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Review

Hypes, hopes, and the way forward for microalgal biotechnology

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The urge for food security and sustainability has advanced the field of microalgal biotechnology. Microalgae are microorganisms able to grow using (sun)light, fertilizers, sugars, CO_2, and seawater. They have high potential as a feedstock for food, feed, energy, and chemicals. Microalgae grow faster and have higher areal productivity than plant crops, without competing for agricultural land and with 100% efficiency uptake of fertilizers. In comparison with bacterial, fungal, and yeast single-cell protein production, based on hydrogen or sugar, microalgae show higher land-use efficiency. New insights are provided regarding the potential of microalgae replacing soy protein, fish oil, and palm oil and being used as cell factories in modern industrial biotechnology to produce designer feed, recombinant proteins, biopharmaceuticals, and vaccines.

Highlights

Microalgae can contribute to food security through the sustainable production of proteins and lipids, which are required to meet population growth and address environmental challenges.

Cellular agriculture is developing with emerging bioprocesses based on solar energy, photovoltaics, H_2, C1 carbon sources, and sugar as feedstocks.

Different trophic modes – autotrophy, heterotrophy, and mixotrophy – have been successfully explored for microalgae.

The production of microalgae has tripled in the last 5 years.

The genetic toolbox for industrially relevant phototrophic strains expanded tremendously in the last 5 years.

Hypes and hopes

During the last 70 years, microalgae have several times attracted attention as promising candidates for industrial exploitation for food and biofuels due to their high areal productivity in comparison with agricultural crops (Figure 1), capacity to grow in seawater, and lack of requirement for fertile land. The first attempts to exploit microalgae industrially as a potential major human food source occurred during World War II in Germany [1]. This first wave of microalgal production development was stopped by the 'green revolution,' which led to a loss of interest in algal production for staple food [2]. In the 1970s, two new applications of microalgae were evaluated: wastewater purification and production of shellfish with algae. Algal ponds and raceways were developed for wastewater purification in the USA and were widely applied [3].

Markets for dry microalgal biomass (mostly Spirulina and Chlorella) and carotenoids (β-carotene and astaxanthin) for human health food and aquaculture feeds developed in the 1990s. In the 'peak oil' days in the 1990s and more recently in 2005–2010, when crude oil prices rose, biofuels were the main driver behind a surge in interest in microalgae production. But, similar to the objective of creating a staple food for the world's population after the Second World War, the objective of creating a biofuel after peak oil also lost industrial relevance when oil prices decreased. Nevertheless, these 'temporary' objectives towards production of bulk products were important for the field because they led to further development of microalgae production technologies.

The current need for sustainable protein sources and food ingredients has led most microalgal production companies to shift towards this sector. For 3–4 years, many microalgal R&D projects and industries have started operating within the rapidly developing markets for meat and soy protein replacement. The worldwide scientific and industrial community working on microalgal applications increased tremendously in the last decade, bringing along technological developments and scale-up of production facilities, unleashing new applications.

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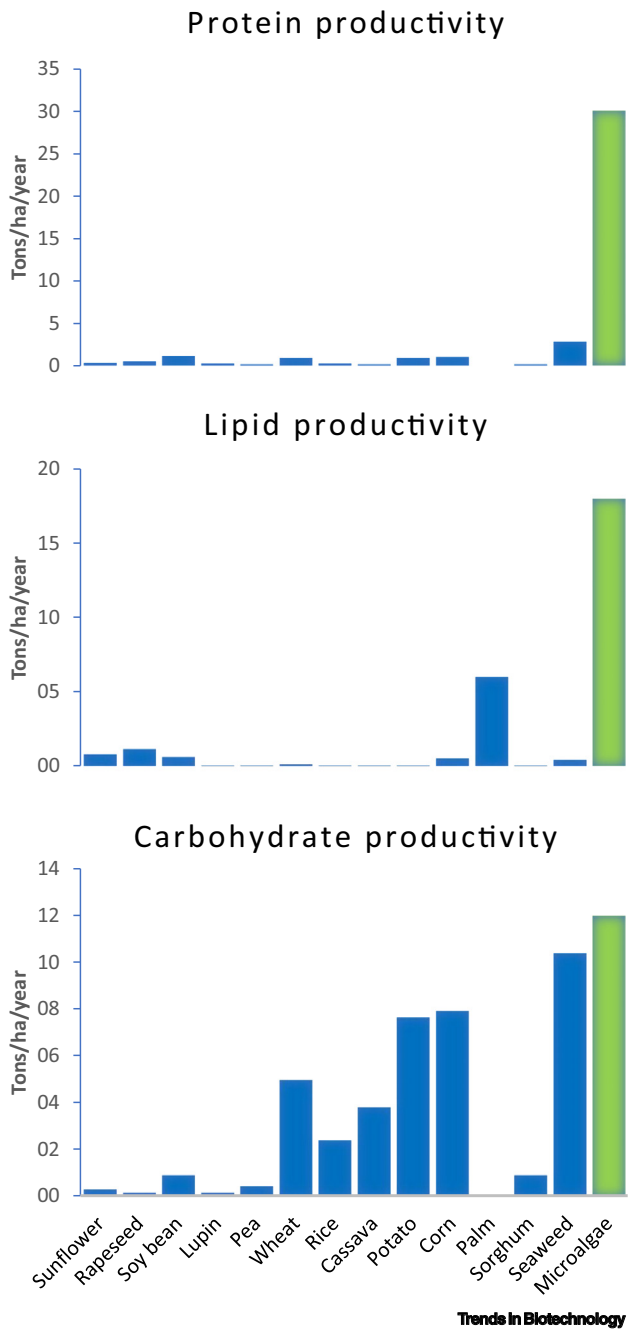

Figure 1. Areal protein, lipids, and carbohydrate productivities on a dry weight basis (when applicable) of agricultural crops, seaweeds, and microalgae. Data on land crops are retrieved from FAOSTAT (https://www.fao.org/faostat/en/#home). Productivities on microalgae are shown by the green bar and were calculated on the basis of a 10-year average yearly radiation in southern Spain (1906 kWh · m^{-2} · y^{-1}); a photosynthetic efficiency of 2.75%; a biomass composition of 50% protein, 30% lipids, and 20% carbohydrates; and assuming 300 days of operation. Note that these values can vary with species and process conditions.

Over the past 10 years, the microalgae food supplement and aquaculture markets have significantly increased [4,5]. Nevertheless, the total microalgae market remains small, considering the large public interest in microalgae. Currently, the global market of microalgae is about 75 000 tons of biomass [4], whereas soybean production was 353 million tonnes in 2020 (https://www.fao.org/faostat/en/#home). Decreasing production costs of microalgae cultivation to competitive costs for bulk products will require economy of scale as shown by Ruiz and colleagues [6].

Bulk production

With bulk production and circularity as targets, three sectors have rapidly evolved in the inclusion of microalgae: production of single-cell protein (SCP), oil production, and nutrient recovery.

SCP

Microbial biomass can be cultivated to yield protein-rich food or feed supplements, better known as 'single-cell protein,' or SCP. Protein formation takes place at high biomass growth rates, which could unleash higher microalgal biomass productivity. Protein contents of microalgae can reach up to 70% of their dry weight, potentially allowing 22 to 44 tons of protein per hectare per year [5]. Despite this, the current production scale is small.

Cultivation and trophic modes

Microalgae can have different trophic modes (Box 1). Among the challenges of phototrophic SCP production are the high costs associated with low biomass concentrations and low volumetric productivities with high energetic demands for mixing, degassing, and harvesting the culture to avoid inhibitory high oxygen concentrations. Mixotrophy combines autotrophic and heterotrophic metabolisms. The presence of both trophic modes in the same strain can increase biomass yield on carbon, up to 0.9 C-mol/C-mol sugar [7], more than double volumetric biomass productivity, and can reach higher cell densities at a given light intensity. By balancing the carbon supply with the autotrophic oxygen production rate, a special cultivation strategy called 'oxygen-balanced mixotrophy' (OBM) can be achieved [7,8]. This recirculation of carbon dioxide and oxygen drastically reduces the need for an external supply and degassing of these compounds, respectively (Figure 2B). Photosynthetic oxygen will be completely recycled in respiration, and CO_2 released from the organic substrate will be almost completely reused by the photosynthetic metabolism within the same cells [7].

SCP can be derived from microalgae, fungi, yeast, or bacteria cultivated with different feedstocks: from agriculture, e.g., sugar beet or sugar cane (Figure 2B,D); fossil-derived, e.g., methane and methanol; by direct use of solar energy for phototrophy (Figure 2A); or using electron donors such as hydrogen generated by electrolysis (Figure 2B) or C1-carbon sources methanol and formate, which we refer to as photovoltaic SCP (PV-SCP). For some of these processes, CO_2 will be required (Figure 2). In a postfossil era, PVs will provide electricity for direct air capture (DAC),

Box 1. Trophic modes and light supply for production

Microalgae are commonly grown by exploiting their photoautotrophic capacity in which cells harvest light energy, use carbon dioxide (CO_2) as a carbon source, and release oxygen (O_2) as a by-product. Alternatives to autotrophic cultures are heterotrophic cultures in which organic carbon, such as sugars and organic acids, are used as carbon sources in the absence of light.

Autotrophic and heterotrophic cultivation of microalgae can be combined in mixotrophic cultivation. In this trophic mode, light and organic carbon are simultaneously provided, and both heterotrophic and autotrophic metabolism operate concurrently within a single microalgal monoculture (Figure I).

Light is an essential substrate for phototrophic and mixotrophic cultivation. There are three operational modes in relation to light: sunlight, artificial light, and a combination of both (Figure II). Although the use of sunlight is the shorter route, this will not suit production in the Northern Hemisphere, where light availability is limited for a large part of the year. The combination of sunlight and artificial light or the use of only artificial light is in full development in northern countries. Artificial light adds to production costs [5,101], but it allows stable production, leads to higher biomass yield on light, and guarantees supply and quality.

Although this could be economically feasible for high-value products, direct use of sunlight is the most attractive route for large-scale production of microalgal bulk products.

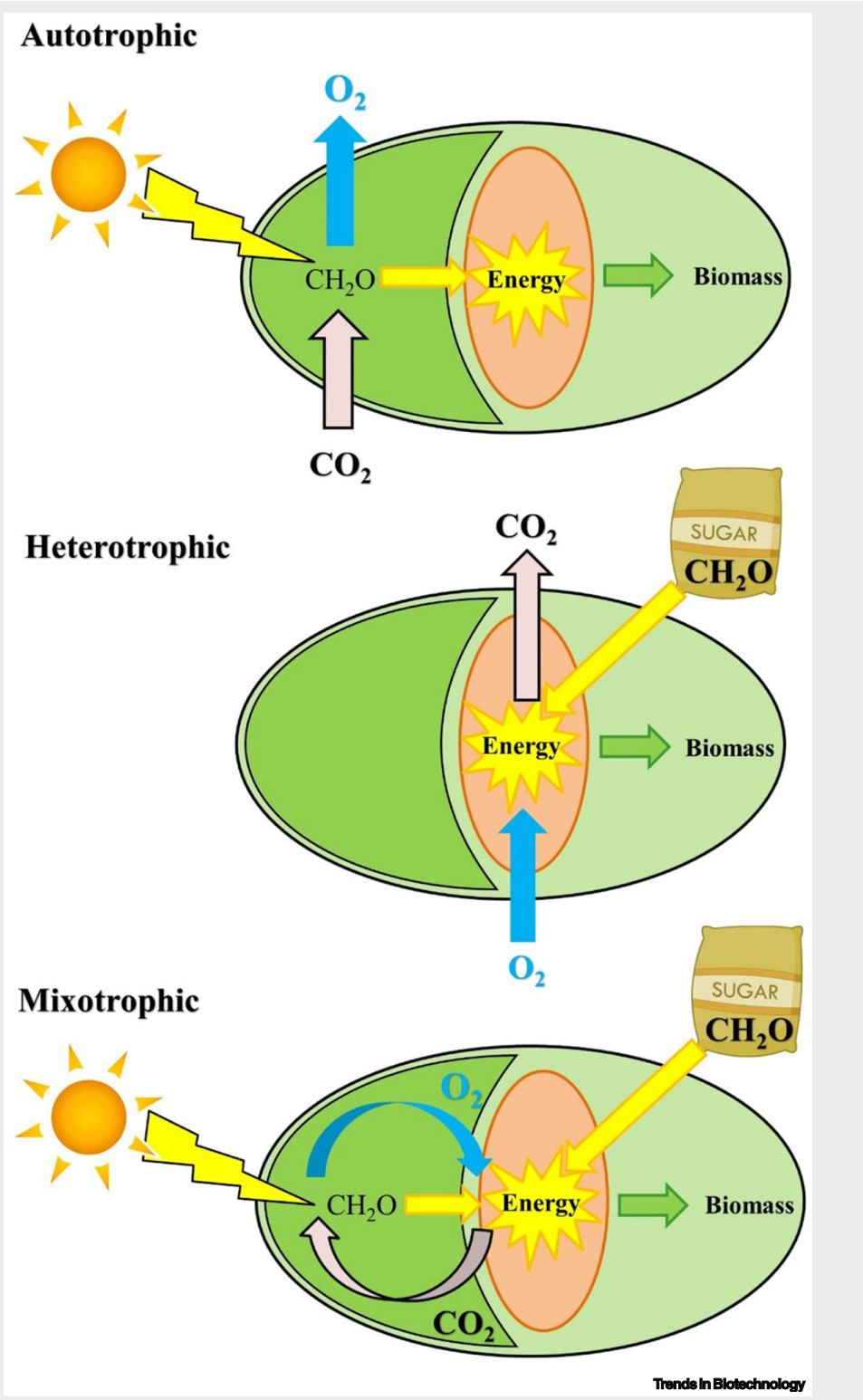

Figure I. Three trophic modes, from top to bottom: autotrophic, heterotrophic and mixotrophic.

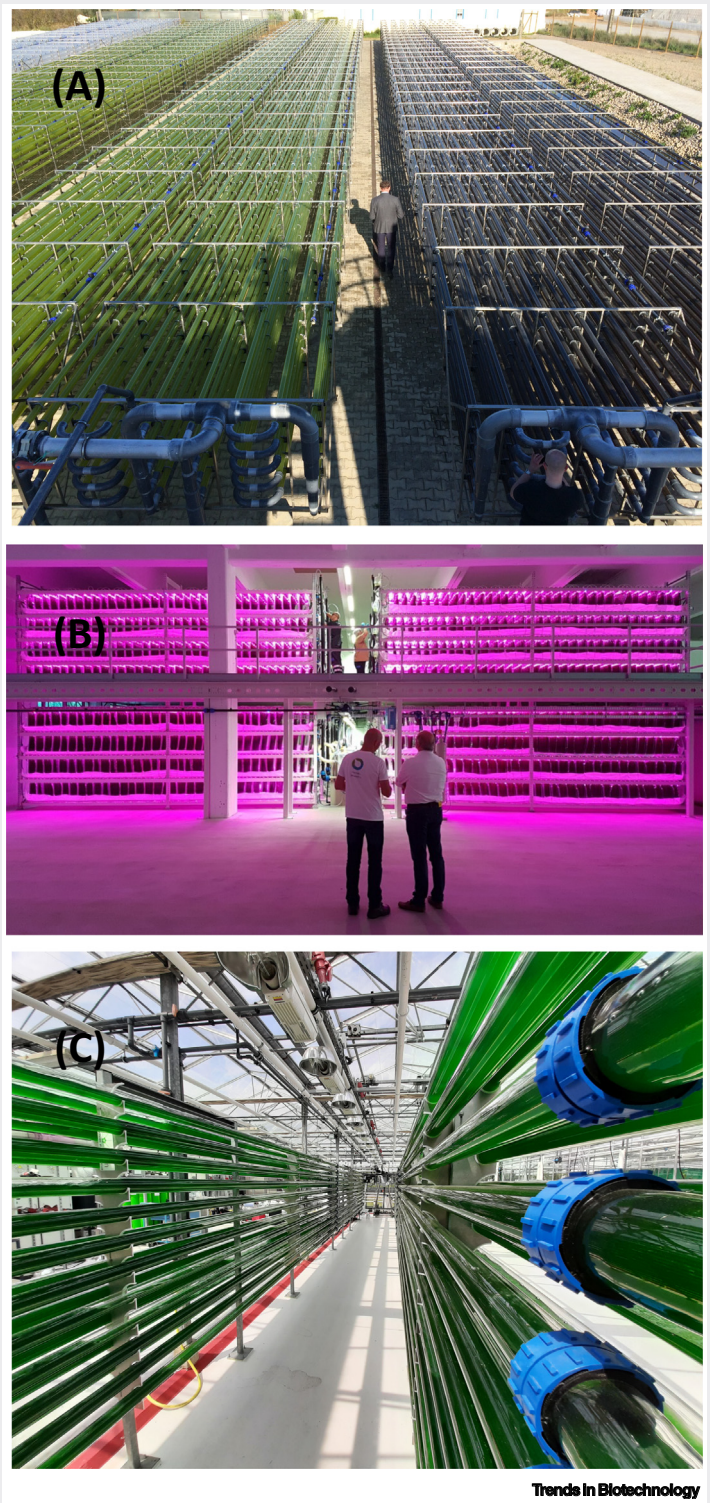

Figure II. Three different modes for autotrophic or mixotrophic cultivation. These modes are based on (A) sunlight at company Necton, Portugal; (B) artificial light at company Proviron, Belgium; and (C) a combination of sun and artificial light in a greenhouse at AlgaePARC, the Netherlands.

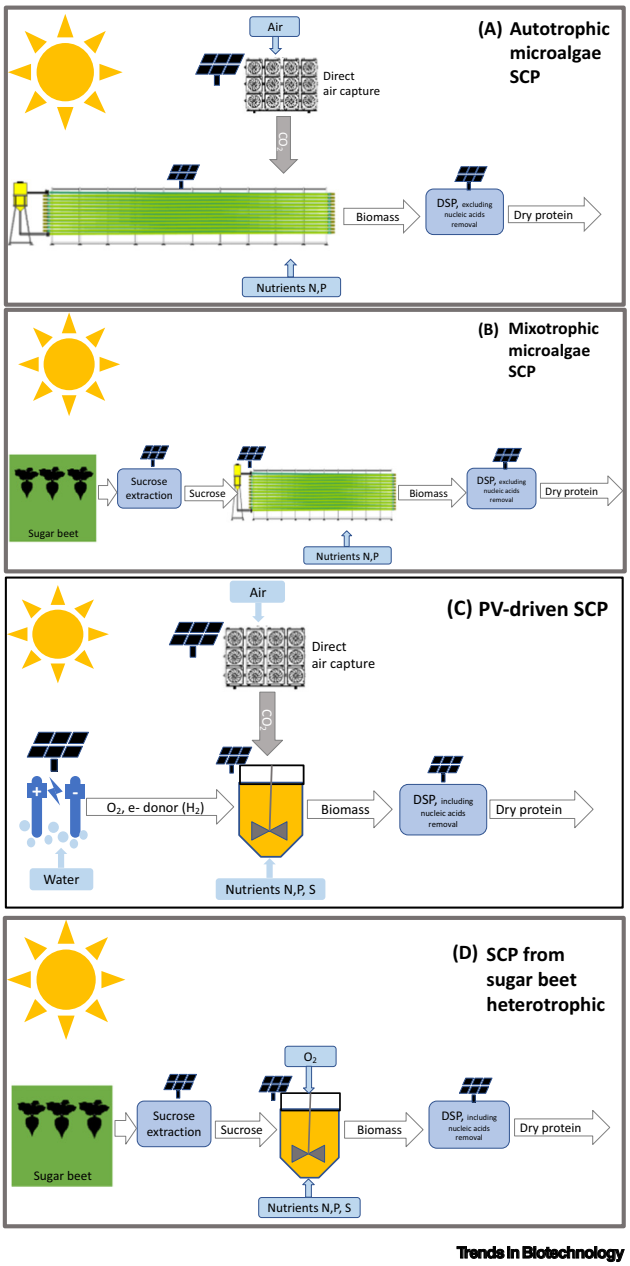

Figure 2. Single-cell protein (SCP) production routes. (A) Microalgae phototrophic SCP production. (B) Microalgae mixotrophic SCP production on sugar beet. (C) Photovoltaic (PV)-driven SCP. (D) Fungi/yeast heterotrophic SCP on sugar beet. (⚡) represents electricity use provided by PV in different process steps. The end product is dry protein and is identical for all scenarios. Downstream processing (DSP) includes harvesting with centrifugation, cell disruption with bead milling, filtration, and spray drying. It is assumed that the level of nucleic acids in microalgae is below the threshold (2%-dw) and does not require any treatment.

which is used to capture and concentrate atmospheric carbon dioxide to operate the (photo)bioreactors and the downstream equipment (Figure 2). A radiation of 2000 KWh · m^{-2} · y^{-1} was assumed. The photosynthetic efficiency on solar energy was considered 2.75% for autotrophic microalgae [5].

Using an identical methodology as described in [9] with specific input data for microalgae (Box 2), we estimated the land use efficiency of SCP produced in two microalgae scenarios (Figure 2A,B). Two additional routes (PV-SCP, illustrated in Figure 3C,D) were calculated in [9]. Figure 3 shows

Box 2. Land use efficiency for SCP production with microalgae: A case study

A radiation of 2000 KWh · m^{-2} · y^{-1} was assumed. The photosynthetic efficiency (PE) on solar energy was 2.75% for autotrophic microalgae [5]. The biomass volumetric productivity in mixotrophic cultivation doubles the autotrophic productivity [8,102]. Assuming identical reactor dimensions, the productivity per ground area is also doubled. The reactor was chosen to be a vertically stacked tubular photobioreactor because this is the most widely available commercial-scale photobioreactor. The energy required for photobioreactor operation includes mixing, degassing, nutrient supply, and temperature control. Temperature is kept between 20°C and 30°C. To calculate the energy required for temperature control, climate conditions of southern Spain were considered, and calculations were made according to [103,104]. Operational energy was calculated to be 18 MJ Kg-dw^{-1}. Energy requirement for nutrient supply was taken from [9], despite a small difference in biomass stoichiometry. The energy demand of DAC was calculated to be 15 MJ Kg-dw^{-1}, following an identical methodology as [9] with a slightly different elemental composition for microalgae ($CH_{1.62}O_{0.41}N_{0.14}P_{0.011}$), which led to a weight fraction of carbon in biomass of 53%. Finally, the downstream costs include harvesting with microfiltration followed by centrifugation, cell disruption using bead milling, and drying with a spray drier.

One of the advantages of microalgae in relation to bacteria is the lower concentration of nucleic acids. High nucleic acid content could lead to gout and kidney stones and therefore needs to be removed before human consumption. Values less than 2% were reported for *Chlorella* and *Spirulina*, which would allow direct use of the biomass [105]. Older studies, however, present 5% to 6% for similar algal species [106], suggesting this value needs closer monitoring. If nucleic acid levels are too high, they should be removed in a dedicated heat treatment step that already is in place for other sources of SCP [107] and requires additional energy.

Leger and colleagues [9] considered a solar-to-electricity energy efficiency using available information on 628 utility-scale (>1 ha) PV solar farms in several countries in the world, resulting in a median of 4.9%. This value is relatively low compared with ~20% solar cell efficiency that PV reaches under standard test conditions. This discrepancy is likely related to numerous factors, most important being PV ground coverage ratio and losses due to power electronics, solar tracking, inverters, and temperature, as well as surface soiling from dust, snow, and other debris [108].

To make the results comparable, the same PV solar efficiency of 4.9% was used to calculate the microalgae-based scenarios, as well as the same sugar beet yield (Table I).

Table I. Input data used to calculate the SCP microalgae-based scenario

	Autotrophic scenario	Refs	Mixotrophic scenario	Refs
Solar radiation	2000 KWh · m^{-2} · y^{-1}	[9]	2000 KWh · m^{-2} · y^{-1}	[9]
PE microalga	2.75 %	[5]	2.75 %	
Biomass areal productivity	8.5 Kg-dw · m^{-2} · y^{-1}		17 Kg-dw · m^{-2} · y^{-1}	[8,102]
Energy required for operation of photobioreactor and nutrient supply	18.7 MJ · Kg-dw^{-1}	[103,104]	11.5 MJ · Kg-dw^{-1}	[6,104]
Energy required for DSP	13.6 MJ · Kg-dw^{-1}		23.4 MJ · Kg-dw^{-1}	[6,104]
DAC energy demand microalgae	14.6 MJ · Kg-dw^{-1}	[9]	N/A[a]	[9]
Sugar beet productivity	N/A		6.5 Kg-fw* · m^{-2} · y^{-1}	[9]
Sucrose content of sugar beet	N/A		16 %	[9]
Energy requirement for sucrose extraction	N/A		1.25 MJ/kg sucrose	[9]
Yield of biomass on sucrose	N/A		0.72 g biomass/g sucrose	[7]
Solar-to-electricity energy efficiency	4.9%	[9]	4.9%	[9]
Microalgae energy combustion	23.3 MJ · Kg-dw^{-1}		23.3 MJ · Kg-dw^{-1}	
Microalgae protein content	57.5%		57.5%	[5]

[a]N/A, not applicable.

how one hectare is distributed, how much protein can be produced per hectare, and the number of people whose protein requirements can be met per hectare.

The land use efficiency of phototrophic microalgae is the highest, leading to the highest production of biomass per hectare per year (Figure 3). The use of land crops as a sugar feedstock for

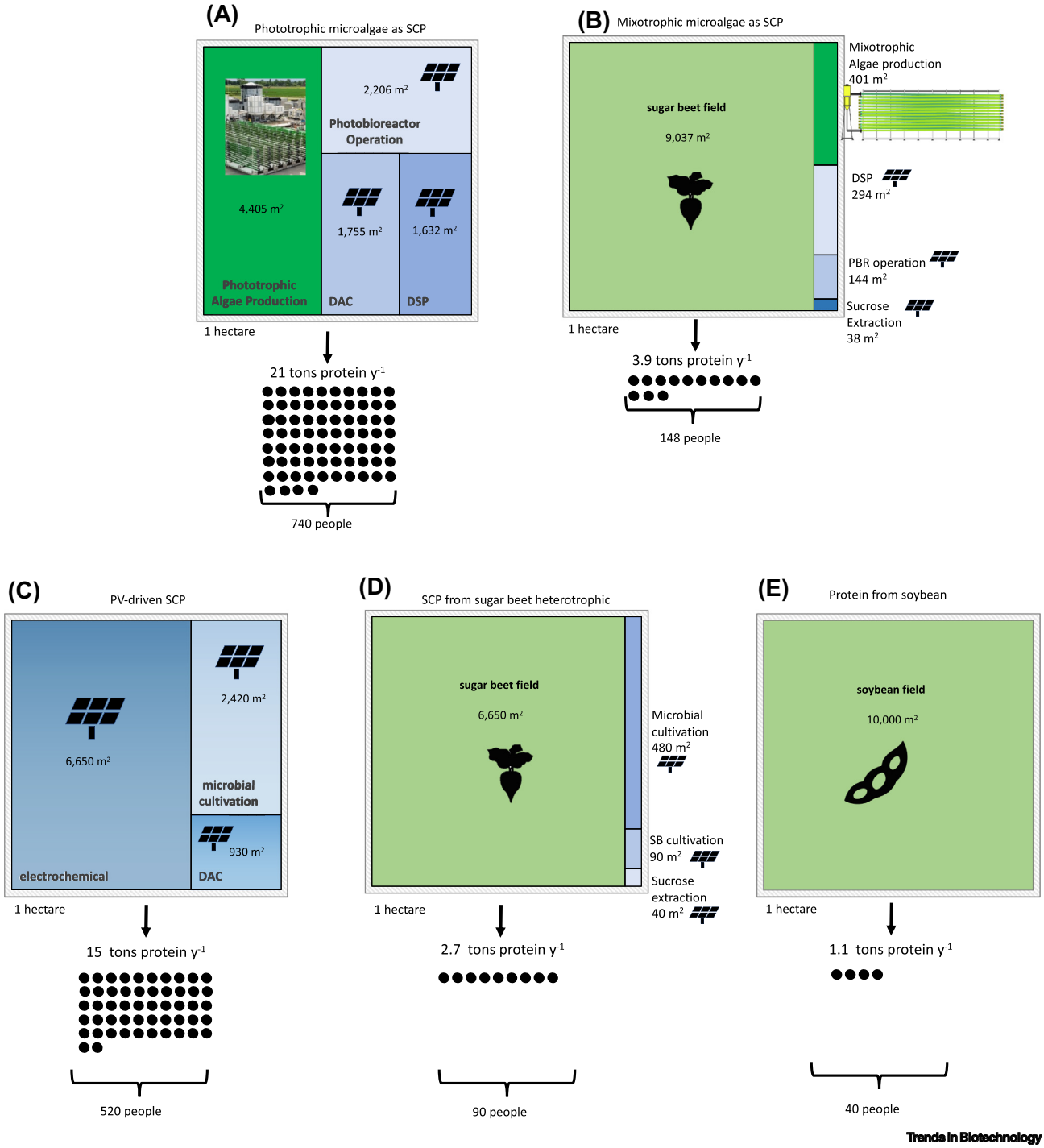

Figure 3. A tailored designer feed that produces and accumulates different functional feed additives. These are compounds that, e.g., enhance nutrient use, disease resistance, or growth performance of animals. Division of land for the production of nutritional protein, using five different production strategies. The protein yields and amount of people who could be fed from 1 ha are shown at the bottom. (A) Photosynthetic production of single-cell protein (SCP) by microalgae. (B) Mixotrophic production of SCP by microalgae. (C) Photovoltaic (PV)-driven production of SCP with hydrogen as the electron donor. (D) Sucrose extracted from sugar beet (SB) used to cultivate microbes heterotrophically for the production of SCP. (E) Proteins from the cultivation of soybean, the staple crop with the highest protein yield,

(Figure legend continued at the bottom of the next page.)

microorganisms, namely sugar beet, which is one of the most productive land crops (Figure 3B, D), led to a lower land use efficiency than the phototrophic microalgae cultivation and PV-driven SCP (Figure 3A,C).

Mixotrophic cultivation shows a 40% decrease in energy requirement for photobioreactor operation compared with phototrophic cultivation, mainly because degassing oxygen and addition of CO_2 are no longer required. However, supplying sugar in a photobioreactor is accompanied by additional costs, dependency on arable land, and a high contamination risk. Clean agrifood residual streams (e.g., brewer's spent grain, molasses, or beet pulp) need to be considered as a sugar source for economic and sustainability reasons and could avoid additional costs. In addition, choosing very fast growers such as *Chlorella sorokiniana* and extremophiles such as *Galdieria sulphuraria* can mitigate contamination. *G. sulphuraria* is a polyextremophile microalgae [10], able to grow at low pH (as low as 0.2) [11], high temperatures (up to 57°C) [12], and high osmotic pressure up to 400 g/L of sugar and 2–3 M of salt [13], which prevents contamination with other microorganisms. In addition, it can take up and metabolise a variety of sugars [13]. *G. sulphuraria* is under evaluation by the European Food Safety Administration for approval as a Novel Food.

Mixotrophic production has been successfully scaled up for the first time to pilot scale in the summer of 2022 at AlgaePARC in the Netherlands. The potential is high, and developments are underway to deploy this technology. Technology maturity decreases in the order of soybean > sugar beet heterotrophic > phototrophic microalgae > mixotrophic microalgae > PV-driven SCP, but there is room for development in all of these.

Particularly for microalgae-based SCP, recent developments have presented the prospect of improved productivities. New, robust strains have been bioprospected, such as *Picochlorum* spp. This strain tolerates high temperatures and high light intensities, has high growth rates with doubling times less than 2 hours [14–17], and has a reported protein content of 40%–55% [18]. The potential for SCP is high, but to establish microalgal species suitable for SCP, manufacturing companies should join forces and cooperate for the expensive and lengthy novel food approval. In order to meet the protein market demands, multiple producers together need to supply larger quantities, similar to what is done in agricultural crop production. This will be crucial to increase market penetration of microalgae-based products as sustainable alternatives to currently available options. Robust and cost-effective production methods with short downtime, automatization, and process control will be required to ensure that microalgae production companies can accept the risks and costs of developing new products from novel species, with a focus on high product quality in terms of digestibility, palatability, and safety.

Fertilizers and nutrient recovery

Food production, including SCP, requires the use of nitrogen (N) and phosphorous (P) fertilizers. N fertilizer is now mainly made from N_2 from the air with the very energy-intensive Haber-Bosch process. Phosphate rock deposits are the major source of phosphate fertilizers, and they are rapidly depleting. The use of energy to fix nitrogen from the air and depletion of phosphorous mines leads to scarcity of the nutrients N and P [19,20].

assuming a yield of 115 g protein · m^{-2} · y^{-1} (a representative average value based on data from the Food and Agricultural Organisation of the United Nations). Irradiance was assumed to be 2000 kWh·m^{-2}·y^{-1} for all scenarios. Phototrophic (A) and mixotrophic (B) cultivations are done in a photobioreactor (PBR). Direct air capture (DAC) corresponds to DAC of CO_2. A daily protein consumption of 80 g per person is assumed. (A) and (B) were calculated using the methodology as described in [9] and microalgae-specific data shown in Box 2. (C), (D), and (E) are reproduced from [9] under a Creative Commons CC-BY licence.

Microalgae production in photobioreactors allows 100% efficiency use of fertilizers, in contrast to current agricultural practice, where nearly 50% of the nutrients in the fertilizer ends up in surface and groundwater, causing pollution and eutrophication [21–23].

Elementary N and P recovery from the ocean water, surface water, and wastewater will be one of the most important global challenges to prevent global fertilizer shortage in the future [19]. Microalgae have gained special attention as a platform for nutrient recovery [24].

Even though many authors propose combining microalgal treatment of inorganic nutrient-rich waste streams with the production of food and feed ingredients, the applicability of this concept is still questionable due to safety concerns. Using waste streams of high quality and consistency typically found in the food and feed producing and processing industry [25] could be a first step. In the meantime, new applications such as biostimulants and fertilizers are rapidly emerging for microalgae grown in waste streams [26].

Lipid production

The ability of microalgae to convert sunlight and CO_2 into valuable lipid compounds has attracted interest from the cosmetic, biofuel, food, and feed industries.

The lipids with the highest interest are triacylglycerols (TAGs), which offer an alternative to palm oil, and omega-3 long-chain polyunsaturated fatty acids (n3 LC-PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are an alternative to fish oil and are essential fatty acids in both aquaculture and human nutrition. In microalgae, n3 LC-PUFAs are produced during growth and accumulate in the cell, mainly in the plastid membranes, in levels of 2%–5% of dry weight [27], whereas TAGs accumulate in lipid bodies under nutrient starvation, leading to growth arrest with lipid contents up to 60% of dry weight [27,28].

The challenge for n3 LC-PUFAs is to increase cell content, whereas for TAGs, the challenge is to increase growth rate during TAG accumulation.

Omega-3 long-chain polyunsaturated fatty acids

Microalgae are the primary EPA and DHA producers that are subsequently consumed and accumulated through the food chain to give the high levels of EPA and DHA in marine oily fish. Currently, and strangely enough, fish oil is still the main dietary source of EPA and DHA in the human diet. There is a current global shortfall in EPA and DHA to supply human requirements and demand [29]. Even though photosynthetic marine microalgae are the primary producers of n3 LC-PUFAs, these fatty acids are present in small concentrations in the cells, up to 5% (wt/wt) EPA on a dry weight basis [30] and even less DHA. Many strains have both EPA and DHA; DHA is only two reaction steps from EPA. Fine-tuning these amounts in photosynthetic cells to produce significantly higher levels of EPA and/or DHA would be a breakthrough in the field of photosynthetic cell factories. DHA can be heterotrophically produced at high levels (Box 1), but only by the thraustochytrid *Schizochytrium*, with DHA content of ~20% (wt/wt) of dry weight biomass [29,31–34]. However, for EPA, efficient microbial sources are limited. Attempts have been made for heterologous expression in the plant *Camelina sativa* [35,36] and in the oleaginous yeast *Yarrowia lipolytica*, resulting in low productivities [37,38]. The development of a photosynthetic cell factory for production of EPA and DHA would address a major global need [29,32–34] in a sustainable way. Even with currently achievable EPA contents and productivities, the production area required to supply the worldwide production of EPA would be equivalent to the land area of the Cape Verde Island Boa Vista (Box 3).

Triglycerides

Triglycerides (TAGs) are used in a wide range of applications from biodiesel to cosmetics, personal care, and as food ingredients. The largest source of TAGs is palm oil, which has led to massive deforestation. Although microalgae have a far higher areal lipid productivity than oil palm (Figure 1), the microalgal TAG yield on light energy is in practice 10 times lower than the theoretical yield [39]. There is an enormous potential to improve lipid productivity, but the scientific and industrial foundations do not yet exist. The technology for production under controlled conditions is well developed, but there is still a major loss in yield under outdoor conditions. Another bottleneck is the lipid production capacity of the strains used. There are many studies of lipid accumulation in algae [28,40–43], and strains have been successfully engineered to increase lipid productivity [44–46, reviewed by 45]. Despite these improvements, productivities are still too low to allow cost-effective production [6], and understanding of the molecular mechanisms of lipid metabolism as well as its transcriptional regulation remains limited [39,47]. In addition, the improved phenotype is very often lost when scaling up to photobioreactors operating under relevant industrial conditions of light and temperature. Improved strains are needed along with more insight into the metabolism during lipid accumulation as well as high-throughput geno- and phenotyping.

Biofactories

Although industrial production of microalgae is expanding around the globe, the variety of microalgal products is still limited to compounds that are naturally produced by wild-type strains, such as enriched biomass with omega-3 fatty acids, vitamins, and pigments [48]. Expanding the palette of microalgal bioproducts requires effective tools for genetic manipulation of industrially relevant strains. Applications of microalgae as designer feed (tailored feed that produces and accumulates different functional feed additives), oral vaccines, or production platforms for pharmaceutical proteins depend on effective genetic toolboxes for genetic manipulation of industrial strains, which are evolving rapidly (Figure 4).

Metabolic engineering tools

Nannochloropsis spp. is emerging as a model industrial platform for microalgae. It is a fast-growing marine species already produced at a commercial scale that can be a rich source of proteins or lipids, depending on cultivation conditions. It produces valuable omega-3 fatty acids, including eicosapentaenoic acid (EPA), and can accumulate up to 60% of TAGs [49,50]. Numerous studies have brought us closer to unlocking this microalga's biotechnological potential by providing vital insights into its complex metabolic network [46,51–60], and the toolset for genetic manipulation of the microalga is expanding. Currently, the commercial applications of *Nannochloropsis* are limited to use as aquaculture feed and production of nutraceuticals [61]. However, *Nannochloropsis* has the potential to become an adaptable microalgal chassis for production of an array of bioproducts.

Other strains resulting from bioprospecting work and not yet produced at large scale, such as *Picochlorum* and *Galdieria*, are emerging as potential industrial platforms due to their metabolic

Box 3. EPA world production: A case study

Total world production of fish oil was between 0.8 million and 1.3 million tons per year in the period 2011–2020 [109], resulting in a median of 1.05 million tons of fish oil produced per year, with 20% EPA and 10 % DHA. Considering an EPA cell content of 5% (wt/wt) [30], an irradiance of 2000 kWh · m^{-2} · y^{-1}, and photosynthetic efficiency of 2.75%, the total area required to supply the total EPA worldwide is 616 km^2. This area is equivalent to the area of the Cape Verde Island Boa Vista (Figure I), showing that EPA production by phototrophic microalgae is a realistic scenario, waiting to be scaled up. After extraction of the lipids of interest, the remaining biomass could still be used as SCP.

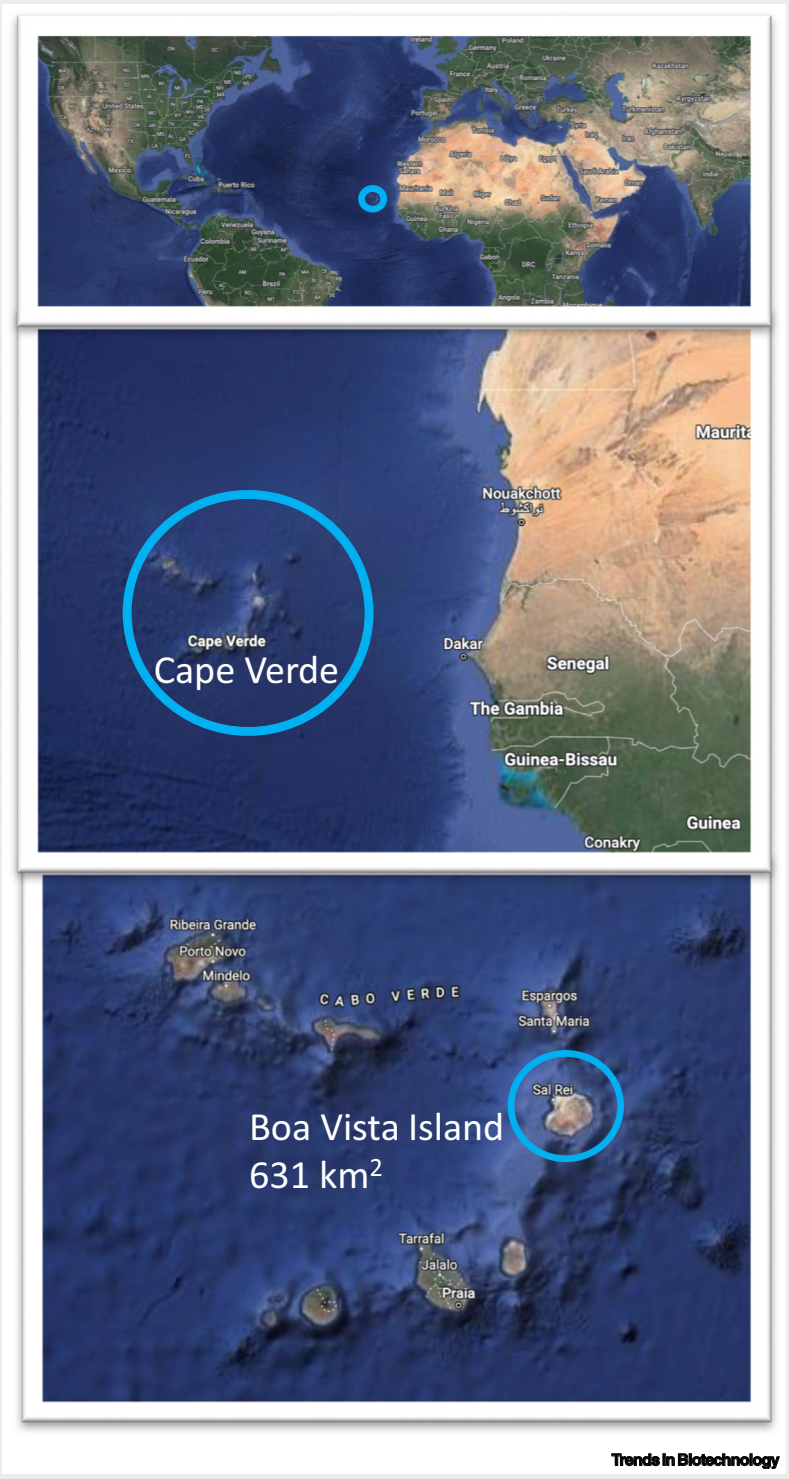

Figure I. World map exemplifying the total area required to meet the worldwide EPA production volume in fish oil between 2011 and 2020 [109] (616 km^2). The required area is equivalent to the land area of the island of Boa Vista (631 km^2), one of the Cape Verde islands. The figure shows the location on a map with different scales.

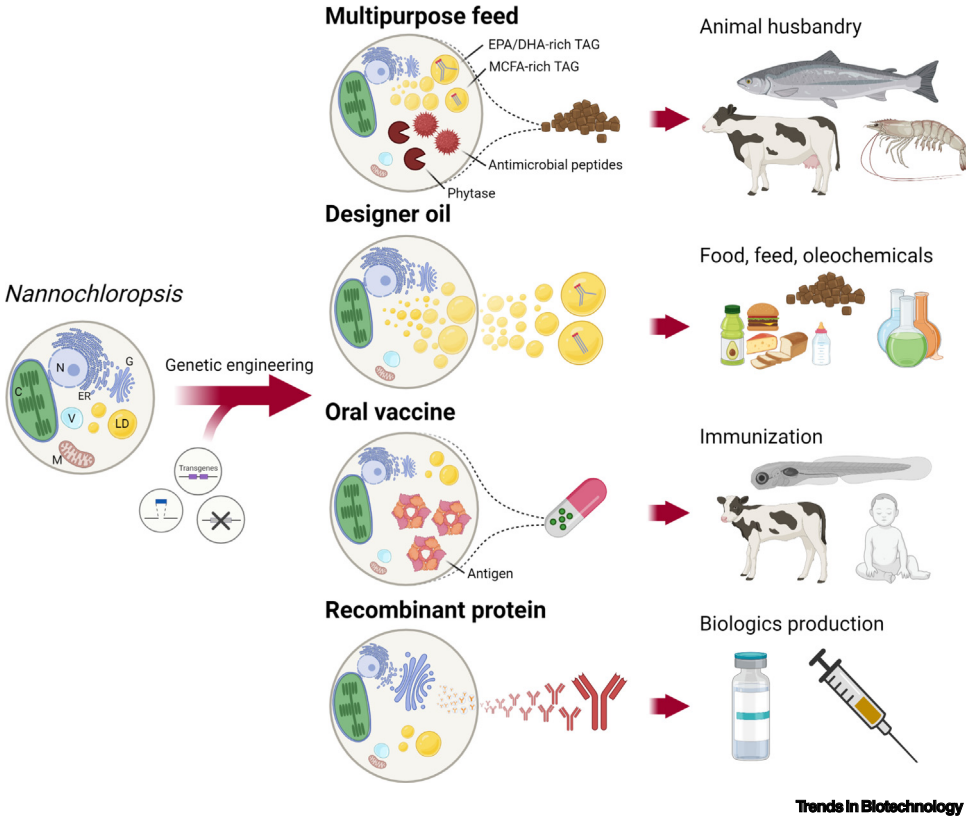

Figure 4. Microalgae as a multipurpose chassis. Depending on the desired application, a microalga can be genetically engineered to accumulate either lipids or proteins or both. High lipid-producing strains could find application in production of oil for food, feed, and oleochemical purposes. For using such a platform as animal feed, cells can be engineered to produce sufficient lipids with a desired fatty acid profile, among bioactive proteins, such as phytase and antimicrobial peptides. To develop a microalgae-based oral vaccine, cells can be manipulated into producing antigen of relevant pathogens, alongside adjuvants to improve immunogenicity. Recombinant protein from microalgae could be relevant for the pharmaceutical sector or as sustainably produced enzymes for industrial purposes. Abbreviations: C, chloroplast; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ER, endoplasmic reticulum; G, Golgi apparatus; LD, lipid droplet; M, mitochondrion; MCFA, medium-chain fatty acid; N, nucleus; TAG, triacylglycerol; V, vacuole. Created with BioRender.com.

flexibility under extreme conditions, and genetic toolboxes for these strains are quickly developing [62,63].

Despite recent advancements, our understanding of the metabolism of industrially relevant strains is still limited. A large fraction of genes do not display sufficient sequence similarity with known entities and cannot be assigned a metabolic function [51]. Uncertainties regarding protein localization, compartmentalization of metabolite pools and metabolite trafficking between organelles further complicate the generation of comprehensive metabolic models. These issues need to be addressed because better understanding of microalgae and their metabolism is required to guide strain engineering designs. In the absence of high-quality metabolic models, genetic engineering strategies are restricted to educated guesses, usually based on knowledge obtained for other organisms.

Forward genetics [46,64–66] combined with efficient genotyping [46]and robust high-throughput screening procedures can help determine gene functions and identify genes that are relevant to the desired trait. Although the promise of forward genetics screens is undeniable, so far only a few

microalgal genes have been linked to a phenotype using random mutagenesis strategies. Moreover, most of these genes await thorough functional characterization.

Another strategy to better understand microalgal metabolism is to modify metabolic network functions and quantify the elicited response. Improved genetic tools and omic technologies have accelerated the pace of these studies in a few microalgae strains, which is reflected in a rapidly growing database of transcriptomic responses to different stimuli [46,67].

An essential requisite for metabolic engineering is an advanced genetic manipulation toolbox. Developing genome editing methods for microalgal species is time-consuming, but it is of utmost importance. The genetic toolbox for industrially relevant microalgae is lacking compared with other microbes, but it is expanding. The inception of transformation protocols and the identification of selectable markers [51,68], implementation of techniques that allow modulation of gene expression [69], genome editing through CRISPR-Cas technology [44,54,59], and gene overexpression are available for several strains [70,71]. Despite this, sophisticated metabolic engineering will require a more complete molecular toolbox.

Vaccines and biopharmaceuticals

Oral vaccines

The discovery of vaccines was undoubtedly one of the most important biotechnological breakthroughs. Subunit vaccines, consisting of, e.g., partial pathogen proteins, are a safe alternative to variants consisting of attenuated or inactivated pathogens [72]. There is a rapidly increasing interest in orally delivered vaccines, particularly in oral subunit vaccines [73,74] due to their several socioeconomic benefits, including reduced need for trained personnel, lower risk of blood-borne disease transmission, and increased patient compliance.

A challenge for oral subunit vaccination is to find antigen delivery systems that can withstand the low pH and proteases of the gastrointestinal tract en route to the gut-associated lymphoid tissue. Current antigen delivery systems include attenuated antigen-expressing bacteria [74] and yeast cell wall shells filled with recombinant antigen and adjuvants [73]. Plant cells are a particularly interesting delivery system because their recalcitrant cell walls provide excellent antigen protection compared with other delivery methods. However, stable antigen production in crop plants has been limited by low content, typically less than 1% of total soluble protein [75]. This is a major drawback because a high antigen content is critical to ensuring immunogenicity of oral vaccines. Moreover, the usefulness of terrestrial crops for production of biologics is debatable due to limited containment options [76].

By contrast, microalgae can be grown in contained closed systems. Microalgae possess rigid cell walls similar to plants, which protect from harsh stomach conditions. Chloroplast-based expression of transgenes in the model microalga *Chlamydomonas reinhardtii* can yield protein contents above 5% of total soluble protein [77], which is substantially higher than for typical plant-based systems. Therefore, microalgae have recently received attention as a potential oral subunit vaccine [78,79]. So far, studies have investigated the immunogenicity of microalgal oral vaccines on fish and shrimp, with promising results [80–83]. Moreover, they may also be interesting for terrestrial animal farming and human medicine. Microalgal oral vaccines can be produced cheaply, they can be administered without training, and they are readily lyophilized, which can protect the antigen payload from degradation for over 1.5 years at room temperature [84]. Accordingly, they are a promising alternative to traditional vaccines, especially in developing regions with less access to trained personnel and infrastructure to ensure refrigerated distribution chains.

So far, most research on microalgal oral vaccines has focused on *C. reinhardtii,* a model organism. However, the new gene expression system developed by [58] for *Nannochloropsis oceanica*, and the genetic toolbox developed by [85] for Spirulina, have revealed an untapped potential for production of recombinant protein in industrially relevant strains, making these microalgae viable candidates for the production of oral subunit vaccines. The versatile genetic engineering methods developed for Spirulina, the most produced and consumed cyanobacteria worldwide, allowed stable, high-level expression of therapeutic proteins (15% of total biomass), including bioactive peptides, single-chain antibodies, enzymes, signalling proteins, and vaccine antigens [85]. The authors showed that oral delivery of a Spirulina-expressed antibody targeting campylobacter – a major cause of infant mortality in the developing world – prevents disease in mice, and a phase 1 clinical trial demonstrated safety for human administration [85].

Biopharmaceuticals

Protein biologics are indispensable therapeutics that are necessary for diagnosis, treatment, and prevention of diseases including cancer and infectious diseases. Currently, they are predominantly produced using mammalian or insect cells, although alternative options, including fungi and plants, are being developed [86,87]. The choice of production system depends mostly on the complexity of the product, but every system has distinct advantages and disadvantages (Table 1). Simple biosimilars such as insulin can be produced in prokaryotic systems, but most protein biologics require a dedicated folding machinery or post-translational modifications, which are only possible in eukaryotic systems. Other disadvantages of prokaryotic systems include presence of proteases and endotoxins. Mammalian cell culture systems offer high-quality products, but they are difficult to scale, and they are time- and cost-expensive due to complex media and cultivation requirements, slow growth, and low product titers. Moreover, genetic manipulation of mammalian cells is difficult, which has led to a search for more readily transfectable production systems. Although insect cells fulfil this requirement, they share the high production costs with mammalian cell systems, and they produce nonmammalian N-glycosylation patterns, which is an important quality criterion for most products. More cost-effective eukaryotic production systems include yeast and filamentous fungi, but these are limited in product quality for complex proteins due to folding and hypermannosidic N-glycosylations, which are undesirable in human therapeutics. Transgenic plants have several advantages compared with other production systems, including inexpensive cultivation, the possibility for scaling up, and the ability to produce correctly folded proteins with complex N-glycosylation patterns that are similar to those of mammalian cells, although not identical [87]. Importantly, plant-derived biologics have no risk of contamination with human viruses, which is a major safety concern and cost factor for animal

Table 1. Qualitative comparison of advantages (+) and disadvantages (–) of different systems for production of protein therapeutics[a]

	Bacteria	Fungi	Microalgae	Plants	Insect cells	Mammalian cells
Genetic accessibility	+++	+++	++	–	–	– –
Growth rate	+++	++	+	– –	-	–
Resource efficiency	+	+	+++	++	– –	– –
Scalability	+++	+++	++	+		– –
Endotoxin or pathogens contamination	–	+	+++	+++	– –	– – –
Protein folding	– –	+	++	++	+++	+++
N-glycosylation	– – –	–	+	+	+	+++

[a]Key references: [95–100].

cell systems [88]. However, genetic manipulation of plants is cumbersome and time-consuming, and product titers are often low. Furthermore, downstream processing is costly because proteins cannot be secreted, and biocontainment is difficult due to pollen.

Microalgae share the benefits of transgenic plants for production of biologics, including high resource efficiency, easy scale-up, complex protein folding, and the potential for plantlike N-glycosylation, and they have further advantages. These include simple biocontainment, higher growth rates, ability to secrete proteins, and rapid genetic engineering. Therefore, microalgae have gathered increasing interest as potential cell factories for production of protein therapeutics. Although microalgae-derived protein biologics are not yet produced on an industrial scale, they are intensively researched. Several protein therapeutics have been expressed in microalgae, including monoclonal antibodies, growth hormones, cytokines, and cytotoxins [89–93]. To allow production of high-quality glycoproteins, N-glycosylation patterns of the microalgae proteins need to be characterized, and adjusted by glycoengineering, to reflect N-glycosylation produced by mammalian cells. Although glycoengineering can be demanding, it was already successfully applied to change N-glycosylation in plants [94]. In addition to glycoengineering, microalgal production of biologics will heavily depend on availability of signal peptides that allow efficient secretion of recombinant protein, because this will be essential to reduce downstream processing costs. Production of complex biologics with mammalian cell–like post-translational modifications in any microalga would be a milestone achievement.

Concluding remarks and future perspectives

Commercial production of microalgae for bulk products such as SCP and lipids is hindered by a lack of scale, which is required to reach competitive production costs (Figure 5, Key figure). The need and potential are clear: The direct use of solar energy for SCP production by microalgae has the highest land use efficiency when compared with other microbial SCP sources using sugar beet production or hydrogen as feedstocks.

Production of microalgae for bulk products will rely on the DAC of carbon dioxide or sugars from agri-industry waste streams, integrated with PV or wind energy supply to meet energy operation requirements, and they will have 100% efficient uptake of nitrogen and phosphorous. For this, further developments need to be made, including integration of DAC and PV with microalgae production facilities, advanced process design, monitoring and control, and digital twins, all rather unexplored areas in microalgal biotechnology (see Outstanding questions). Combining this with fast-growing industrial strains that can cope with dynamic conditions outdoors could lead to photosynthetic efficiencies closer to the ones measured in controlled lab-scale reactors (6.5%), which would be a breakthrough in the field. Selection and scale-up of new strains need to be faster through the development of high-throughput genotyping and phenotyping platforms.

There have been exciting developments in genetic toolboxes for industrially relevant strains, unleashing the expansion of microalgae bioproducts for designer feed and food, oral vaccines, and pharmaceutical proteins. Despite this, sophisticated metabolic engineering will require a more complete molecular toolbox (Figure 5) containing several transcriptional promoter elements of varying strengths, including inducible promoters, transcriptional terminators, selection markers, genetic switches, and translational elements such as internal ribosome entry sites and riboswitches. In addition, virus-mediated technologies are opening the door to a faster, highly programmable genetic engineering of eukaryotic cells. Only then can a microalgae species be manipulated into producing optimal quantities of a desired product. In parallel to this, knowledge needs to be gained in several areas, such as secretion of recombinant protein and

Outstanding questions

What is required to scale up production to a scale of hundreds of hectares?

How can microalgae be used as a CO_2 fixation technology?

How can photovoltaics be integrated in a production facility to make production independent of energy supply from the grid?

How can agrifood side products be used for mixotrophic production of microalgae?

How can N and P from surface water, seawater, and waste streams be used to grow microalgae for food applications?

How can the potential of genetic engineering be efficiently unlocked to transform and domesticate photosynthetic platforms and let them become more competitive?

How can proteins be excreted from microalgae in a continuous production system?

How can microalgae be used as an expression system for biopharmaceutical proteins?

How can algae protein products be made white?

What is the potential of using nitrogen-fixing microalgae and cyanobacteria?

Are microalgae an effective expression system for viral proteins?

Key figure

Main applications and specific challenges of microalgae

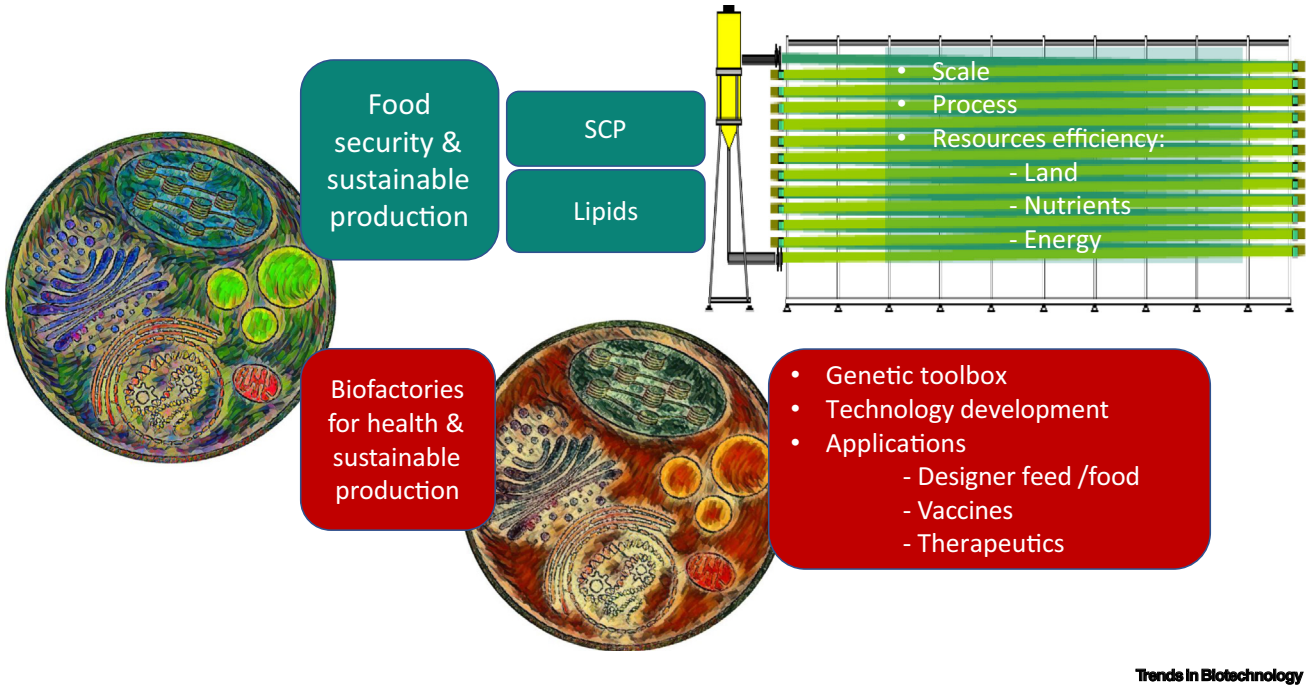

Figure 5. This figure depicts some of the main products that can be sustainably derived from microalgae, including food and health applications. Abbreviation: SCP, single-cell protein.

immunogenicity of microalgal oral vaccines, and glycosylation patterns of the microalgae proteins need to characterize.

Hype has led to technology development, hopes are coming true, and the field of microalgal biotechnology is moving forward.

Declaration of interests

The authors have no interests to declare.

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