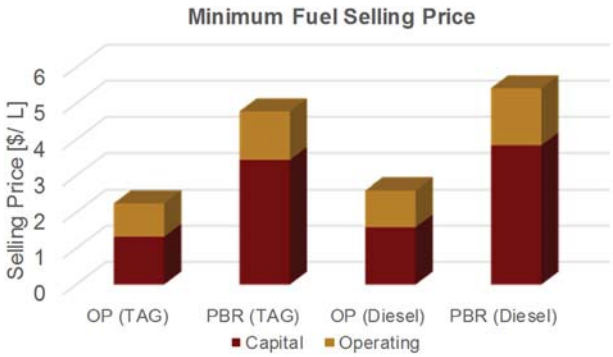

Figure 9. Minimum selling price required to achieve 10% rate of return for algal TAG and Diesel production (TAG, triacylglycerides; OP, open ponds; PBR, photobioreactors). Adapted with permission.[187] Copyright 2012, Elsevier.

cultivated in open raceway ponds of around 400.000 m^2 in the Chihuahuan desert outside of Columbus, New Mexico. In order to harvest the cells, they are first collected with a flocculent and pumped into a settling tank where dewatering takes place. The suspension is dragged via vacuum through Zeeweed membranes, thereby accumulating the algae cells in the process tank. The resulting concentrated suspension is fed to a disc stack centrifuge to remove more water. The water is recovered and pumped back into the raceway ponds. The algae oil is extracted with hexane and a conditioning agent in a high shear reactor. The hexane phase containing the algae oil is separated from the water phase containing the cell debris and algae solids. The solids from the water phase are concentrated in a centrifuge and further processed to animal feed. The hexane of the organic phase is distilled off and reused. The resulting algae oil is transported to the refinery, where the oil is converted to "Green Crude" [239].

The fatty acids, mainly C_{15}-C_{18}, and glycerides are transesterified and further subjected to established processes for refining of crude oil, namely hydrotreating. This ultimately hydrogenates and deoxygenates the fatty acids to hydrocarbons [239]. The process is standardized from the Honeywell UOP Ecofining^{TM} process [240, 241].

The resulting long-chain linear paraffins are hydrocracked into shorter branched paraffins, with boiling points in the range of a kerosene fraction (Fig. 11). The blending of this algae based kerosene with conventional kerosene has been cited as means to fine-tune the density to existing specifications [239]. Sapphire Energy has supplied its algae-derived hydrocarbons to the refiner Tesoro Cooperation in 2013, which blends its petroleum with 0.0003% of "Green Crude" [242].

A different approach has been demonstrated by the Solazyme company, which cultivated their algae heterotrophically in fermenters on sugarcane as a carbon source. The produced fuel resembles conventional Biodiesel in that it consists of fatty acid methyl esters. This differs from the aforementioned "Green Crude" hydrocarbons in that the latter possesses higher cetane values and is more stable due to a lower oxygen content [239]. Since 2016, Solazyme has focused its business on nutraceutical supplements and renamed the company to Terra Via^{TM} [235].

The demonstration plants for both aforementioned approaches were supported by Government funding. Currently, fuel production from microalgae is not economically viable.

7 Case study: Docosahexaenoic acid production

The production of docosahexaenoic acid (DHA or 22:6) is an illustrative example for the production of a very high-value, small scale product.

The *ω3* fatty acid (Fig. 4) is essential for humans, as it amounts to 20–25% of the total fatty acid content in the gray matter of the brain and is also the main *ω3* fatty acid in the retina [153]. Humans are not capable of synthesizing DHA de novo, and synthesis from its precursor α-linolenic acid is relatively inefficient. Consequently, DHA must be acquired from diet. Fish and fish oil are long known for their high *ω3* fatty acid content, but they contain an increasing heavy metal content and are not suitable for some vegetarians. Microalgae are the primary producers of DHA, and suited for commercial production [243], as a part of infant formulae and human nutrition supplements.

Martek Biosciences (since 2010 a part of DSM) was one of the first companies to sell DHA as a health supplement, employing the heterotrophic dinoflagellate *Crypthecodinium cohnii* as a source [244, 245]. In *C. cohnii* 40–45% of the total amount of fatty acids is DHA.

Crypthecodinium cohnii is cultivated heterotrophically in the dark with a mixture of glucose and yeast extract as a carbon source for 3–5 days under nitrogen starvation; a classical approach to increase the cellular lipid content (chapter 3.1). The cells are then harvested via centrifugation and dehydrated by spray drying. After disrupting the cells with a colloid mill, the algae oil is extracted with hexane. The oil is refined in phosphoric acid and oleic acid and kept at 21°C, resulting in the precipitation of DHA. The DHA is isolated by centrifugation and spray dried. The resulting oil is refined, bleached, winterized (removing higher melting compounds like waxes by slow cooling), deodorized, and stabilized with tocopherols as antioxidants. The two additional centrifugation and drying steps increase costs for the process drastically (Fig. 12). After blending with high oleic sunflower oil, the standard 40% w/w DHA product is obtained [246]. DSM offers DHA as "life's DHA" capsules (with 200 mg per capsule) for an end consumer price of ca. $3 per g DHA [247].

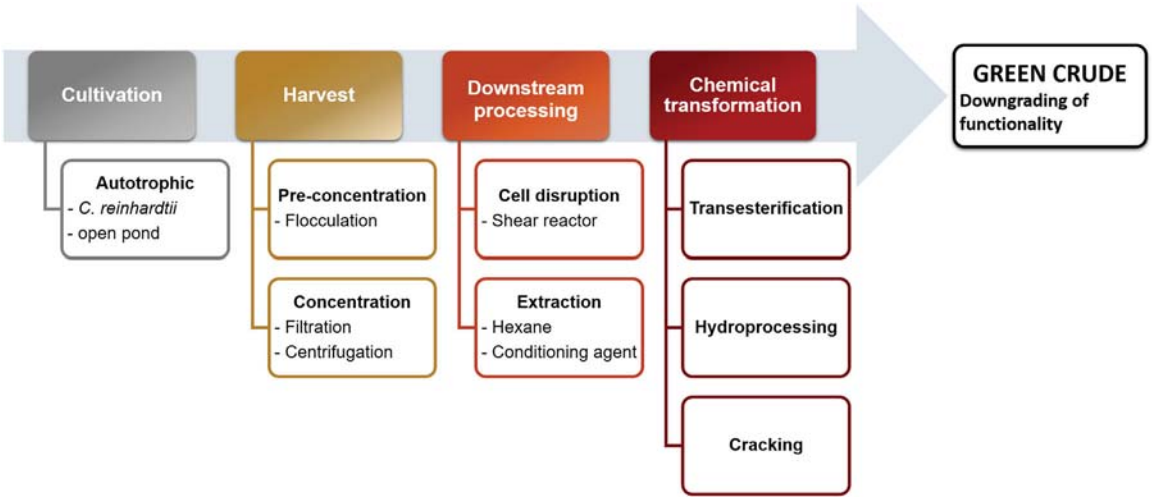

Figure 10. Flow chart of the Diesel and Jet fuel production of Sapphire Energy, Inc., USA in the green microalgae *C. reinhardtii*.

8 Case study: Dehyton® AO 45 surfactant production

Dehyton® AO 45 is an amphoteric surfactant produced commercially by BASF in cooperation with TerraVia, formerly Solazyme [248]. The surfactant is a mixture of capric, lauric, myristic and oleic amido propyl betaine (Fig. 13). Dehyton® 45 AO is considered as an alternative to Cocamidopropyl betaine gained from coconut oil, which contains lauric amido propyl betaine as the main component. Dehyton® 45 AO is used in hair care and toiletries or as a viscosity builder.

The surfactant is produced by BASF from algae oil ("AlgaPūr™") provided by TerraVia (Fig. 13) [249]. The oil is produced from the genetically modified green alga *C. protothecoides*, which is grown in fermenters ($\sim$100 m^3) under heterotrophic conditions [185]. Sugarcane is applied as a carbon source, and the cells are grown under nitrogen starvation conditions.

The cells are harvested via centrifugation and dried in a drum dryer. The dried algae are pressed with an expeller press to release the oil from the cells. The obtained oil is refined via vacuum treatment combined with a steam distillation to remove free fatty acids and odor substances, respectively. In a degumming step, phospholipids are removed with acid followed by a bleaching step in which colored components are removed with bleaching clay [249, 250] (Fig. 14).

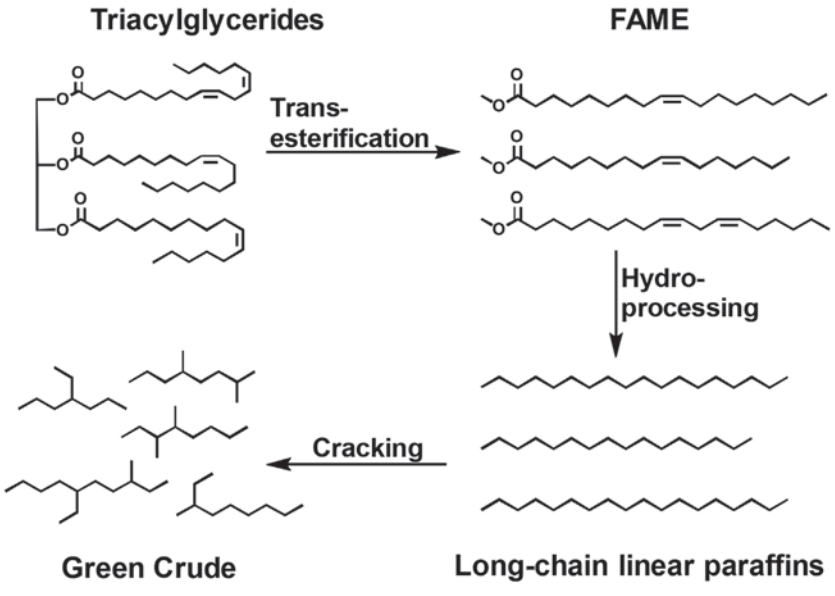

Figure 11. Schematic chemical conversions in Green Crude production by Sapphire Energy.

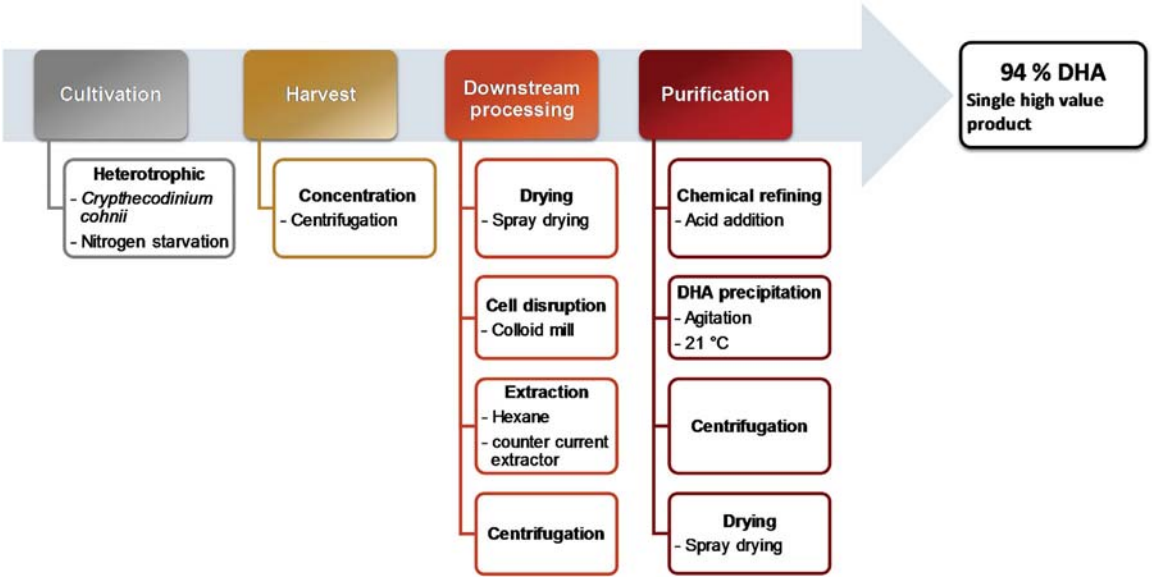

Figure 12. DHA production developed by Martek Biosciences, now part of DSM.

The remaining triglycerides, mainly C_{12}, C_{14}, and C_{18}, are then further processed by BASF to the amido propyl betaine. The mild zwitterionic surfactant is marketed as a ca. 50% aqueous solution. The product has been launched on the US market in 2015 as the World's first commercial algal derived betaine for personal care applications (shampoos, liquid facial and body wash soaps, and hand dishwashing liquids). The price of Dehyton® 45 AO ($1.50/kg)[2] is comparable to other surfactants on the market. Evidently, pricing is influenced by marketing considerations and scale, and the long-term success of this recently introduced process remains to be proven. Irrespectively, this example shows that the production of chemical intermediates from algae oil is viable and the commercial utilization of algae is not restricted to very high-value pharma- and nutraceuticals. Different from the production of fuels, here the specific features of algae lipids are taken advantage of, in this case, the unique spectrum of chain lengths in combination with the terminal functional carboxylic group.

9 Chemical functionalization of algae oil

Like most classical oleochemical conversions of seed oils from higher plants, the aforementioned process for Dehyton® AO 45 converts the fatty acids' carboxylic acid groups. Additional functionalities can be introduced by conversions of unsaturated fatty acids' double bonds. Established examples are ozonolysis and dimerization of fatty acids or epoxidation of triglycerides. While these methods in principle can be useful for the upgrading of algae oils, further-reaching catalytic tools are desirable to make use of the specific molecular structure.

9.1 Carbonylation

Carbonylation reactions are an established industrial technique to introduce carboxylgroups by reaction of olefinic substrates with CO as a carbonyl source and an alcohol or water as a nucleophil [251–254].

An extraordinary example is the isomerizing alkoxycarbonylation, which converts the internal double bond buried deeply in the fatty acid chain to an ω-ester group, resulting in the corresponding α,ω-diester [255, 256]. Very high conversions and selectivities of over 90% can be achieved [257, 258]. Additionally, alkoxycarbonylation has proven to be quite tolerant toward functional groups. This is an essential feature for reactions with multicomponent mixtures like algae oil [23].

In laboratory scale studies, the diatom *P. tricornutum* was cultivated in aerated 10 L round bottom flasks, the cells were disrupted via ultrasonication and the lipids extracted with a

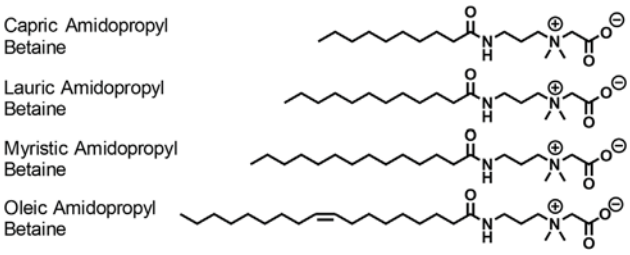

Figure 13. Dehyton® 45 AO consists of capric/lauric/myristic/oleic amido propyl betaine.

[2] Information provided by BASF.

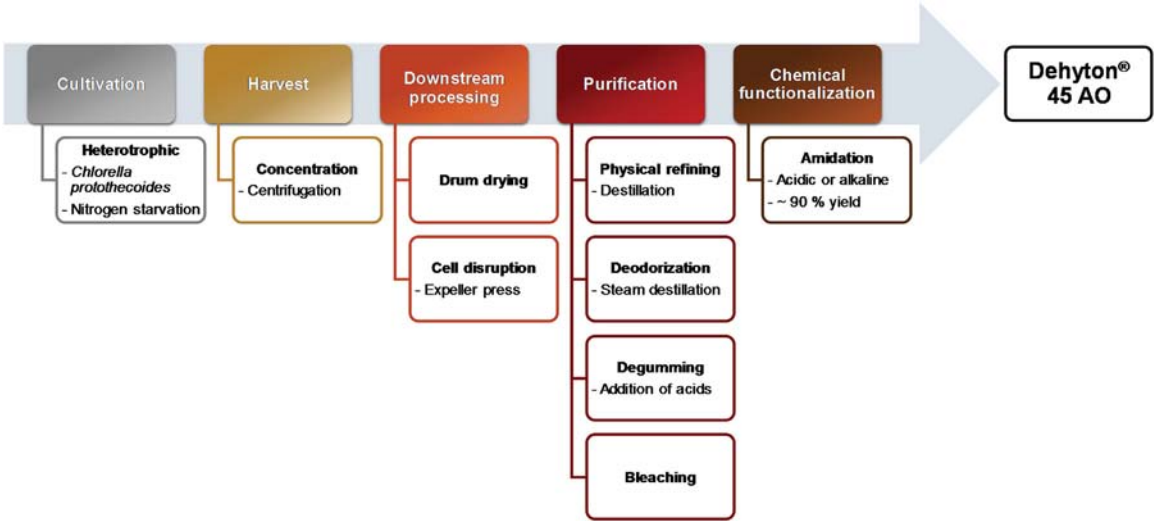

Figure 14. Flow chart of the production of a microalgae-based surfactant from oil produced by the green microalgae *C. protothecoides* by Solazyme and the BASF.

$CHCl_3$/MeOH mixture according to Folch et al. without any additional drying steps [259]. The resulting algae oil (gas chromatogram in Fig. 15, top) was not purified further and directly converted via isomerizing alkoxycarbonylation to the corresponding α,ω-diesters with $[(dtbpx)Pd(OTf)_2]$ as a catalyst precursor. Hereby, the monounsaturated methyl palmitoleate (16:1) is converted to the 1,17-diester, and methyl oleate (18:1) to the 1,19-diester (Fig. 16). The saturated fatty acids do not disturb the reaction. The carbonylation of the polyunsaturated fatty acids is unselective [260] resulting in a broad product spectrum. The byproducts can be removed via recrystallization in heptane. The crude algae oil additionally contains carotenoids, chlorophylls, carbohydrates, and phosphocholines. These do not shut down the desired catalytic carbonylation that is the catalyst is reasonably robust to this end.

The diester mixture can be used to prepare an algae-based polyester with high molecular weight ($>M_n$ 10 000 g/mol) and a crystalline structure that is comparable to high density polyethylene [23].

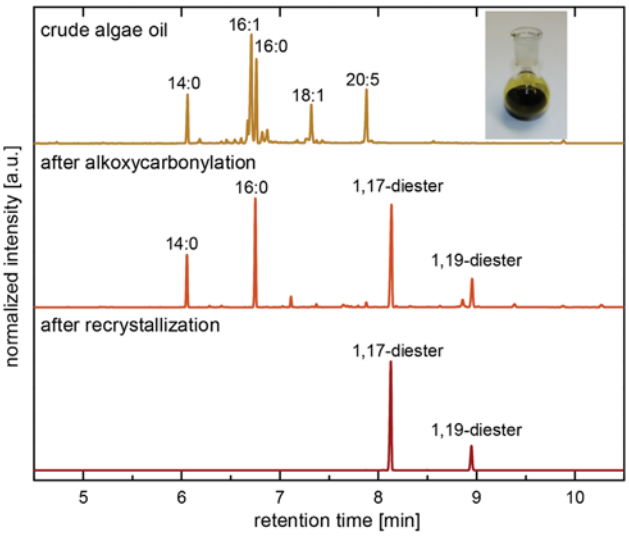

Figure 15. Top: Algae oil composition of *P. tricornutum* (yellow) and algae oil after extraction still containing odorous and coloring compounds. Center: Crude reaction mixture after isomerizing alkoxycarbonylation (orange) and after recrystallization, >99% purity (red).

9.2 Olefin metathesis

Olefin metathesis has recently been introduced as an oleochemical conversion practiced commercially on palm oil. In a joint venture, Elevance and Wilmad operate a plant for the butenolysis of palm oil to afford chemical intermediates, difunctional monomers, and hydrocarbons for fuel use [261]. Metathesis is usually an equilibrium reaction and results in a mixture of products (Fig. 16).

This differs from, for example, the kinetically controlled selective carbonylation described in section 9.1. Ethenolysis with a ruthenium alkylidene catalyst has been shown to be applicable to microbial oils on a laboratory scale, to generate hydrocarbon fractions for use as fuels [262].

For the case of algae lipid feedstocks with their different fatty acid chain lengths and number of double bonds, a spectrum of unsaturated products, and carboxylates will be obtained. This is illustrated for the case of eicosapentaenoic acid, present in, for example, *P. tricornutum*, in Fig. 17. The resulting olefins can serve as building blocks for short-, mid-, and long-chain mono- and diesters useful as viscosity modifiers, lubricants, plasticizers, surfactants, or monomers

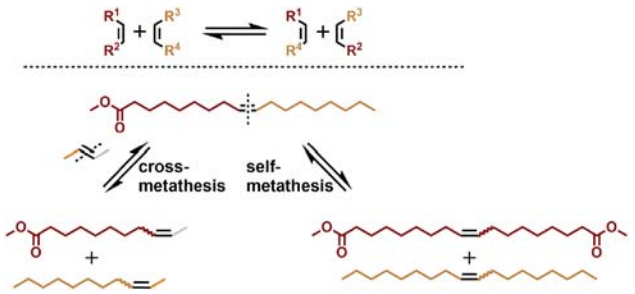

Figure 16. General Scheme of cross- and self-metathesis (top), and butenolysis and self-metathesis of methyloleate (bottom).

[264] (Julia Zimmerer, Lara Williams, Dennis Pingen, Stefan Mecking, unpublished results).

10 Conclusions

Microalgae produce a unique spectrum of fatty acids regarding chain lengths and degrees of unsaturation. To use this potential for the production of chemicals, viability of the production of sufficient amounts of algae oils is a first prerequisite. To this end, existing processes for the smaller scale production of very high-value pharmaceuticals and nutrients on the one hand, and recent insights from the operation of sizeable scale demonstration plants for biofuel production on the other hand are instructive.

As a general picture, estimations of the economics of autotrophic growth suggest the effort required is not fundamentally prohibitive to be considered for production of chemicals on the mid or long term. Key issues are achievable cell densities, and also the efficiency of isolation from the diluted cultures. These can be improved by genetic engineering, and gradual development and optimization of the work up equipment and procedures. At current and for the near future, heterotrophic growth is superior economically. It can also be implemented more rapidly for a given target product, as established fermentation and corresponding workup technology can be used. Notably, here other single-cell organisms like yeast can be competitive to microalgae as illustrated by DuPont's recently introduced production of eicosapentaenoic acid by engineered *Yarrowia lipolytica* [263].

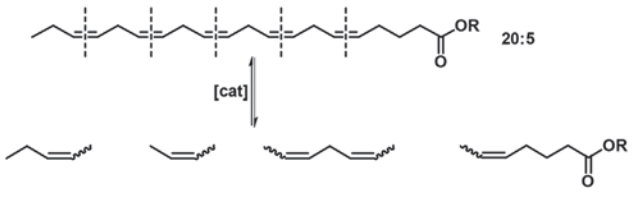

Figure 17. Metathesis (butenolysis) products of methyl eicosapentaenoate.

The recent introduction of surfactants prepared by amidation from oil produced heterotrophically by *C. protothecoides* underlines the feasibility of microalgae as a source for typical chemical intermediates. This background encourages an exploration of advanced catalytic methods to utilize microalgae oils as alternative sources for compounds which are in demand but difficult to access, like dicarboxylic acids of particular chain lengths, and moreover to explore novel entities not available to date.

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