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Phaeodactylum tricornutum: a diatom cell factory

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Abstract

A switch from a petro-based to a bio-based economy requires the capacity to produce both high-value low-volume, as well as low-value high volume products. Recent trends point to the case for developing microalgae based microbial cell factories, with the objective of establishing environmentally sustainable manufacturing solutions. Diatoms display rich diversity and potential in this regard. Here, we focus on *Phaeodactylum tricornutum*, a pennate diatom commonly found in marine ecosystems and discuss recent trends in developing the chassis for a suite of natural and genetically engineered products. Both upstream and downstream developments are reviewed for the commercial development of *P. tricornutum* as a cell factory for a spectrum of marketable products.

Microalgae chassis in the biobased economy

The growing world population is constantly placing increasing demands on available resources that need to be utilised in innovative and environmentally sustainable ways for providing us with food security, energy, and chemicals [1]. A transition to the **bio-based economy (see Glossary)** from an environmentally unsustainable petroleum-based economy will require the capacity to incorporate low-value, high volume productions, in addition to high-value low volume manufacturing, which remains a techno-economic challenge. Nevertheless, there is an increasing desire to create microbial cell factories that can address this capability. Microbial cell factory hosts have predominantly been proposed for high-value products, such as the production of recombinant vaccines and therapeutics for the prevention and treatment of diseases. *Escherichia coli* has often remained the choice for development as a microbial **cell chassis**, but eukaryotic platforms, such as *Saccharomyces cerevisiae* [2], Chinese hamster ovary (CHO) cell lines [3], and plants [4] are also well developed. However, with the ever-increasing need to provide environmentally sustainable solutions to manufacturing that are also economically viable for a multitude of products including low-value high-volume products, alternative platforms with diverse capabilities are required. In this regard, microalgae offer promise.

Microalgae are photosynthetic organisms that possess the machinery to produce high-value phytochemicals, and have the capacity to utilise carbon dioxide as a feedstock, potentially enabling economic and environmentally sustainable manufacturing solutions. Compared to plant cells, they can be relatively quick to grow (doubling times in hours typically, and biomass productivities in days as opposed to months or years for plants), can be grown on non-arable land with

potential for reclamation of nutrients from waste resources (wastewaters and waste CO_2 sources), offering higher process turnover and economically viable processing alternatives. Their ability to grow in a multitude of niches including marginal land, desert land, brackish water or the open ocean, not only offers effective resource utilisation, but also indicates an adaptive lifestyle with a diversity of metabolic capabilities that could be tapped into. Industrial scale cultivations for commercial manufacturing have largely focussed on harnessing the natural capabilities of selected species, such as ß-carotene from *Dunaliella salina*, phycocyanin from *Arthrospira* spp., and astaxanthin from *Haematococcus pluvialis*. However, this trend is slowly changing. The genomes of >40 microalgae species are now publically accessible and more are in the pipeline, with an increasing array of tools being developed to engineer metabolic pathways towards the development of cell factories that are tractable and functionally diverse. There is a growing body of knowledge on the biochemical makeup, metabolic pathways, and functional capabilities of a handful of species. However, given the vast diversity of microalgae, they still remain a largely untapped resource. Much of our knowledge on microalgae metabolism and tools development in a manufacturing context, in the recent years, has been driven by the need to develop microalgae for biofuel production. Current consensus points towards a biorefinery concept in manufacturing, with multiple product streams [5-7], especially for producing low-value high-volume products. Alternative product streams for productions from the same microalgae chassis will be needed. In addition to harnessing the natural product spectrum from microalgae that include bioactives with nutraceutical and cosmeceutical properties, there is also a drive to develop engineered microalgae towards therapeutic protein production [8, 9], bioplastic precursors [10] and plant terpenoids [11]. Moving forward, the challenge is in developing microalgae

chassis to accommodate the biorefinery concept, for economically viable propositions that can harness potential environmental sustainability offered by using a microalgae chassis for biomanufacturing. Whilst several microalgae are at various stages of development as cell factories (Box 1), there is a growing body of knowledge on the pennate diatom, *Phaedactylum tricornutum,* and its development as a microalgae cell chassis, which we discuss here.

P. tricornutum – a well-studied diatom

P. tricornutum is a model diatom (Box 2) that exhibits a complex sexual life cycle and is perhaps the most characterised of all diatoms so far. It is a robust laboratory species and can be grown in a range of culture media. It is known to accumulate a spectrum of marketable products, and is a commercially viable species for large-scale cultivation (Table 1). Its potential for being a suitable chassis can be attributed to its ability to grow in seawater, a tailored molecular toolbox, and the ability for multiple products of interest to be extracted sequentially [12, 13]. In outdoor mass culture systems, it has been shown to dominate and outcompete other microalgae species with a tolerance for high pH and an ability to grow under low light [14, 15].

P. tricornutum has a unique biology amongst diatoms (Box 3). **Gene knockout tools** have been developed [16, 17] and the genome can now be edited by CRISPR/Cas-9 for gene-targeted mutations [18-20]. The advances in genetic knowledge of *P. tricornutum* has contributed to its development as a chassis for the production of non-native components, including polyhydroxybutyrates (PHBs) for bioplastics [10], monoclonal antibodies [8] and plant triterpenoids, which are not

endogenous to the organism [11] and levels of DHA which are low in native strains have been elevated [21].

The biochemical characteristics, robust growth in mass culture systems, tolerance of low-light levels and high pH, and the well characterised genome and engineering tools available suggest *P. tricornutum* could be an important microalgae candidate as a diatom cell factory. For this platform organism to produce products of interest on a commercial scale, process optimisations based on developments upstream and downstream are essential.

Cultivation of *P. tricornutum*

Both indoor and outdoor settings in photobioreactors (PBRs) (tubular, column, flat-panel, and proprietary types) and open (raceway) ponds have been used to cultivate *P. tricornutum*, with varying product yields and productivities (Table 1). Flat-panel PBR configurations have shown improved biomass, EPA and fucoxanthin productivities, attributable to low shear stress and effective illumination [22]. High energy requirement for temperature maintenance [23], and temperature dependent loss of biomass to photorespiration [24] are challenges to address with the use of PBRs. Light is a major influencing factor in product accumulations. Current photosynthetic productivity in microalgae, in general, is lower than theoretically attainable levels in the photoautotrophic mode [12]. Intensity of light [25], wavelength [26] and photoperiod [27] are all known to have an influence on *P. tricornutum* metabolism and in turn affect productivities. In terms of product accumulations, low light intensities appear to favour the accumulation of fucoxanthin [28, 29] and EPA [30], whilst higher light intensities appear to favour accumulation of storage components, chrysolaminarin [31] and triacylglycerides (TAGs) [32], with

degradation of synthesised chrysolaminarin in the dark [31], under N-limitation. However, under nutrient rich conditions, TAG and EPA contents were found to be independent of the applied light intensity [14].

P. tricornutum is a successful **photoautotroph** but is also capable of mixotrophic growth on glucose, acetate, fructose and glycerol. Based on various reports, glycerol appears to be the most suitable organic carbon source for mixotrophic growth, resulting in the highest biomass and EPA productivities to date (Table 1), but it has not been explored for other products of interest. **Mixotrophy** with glycerol mimics typical responses with nitrogen limitation resulting in increases in TAGs but there are also resultant increases in biomass without loss of photosynthetic capacity of the cell [33].

Nitrogen and phosphorus limitations have been successfully utilised to alter product accumulations in *P. tricornutum* [32, 34-37], and silicon is known to be a non-obligatory requirement for growth of the organism, although it may have a positive influence on some growth processes as found under low temperature and green light [38], so can also be used to alter product accumulations [37]. Whilst nitrate is a commonly examined nitrogen substrate, a switch to ammonium can lead to increased lipid productivities [32], and encouraging environmental benefits could be attained by switching to urea, or ammonium nitrate [39], in large scale cultivations.

It is anticipated that developments in PBR configuration, lighting, media formulation, carbon assimilation/utilisation, in combination with bioprospecting can result in significantly increased biomass and product yields. However, to elevate intrinsic yields, upstream developments through the manipulation of product pathways may be required.

Upstream developments

Toolkits for a tractable chassis

Domestication of microalgae is often hindered by the absence of controllable sexual cycles that would allow combining desirable traits and the removal of unwanted alleles. To date, in microalgae, the genetic toolkit developed for *P. tricornutum* is one of the most advanced, and both nuclear [40] and chloroplast [41] transformations have been achieved. Identifying endogenous promoters, well mapped plasmids, antibiotic-free **selection markers** and successful modes of DNA delivery will not only enable the development of an appropriate understanding of the chassis but also a tractable chassis fit for purpose. For the effective expression of recombinant proteins adapting DNA sequences to host specific codon usage whilst avoiding weakly translated codons is beneficial for protein translation, but as *P. tricornutum* possesses a typical GC-content of 48 %, this might be less critical and foreign genes and enzymes can be expressed without codon optimisation [42, 43].

The pPhaT-1 plasmid has been the most frequently used vector and contains the *sh-ble* gene that confers resistance to the antibiotics zeocin and phleomycin [44]. Alternatively, the pPhAP1 vector with an alkaline phosphatase promoter has been shown to be effective with enhanced green fluorescent protein (EGFP) expression at 9.3-10.5 times greater than other diatom vectors showcasing the potential of the vector in obtaining high yields of recombinant protein [45]. In terms of promoters, the light regulated fcpA (LHCF1) and fcpB (LHCF2) promoters are the most frequently used but they are constrained by being light dependent and inactive in the dark [46]. A nitrate reductase (NR) promoter has been used in *P. tricornutum*, that is reportedly better than LHCF1 in green fluorescent protein (GFP) expression [47]. The NR promoter inhibits transcription in ammonium, but this has been reported to be 'leaky'

[47]. Alternatively, EF2 that encodes elongation factor 2 is a possible new constitutive promoter with higher expression than LHCF2 that is not significantly affected by light [48]. Other endogenous promoters that have been shown to have good performance characteristics include the alkaline phosphatase promoter [45] and glutamine synthetase promoter [49]. In addition, potential promoter regions identified from diatom-infecting-viruses (DIVs) have also been shown to function effectively [50]. Constitutive promoters to express multiple target genes have also been recently demonstrated [48, 51]. Introns can also be included in the expression cassette that contain transcriptional enhancers or sequences conferring enhanced mRNA stability or enhanced mRNA turnover.

The availability of bacterial conjugation [18, 52] for economic genetic transformations provides a viable solution to the more expensive alternatives of biolistics and electroporation. In bacterial conjugation, DNA is replicated stably within the nucleus as an episome. This presents an opportunity for artificial chromosome transfer into diatoms and could result in a one-step transfer of whole metabolic pathways, as has been shown for vanillin biosynthesis [18], where a plasmid encoding eight genes involved in vanillin biosynthesis has been shown to be successfully propagated in *P. tricornutum* over four months with no evidence of rearrangements.

Genome editing tools offer a breakthrough in targeted mutagenesis for the development of *P. tricornutum* as a cell factory, given that the organism is diploid and sexual reproduction cannot be controlled in the laboratory, limiting random mutagenesis as a useful tool for trait improvements. From an industrial perspective, genome editing is not yet subject to regulatory approval as the mutants are not stated as genetically modified (GM) [53], and more importantly the changes introduced are

believed to be more tractable. Meganucleases [54], TALENS [16, 17], and CRISPR/Cas9 with an optimised Cas9 [18-20] have all been shown to work in *P. tricornutum* for targeted genetic engineering and the generation of stable knockouts. Using these techniques can result in marker free transgenic types.

Using a non-genetically modified organism approach, **adaptive laboratory evolution** has been shown to result in a two-fold increase in growth and fucoxanthin content in semi-continuous cultivation over only 11 generations with red and blue light [55]. Adaptations to elevated carbon dioxide levels over nearly 2000 generations [56] have shown to evolve lower mitochondrial respiration.

Genome scale metabolic models (GSM) have been developed for *P. tricornutum* [57]. Further developments in pathway elucidation can result in developments using a top down or bottom up approach for elevating products of interest and synthesising heterologous compounds.

Improved knowledge of product biosynthetic pathways and their regulation

By far the most widely investigated process, driven by the need to develop alternative renewable strategies for meeting our energy demands, is the accumulation of storage neutral lipids (TAGs), which can be converted to biodiesel. Nitrogen depletion is the most widely employed strategy to induce TAG accumulation in microalgae, and this is no different in *P. tricornutum* [14, 58, 59]. Briefly, *de novo* fatty acid synthesis occurs in the plastid, followed by two known routes to TAG synthesis: (a) acyl lipid assembly in the endoplasmic reticulum (Kennedy pathway) and (b) the acyl-CoA independent pathway involving phospholipid:diacylglycerol acyl transferase (PDAT) which involves the chloroplast membrane lipids (Figure 1).

In more recent years, the role of Methylcrotonyl-CoA Carboxylase [60], phosphoenolpyruvate carboxykinase (PEPCK) [61], Diacylglycerol Acyltransferase (DGAT) [62], 1-Acylglycerol-3-Phosphate O-Acyltransferase 1 (AGPAT1) [63], and Malic enzyme (ME) [64] have all been implicated in TAG synthesis in *P. tricornutum*, in addition to the identification of lipases [65], which play a role in TAG degradation and reallocation of fatty acids to maintain lipid homeostasis in the plastids (Figure 1). TAG accumulation involves remodeling the intermediate metabolism, especially reactions in the tricarboxylic acid (TCA) and the urea cycles [66], with restructuring of carbon metabolism through down regulation of the Calvin cycle, chrysolaminarin biosynthesis and up-regulation of the TCA cycle, and pyruvate metabolism to reroute the carbon to lipids [35, 59, 67]. This has also been linked to cell cycle arrest [68]. With regards to EPA, the involvement of elongases and desaturases in EPA synthesis has been demonstrated, both through homologous and heterologous expression [69, 70]. For fucoxanthin synthesis, phytoene synthase (psy) regulation has been shown to be involved [50]. The metabolic pathways and regulatory nodes for chrysolaminarin synthesis are not yet as well investigated. Two potential 1,6-beta transglycolases involved in the branching of 1,3 beta glucan chains have been characterised and found to be associated with the vacuole, suggesting branching of chrysolaminarin may occur in these organelles [71]. It appears that the carbohydrate storage in the vacuoles is intertwined with carbohydrate metabolism, photosynthetic homeostasis and plastid morphology [72]. UDP-glucose pyrophosphorylase (UGPase) has been identified as a rate limiting enzyme that might play an important role in chrysolaminarin biosynthesis [73].

Carbon capture and photosynthesis in P. tricornutum

Photoautotrophic growth is a key advantage of this microbial chassis and an understanding of carbon capture and photosynthesis is essential in developing the chassis. Compared to plant systems, the understanding of this aspect is still fragmentary, but recent developments are encouraging. *P. tricornutum* is known to take up bicarbonate, a 'chloroplast pump' acting as the primary active transporter [74]. Several carbonic anhydrases (CAs) have been identified in *P. tricornutum* that participate in the carbon capture mechanisms to maintain sufficient partial pressure in the **pyrenoid** for CO_2 fixation by Rubisco. *P. tricornutum* appears to lack external CAs and CAs localised in the cytoplasm [74]. However, recently a theta-type CA localised in the thylakoid lumen with essential roles in photosynthesis and growth of the organism has been reported [75]. CCM mechanisms in diatoms appear to be diverse between species [76]. Plasma membrane type aquaporins are known to function in CO_2/NH_3 transport and provide photoprotection [77].

Photosynthetic efficiencies in diatoms, although higher than in plants, can still be improved with appropriate knowledge of light harvesting and utilisation in diatoms. There is still substantial scope in bridging the gap between currently achievable and theoretical conversion yields [12]. The fucoxanthin-chlorophyll proteins (FCPs) are the most characterised light harvesting complexes in diatoms. In *P. tricornutum*, FCPs associated with both photosystems I and II have been characterised [78]. There are similarities with plant systems, but differences in pigmentation exist [78]. The photosystems are segregated in subdomains, which minimise physical contact, as required for improved light utilisation, but are interconnected, ensuring fast equilibration of electron carriers for efficient optimal photosynthesis [79]. More recently, the efficacy of light harvest and energy transfer in these systems is becoming clearer with the structural elucidation of the complex [80].

Downstream considerations and challenges

P. tricornutum cultivations are typically suspended cultures, requiring cell harvest and extraction stages, which represent a target for increasing the cost competitiveness of *P. tricornutum* as a cell factory. **Downstream processing** costs have a significant contribution to overall process costs (typically ranging between 20-60%) [7]. Harvesting alone can contribute to 23% of the cultivation cost with raceway cultivations, but can be as low as 5-7% with photobioreactors, due to the potential for higher biomass concentrations [81].

The aim of harvesting is to concentrate the biomass by 10-300 fold (typically from 0.05-0.5% to 5-15% total solids) for subsequent drying/extraction or direct extraction of wet biomass in the shortest period to avoid spoilage, especially in warm climates [82]. It is essential for the harvesting technique to be quick, have good harvesting efficiency (>90%), inexpensive, leave behind little or no toxic residues, and not affect the quality of the biomass [7]. Traditionally, centrifugation has been used for harvesting, and the apparatus include conventional cream separators, bucket centrifuges, disk-stack, and super-centrifuges. Nevertheless, centrifugation is energy intensive and can account for 20-25 % of the cultivation costs [7].

A wide variety of harvesting methods have been suggested for *P. tricornutum* (Figure 2). Novel methods include the use of nanoparticles, ultrasonic harvesting, flotation, and tangential flow membrane filtration. However, the most well researched area for harvesting *P. tricornutum* have centered around flocculation with this method being scalable. Flocculants are well characterised and are effective through different modes; charge neutralisation (inorganic flocculants), sweeping, polymeric bridging (organic flocculants) and electrostatic patch mechanisms [83].

Alternatively, 'auto'flocculation (alkaline flocculation) can be effective in a narrow pH window (10-10.5) in the presence of magnesium with flocculation induced by the precipitation of brucite (magnesium hydroxide), which causes charge neutralisation and adsorption of hydroxide particles onto the cell wall of *P. tricornutum* [84, 85]. To date many flocculants have been tested (cationic, anionic and nonionic) but there has been an inconsistency with the setup design, flocculant concentrations used, the initial biomass density, optimal pH and the time required for optimal harvesting. Bioflocculation using closely associated bacteria within the culture, and the influence of extracellular proteins on the harvesting efficiency [86], are innovative, environmentally friendly and potentially cost-effective approaches that warrant further investigation.

The range of extraction techniques investigated in *P. tricornutum* is showcased in Figure 2 and comprises biochemical, mechanical and physical methods. The conventional scalable methods (e.g., bead milling for cell disruption followed by solvent extraction) are mainly constrained by economic and environmental drawbacks associated with the extraction and purification of bioactives, in conjunction with degradation issues [87]. Modern green extraction techniques mitigate most concerns of traditional techniques and warrant further investigation. These green solutions include microwave assisted extraction (MAE), pulsed electric field (PEF), ultrasound assisted extraction (UAE), pressurized liquid extraction (PLE), supercritical fluid extraction (SFE) and the use of enzymes and ionic liquids [88]. Ethanol has been suggested as a green solvent for the extraction of fucoxanthin and EPA from *P. tricornutum* biomass [89] that doesn't require a drying step when used as a water-miscible solvent in PLE [90]. Lipid extractions can also be enhanced with microwaves (MWs) or the application of deep eutectic solvents (DESs) for cell wall

disruption, followed by supercritical CO_2 ($scCO_2$) and dimethyl carbonate (DMC) extractions. The fatty acid profiles from such extractions can be similar to the traditional Bligh and Dyer extraction method albeit with higher selectivity [91]. More detailed knowledge is required from the cell walls of microalgae to facilitate successful cell disruption and extraction approaches. There is still a requirement to develop these environmentally friendly methods at pilot scale with an emphasis on MAE and PLE due to the wide spectrum of microalgal metabolites that can be extracted [49].

Whole cell formulations for end-use could be a cost-effective approach that would obviate the need for extraction steps and alleviate product stability/storage concerns. This is in particular useful in the delivery of bioactives in food/feed formulations, as has been demonstrated for effective delivery of omega-3 fatty acids in Salmon feed [92].

Towards a diatom biorefinery

The biorefinery principle is well defined but has very rarely been put into practice. t'Lam and colleagues [7] evaluated that a multi-product biorefinery is not currently feasible with downstream processing accounting for 50-60 % of the process. Currently there is too much emphasis on inflated prices of microalgal products such as pigments as has been observed in the *H. pluvialis* derived astaxanthin industry. Inflated prices may work for fucoxanthin from *P. tricornutum*, which relies on clinical backing for the associated health claims. Few studies have fully addressed the options of sequentially extracting products from *P. tricornutum* biomass in an integrated biorefinery (Figure 2). Sequential extraction of fucoxanthin, followed by

EPA and chrysolaminarin, in turn has been suggested as a possibility [13]. However, the economic viability of this proposition is yet to be investigated.

The economic feasibility of a microalgae biorefinery has been argued for [81] with production costs of €6-7/kg DW and a resulting revenue of €31/kg DW including cultivation and downstream processing. Production costs of €3-6/kg DW over the same scale can be achieved even with closed tubular PBRs [93], but the electricity costs required for cultivation are high (62 % of the costs of cultivation in an indoor setting) [39] and consequently there is a necessity to produce microalgal products in an outdoor setting. More careful integration of the downstream processing units could result in a cost-effective solution [7] Using bioflocculation in combination with PEF for the 'milking' of *P. tricornutum* cells (Figure 2) through a wet processing method could result in the release of multiple products of interest without killing the cells, but this remains to be tested.

Concluding remarks

As showcased above, *P. tricornutum* has great potential as a microbial cell chassis for homologous and heterologous compounds. The high photosynthetic efficiency, a detailed understanding of product pathways, a well-developed suite of molecular tools, in conjunction with the ability to produce a spectrum of marketable products, all point to great promise for the future development of the chassis. *P. tricornutum* has been explored to produce single products at pilot scale, including TAGs for biodiesel and fatty acids of nutraceutical value (EPA and DHA), but products such as recombinant proteins and chysolaminarin are yet to see large scale trials. Fucoxanthin appears to be the only product that has been shown to be commercially viable currently, due to its high selling price (for the pure component),

but as product supply increases, selling prices may fall as observed in the astaxanthin industry. There is also considerable potential for the development of more products of value using the chassis as demonstrated with heterologous compounds and scope for reallocation of carbon to maximize specific productivities. Nevertheless, long-term sustainable commercial productions from *P. tricornutum* will require development of a biorefinery approach to valorise cultivations with incorporation of multiple product streams, especially when aiming for low-value high volume products. Despite the promise, challenges remain that require attention. A primary challenge is in maximising biomass productivities, in a way to make the most of photoautotrophy and develop processes that are cost effective and competitive compared to other heterotrophic hosts. Learning to make the most of the photosynthetic capacity and increasing carbon uptake and routing to products of value are desirable. Environmentally sustainable developments in PBR technology and innovative downstream processing options that reduce costs for enabling economic extraction of multiple products of interest are required within a biorefinery approach (see Outstanding questions).

Industrial cultivation of genetically engineered *P. tricornutum* will require secondary containment such as glass houses or polythene tunnels, antibiotic resistance needs to be replaced with other selection markers such as fluorescent proteins and legislation will have to be complied with in terms of genetically modified organisms especially as the regulations surrounding CRISPR-Cas 9 are as yet bordering on uncharted waters in the EU. Nevertheless, the future for *P. tricornutum* as a microalgal cell chassis is certainly bright.

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Glossary

Adaptive laboratory evolution: is a method employed to gain insights into the fundamentals of molecular evolution and adaptive changes that occurs in populations during long term selection under specific growth conditions. The organism is repeatedly cultured by exposure to pre-defined conditions to accelerate evolution in the laboratory.

Allele specific expression (also known as allelic bias): is where one allele is expressed at levels higher than the other.

Bio-based economy: is where biological feedstock and/or bioprocesses are employed to derive products of value for economic gain, as sustainable solutions to the challenges we face (food, energy, chemicals, health, materials, and environmental protection).

Bio-refinery: is a process which aims to replace oil with biomass as a feedstock for fuel and chemical production through the integration of green chemistry and sustainable production. The aim is to produce multiple products from biomass to add value to the process (in a way akin to the chemical refinery).

Cell chassis: can refer to a cell host or an organism with a genome that can be altered to maintain heterologous DNA parts for gene expression.

Downstream processing: refers to steps involved downstream of cultivation to recover products of interest, and can encompass cell harvest and the extraction of products of interest from natural sources followed by product purification or formulation.

Gene knockout tools: are tools used to make an organism's gene inoperative. These are usually developed to study gene function and to investigate the phenotype after gene loss.

Genome editing: targets precise sites within a genome by introducing double strand breaks into a specific guided site that is repaired, resulting in a modest deletion of a few nucleotides of the native gene. Insertional activation is not required for mutations to be created through incorporation of a non-native gene encoding a selectable marker.

Gibson and Golden Gate assembly: are assembly methods used across the synthetic biology community which employ a 'one-pot/tube' method and do not rely on restriction sites. Gibson assembly involves long-overlap assembly (exonuclease for annealing fragments, polymerase for filling the gaps and DNA ligase for sealing the nicks in the assembled DNA) where multiple parts can be assembled (promoters, terminators and other short sequences). Golden Gate is based on Type II S restriction endonucleases, which cleaves double-stranded DNA outside their recognition sites resulting in a short single-stranded overhang with Bsal restriction sites utilised for simultaneous digestion and ligation preventing re-ligation.

Mixotrophy: a mode of cultivation where the culture utilises both carbon dioxide and organic carbon supplemented in the nutrient medium.

Photoautotroph: is an organism that can make energy from sunlight and carbon dioxide through photosynthesis to synthesise organic compounds for nutrition.

Pyrenoid: are sub-cellular compartments in the chloroplast of most algae and are associated with the carbon concentrating mechanism (augments photosynthetic productivity by increasing inorganic carbon).

Secondary endosymbiotic events: are events which occurred when a living cell engulfed another eukaryotic cell that had undertaken primary endosymbiosis resulting in a cell with a double phospholipid bilayer around the mitochondria and chloroplasts.

Selection markers: are sequences that are expressed for a specific set of traits that provide the transformed cells with different properties from untransformed cells. Selection markers affect the condition under which the transformed cell can grow such as, antibiotic resistant transformed cells growing in medium with antibiotics.

Transposon (also known as a jumping gene): is a DNA sequence that can alter its position within a genome, occasionally resulting in a reverse mutation, altering the cells genetics and genome size.

Text Box 1: Microalgae cell factories

Microalgae are a taxonomically diverse group of organisms that are spread across the eukaryotic tree of classification (Figure I). We have some knowledge of a few representatives and are developing an improved understanding of a handful of species with biotechnological potential (Table I).

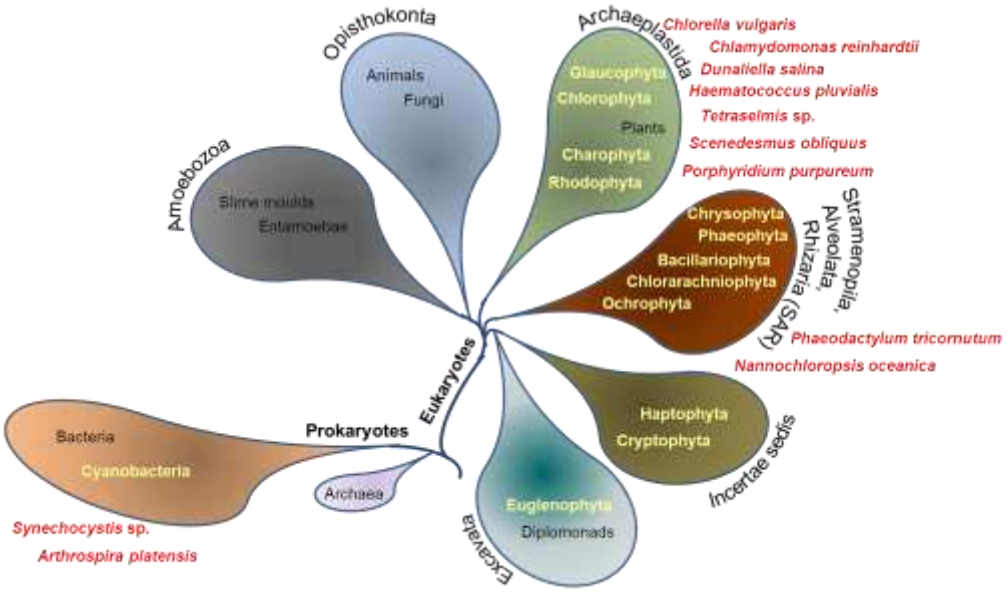

Figure I: A cartoon representation of the phylogenetic diversity of microalgae phyla (in yellow) and representative species being developed (in red). Other representative lineages, such as animals and plants are also provided (in black).

Table I: Alternative microalgae (genome sequenced) cell factories under development and their biotechnological potential

Organism	Genome size (MB)	Biotechnology potential	Opportunities	Challenges
Chlamydomonas reinhardtii CC-503 cw92 mt+	120 (chloroplast and mitochondria sequenced)	Biofuels, recombinant proteins, model chassis	Fast growth (6-8 h doubling time); all three genomes completed and well annotated; easily transformable with inducible and constitutive promoters; cloning methods developed for insertion of multiple genes (Golden Gate, Gibson Assembly); range of plasmids and strains (>300 and >2700 respectively); advanced genome	Low oil content; poor growth in open ponds, sensitive to solar irradiance, internal RNA silencing mechanism preventing gene expression, no standardised expression strain, poor expression of heterologous proteins, relatively few protein coding genes from the genome have been fully functionally validated, number of transgenes to

			editing (CRISPR Cas-9, ZFNs, TALENS); detailed omics studies/data.	regulate expression is low; gene silencing reported; requirement for freshwater.
Chlorella vulgaris C-27	Only the chloroplast has been sequenced	Biodiesel, proteins, wastewater treatment	Rapidly consumes nitrate and phosphate; oil and starch accumulation (more in brackish water strains).	Most strains are freshwater; small cells are difficult to harvest; algaenan cell wall makes extraction difficult; the nuclear genome remains to be sequenced; difficulties noted in transforming cells even by biolistics.
Nannochloropsis oceanica CCAP 211/46 (CCMP 1779)	28.7	Biodiesel, EPA, violaxanthin	Optimal techniques for homologous recombination have been developed; genetically tractable chassis; plastid transformation through electroporation; advanced genome editing tools (CRISPR cas-9).	Small cells are difficult to harvest; algaenan cell wall makes extraction difficult; vulnerable to contamination in large scale culture.
Porphyridium purpureum CCMP 1328	19.7	PUFAs - EPA and ARA, exopolysaccarides	A wide spectrum of products; exopolysaccharides secreted into the medium; successful transformation through biolistics.	Not been cultivated outdoors in large scale; poor genome annotation.
Tetraselmis sp. CCMP 881	Only the chloroplast has been sequenced	Proteins, PUFAs, carbohydrates	Cultivated at scale without culture collapse in semi-continuous mode; easy to harvest (gravitational settlement).	The nuclear genome has not been sequenced; little or no annotation of chloroplast; transformation procedures not well studied.
Synechocystis sp. PCC 6803	3.57	Terpenoids, PHB, biomethane, phycocyanin	Molecular toolbox for genetic modifications developed; high-throughput system biology for genome wide analysis (omics approaches) shown; produces a diverse range of products; green light inducible lytic system for production of products of interest.	Low product titers; biofilm fouling; high production cost; difficulties in outdoor cultivation and harvesting.
Scenedesmus obliquus UTEX 393	Only the chloroplast has been sequenced	Lutein, beta-carotene, biodiesel, wastewater treatment	High lipid productivity; efficient nutrient assimilation; ability to alter morphology through agglomeration that improves resilience against grazers.	Tools for genetic manipulation need development.
Haematococcus pluvialis	Only the chloroplast has been sequenced	Astaxanthin, biodiesel, PUFAs	Detailed investigations on cultivation and elevating astaxanthin productivity; easy to harvest (gravitational settlement); detailed techno-economic assessments conducted; conventional mutagenesis well established.	Vulnerable to contamination; nuclear genome unsequenced; the chloroplast is the largest sequenced and is poorly annotated; high cost of production, vulnerable to photobleaching under high light; only been produced at large scale for astaxanthin; only the biosynthetic pathway of astaxanthin is well understood; thick walled-aplanospore makes product extractions difficult; genetic manipulation tools poorly developed; low biomass and product yields.
Dunaliella Salina CCAP 19/18	300 (chloroplast and mitochondria sequenced)	Beta-carotene, biodiesel	Extremophile (salt tolerant); lack of cell wall makes it suitable for extractions; easily transformable; lower risk of contamination	Slow growing; shear sensitive and difficult to harvest.
Arthrospira platensis YZ	6.62 MB (still under completion)	Phycocyanin, protein, PUFAs, vitamins, minerals, bioethanol	Extremophile (high alkalinity); detailed cultivation studies and at scale; easy to harvest; phycocyanin is water soluble and easy to extract.	Genetic manipulation tools poorly developed; low biomass and product yields.

Text Box 2: Diatoms and their biotechnology potential

Diatoms are among the most successful and productive photoautotrophs in the marine environment, responsible for about 40% of primary productivity in the oceans and 20% of global CO_2 fixation, with biogeochemical transfer of important nutrients such as nitrogen, carbon, and silicon [94]. They dominate the phytoplankton community in the oceans under nutrient replete conditions, rapidly growing and dividing, and have the ability to survive long periods of limitations in light and nutrients [95]. They have been shown to possess more competitive traits compared to other phytoplankton groups [96]. Diatom genomes harbour a combination of genes and metabolic pathways first thought to be exclusive to plants and animals. Diatoms have the urea cycle and the ability to generate chemical energy from the breakdown of lipids that were considered distinctive animal features, and also have evidence of a C4 photosynthetic pathway that has previously been only recorded in plants. Their evolution can be traced to **secondary endosymbiotic events**, with red and green microalgae, in addition to exosymbiotic gene acquisitions [37, 95]. This provides them with a gene pool with diverse metabolic potential. **Allele specific expression** is an additional feature that may permit further phenotypic plasticity and thus help diatoms to thrive in dynamic highly unstable environments [97]. Sequenced diatoms (Table I) provide the foundations for understanding their cellular makeup, with their genomic and epigenomic characteristics indicating a high versatility and resilience for survival under demanding environments [95, 97]. Some of these traits also bear the potential for serving as robust production vehicles.

Table I (Text Box 2): Sequenced diatoms, developmental status and their biotechnological relevance.

Species/strain	Structural Type	Genome size and composition	Genetic toolkits & biochemical knowledge	Biotechnological relevance
Phaeodactylum tricornutum CCAP 1055/1	Raphid pennate	27.4 MB. 88 chromosomes. Transposable elements make up 6.4 % of the genome.	High frequency of targeted mutagenesis possible with meganucleases, TALENS, CRISPR-Cas9; nuclear and chloroplast transformations demonstrated; multiple plasmids can be co-transformed; Golden Gate Assembly possible for cloning multiple genes of interest; several constitutive and inducible promoters are known to function well; a wide range of reporters (LUC, GUS, GFP, YFP, CFP) have been shown to work; overexpression and gene silencing are well developed. (see this review)	Production of several endogenous and heterogenous biochemicals including TAGs for biodiesel, fucoxanthin, EPA, chrysolaminarin, sterols, recombinant proteins, vanillin, PHBs.
Thalassiosira pseudonana CCMP 1335	Polar centric	32.4 MB. 65 chromosomes. Transposable elements make up 1.9 % of the genome.	Transformation by biolistics and conjugation shown to work. Gene silencing and gene knock-outs established. Secretion of recombinant proteins shown. Multiple plasmids can be co-transformed. Golden Gate Assembly possible for cloning multiple genes of interest. Overexpression and gene silencing are developed.	Silica biomineralisation; model for understanding the mechanisms behind silicification and TAG accumulation.
Thalassiosira oceanica CCMP 1005	Polar centric	81.6 MB. 51 chromosomes.	Transformation by biolistics. High genomic plasticity as shown from horizontal gene transfer.	Highly tolerant to low iron levels, reaching near maximal growth, might be a good biological indicator for iron stress; model for iron uptake studies.
Fragilariopsis cylindrus CCMP 1102	Raphid pennate	61.1 MB.	No known methods of transformations published.	Adaptations to polar conditions and survival in sea ice; antifreeze proteins.
Pseudo-nitzschia multiseries CLN-47	Raphid pennate	281.7 MB.	No known methods of transformations published.	Produces domoic acid, analogue for glutamic acid which causes Amnesic Shellfish Poisoning (ASP).
Pseudo-nitzschia multistriata B856	Raphid pennate	59 MB.	Biolistics transformation, h4 (constitutive) promoter, *sh-ble* gene for resistance to antibiotic zeocin. Meiotic toolkit with 42 potential genes involved in meiosis.	Domoic acid production. Ability to reproduce sexually for classical loss of function screens and different combinations of double transformants.
Pseudo-nitzschia australis HAB 200	Raphid pennate		No known methods of transformations published.	Contaminated genome with bacterial sequences-potential obligate symbiotic relationship; produces Isodomoic acid, unusual amnesic shellfish poisoning toxin.
Seminavis robusta D6	Pennate	Only the chloroplast has been sequenced	Meiotic toolkit with 42 potential genes involved in meiosis; the mating system is heterothallic so sex can be controlled reliably as sexual reproduction cannot begin until compatible clones are mixed, best success in mating and F1 development of the diatoms.	Sexual crosses can be made routinely, offering the potential for forwards genetics.
Fistulifera sp. JPCC DA058	Raphid pennate	Chloroplast sequenced	Biolistics; endogenous and heterogenous promoters.	High concentrations of EPA; high biomass productivity.
Odontella sinensis	Polar centric	Chloroplast sequenced	No known methods of transformations published.	Brevetoxin interactions at the cellular and subcellular level.

Text Box 3: Biology of *P. tricornutum*

P. tricornutum is a marine species including brackish water strains (Figure I)), such as CCAP 1052/6 (UTEX 646) and CCAP 1052/1B (UTEX 640), but only CCAP 1052/1B has been reported to grow in freshwater [98]. To date UTEX 640 has been the workhorse for high biomass and EPA productivities, CCAP 1055/1 for genetically engineered products of interest and UTEX 646 for monoclonal antibodies (Table 1). Strain UTEX 640 has the highest natural EPA content of 5.14 % DW [90] but a genetically engineered CCAP 1055/1 has an EPA content of 8.54 % DW [99]. For the commonly used strains for maximising lipid productivities it has been identified that UTEX 640 is better than UTEX 646 in terms of biomass and lipid productivities [100]. Bioprospecting can lead to the isolation of strains with biotechnological significance, for example, three new strains (M26, M28, and M29) were isolated from two fjords in Norway and M28 had a high TFA content of 42.9 % DW [101]. Strain M21 was of particular interest because it had a high growth rate at 10°C and a high EPA content which increased in the stationary phase to 4.6 % DW [102].

The great adaptability of *P. tricornutum* has been attributed to its pleiomorphism (four different morphotypes known - oval, fusiform, triradiate, and cruciform, and the predominating morphotype appears strain specific [101, 103, 104]). Most *P. tricornutum* strains predominate as fusiforms but some strains have been found to predominate as oval forms (CCAP 1052/1B and CCAP 1055/5) and some as triradiates (CCAP 1055/7) [103]. From a biotechnological point of view the fusiform morphotype appears most commercially relevant due to its growth rate (~1.4 times higher than comparable oval cells) [103] and a greater antibacterial activity (twice as much as the ovals) attributable to the EPA, hexadecatrienoic acid, and palmitoleic acid content [105].

The main polysaccharide in the fusiform cell morphotype is a sulphated glucuronomannan and its role in cell-wall biogenesis and the frustule architecture have been suggested [106]. It has been found that the carbohydrate composition differs between morphotypes but the structure of glucoronomannan appears conserved in fusiform and oval morphotypes [107]. This might have biotechnological implications with respect to product extractions.

P. tricornutum has a small genome of 27 Mbs that nevertheless encodes a plethora of genes, around 12,000, with 26 % of the genes being species-specific [108, 109]. It has been found to contain class I retrotransposons and a few class II **transposons**, which appear to be modulated by different stressors, such as nitrogen depletion [110-112]. The transposons contribute to 6.4 % of the genome [104, 110], which offers potential for creating or reversing mutations in the cells for biotechnological exploitation. It is less silicified among diatoms and the requirement for silicon is not obligatory. This not only favours large-scale cultivations, but also enables ease of introducing DNA into the cell. It is now routine to express multiple transgenes in *P. tricornutum* through the **Gibson and Golden Gate assembly** [11, 17].

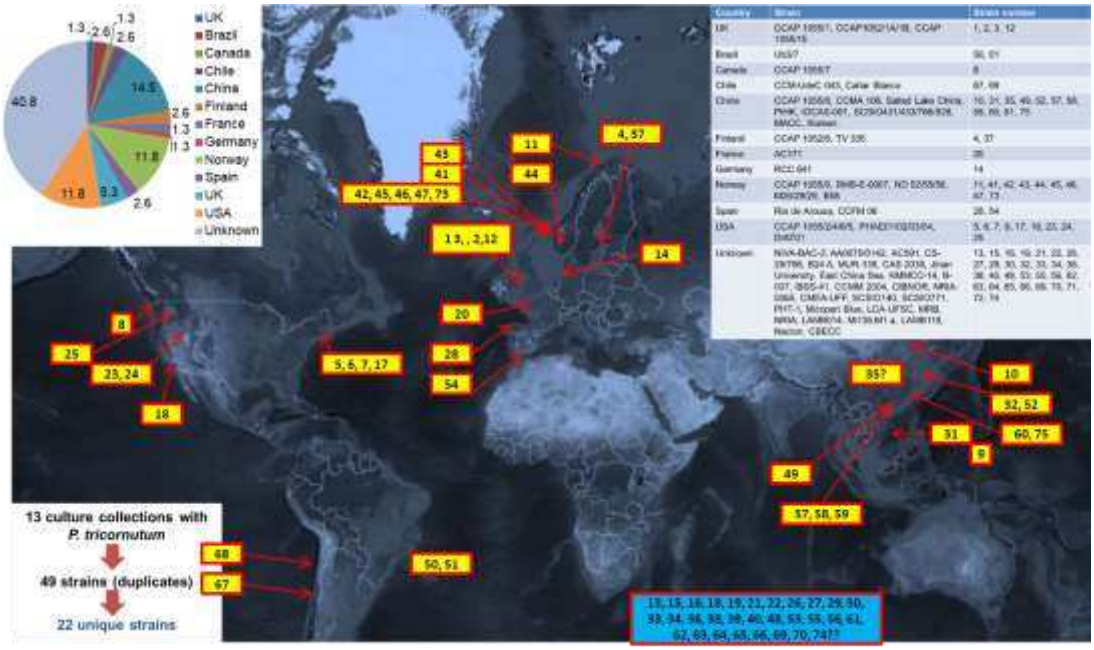

Figure I: Global distribution of *P. tricornutum* strains, showcasing 75 isolates reported in the literature (including synonymous ones). These include 22 unique strains held in 13 culture collections. A high proportion of these is of unknown origin (40.8 %).

Table 1: Products derived from *P. tricornutum* cultivation. Details given are for the most advanced productions scales reported in the literature. PBR - photobioreactor; (R) - recombinant strain; TSP - total soluble protein.

Product class	Product	Strain	Product yield/ productivity reported	Functional end-use	Operational conditions	Ref.
Lipids	EPA	UTEX 640	3% DW; 56 mg/L/d	Nutraceutical, cardiovascular health, precursor for prostaglandin-3, thromboxane-3 and leukotriene-5 eicosanoids	Outdoor chemostat split-cylinder airlift PBR (50-60L), Almeria, Spain, photoautotrophic/mixotrophic with glycerol	[113]
	DHA	CCAP 1055/1 (R) - Pt_El05	0.64% DW	Nutraceutical, primary structural component of the human brain, cerebral cortex, skin and retina	Indoor horizontal fence PBR (550 L), photoautotrophic	[21]
		CCAP 1055/1 (R) - Pt_El05	0.26 % DW		Indoor raceway (1250 L), photoautotrophic	
		CCAP 1055/1 (R) - Pt_MCAT_PtD5b	0.92 % DW		Indoor flasks, photoautotrophic	[99]
	ARA	CCAP 1055/1 (R) - Pt_MCAT_PtD5b	1.89 % DW	Nutraceutical, prostaglandin precursor	Indoor flasks, photoautotrophic	[99]
	TAG	UTEX 640	58.5 mg/L/d, 45 % DW	Biodiesel	Green Wall Panel III (≤40 L), outdoor, photoautotrophic	[100]
	Brassicosterol	CCAP 1055/1 (R) - LjLUS-25		Decreased risk of coronary heart disease, anti-inflammatory activities	Lab PBR (≤1L), photoautotrophic	[11]
Carbohydrates	Chrysolaminarin	CAS	14% DW; 94 mg/L/d	Antioxidant	Indoor flat-plate PBR (50 L), photoautotrophic	[114]
Terpenoids	Fucoxanthin	CAS	0.7% DW; 4.7 mg/L/d	Antioxidant, anti-obesity, anticancer, anti-inflammatory	Indoor flat-plate PBR (50 L), photoautotrophic	[114]
	Lupeol	CCAP 1055/1 (R) - LjLUS-25	0.01% DW	Antiprotozoal, antimicrobial, antitumour, chemopreventative	Lab Algem PBR (≤1L), photoautotrophic	[11]
		CCAP 1055/1 (R) - AtLUS-6	0.0013% DW		Indoor horizontal fence PBR (550 L), photoautotrophic	[11]
	Betulin	CCAP 1055/1 (R) - LjLUS-25	Detectable levels	Antitumour	Lab Algem PBR (≤1L), photoautotrophic	[11]
Heterologous compounds &	Polyhydroxybutyrate (PHB)	CCAP 1055/1	10.6% DW	Bioplastics	Indoor flasks (≤1L), photoautotrophic	[10]

Proteins		(R)				
	Human IgGαHBsAg. Ab against Hepatitis B virus surface protein	UTEX 646 (R)	0.0021% DW; 8.7% TSP	Monoclonal antibody	Indoor flasks (≤1L), photoautotrophic	[115]
	IgG1/kappa Ab CL4mAb. Hepatitis B Virus surface protein without the ER retention signal (DDEL) at the C-terminus of both antibody chains	UTEX 646 (R)	2.5 mg/L (secreted)	Monoclonal antibody	Indoor flasks (≤1L), photoautotrophic	[116]
	Monoclonal IgG antibodies against the nucleoprotein of Marburg virus (close relative of Ebola virus)	UTEX 646 (R)	2 mg/L (secreted)	Monoclonal antibody	Indoor flasks (≤1L), photoautotrophic	[8]
Whole cell	Biomass	UTEX 640	25.4 g/L, 1.7 g/L/d	Aquaculture/animal feed	Outdoor split-cylinder airlift PBR (60 L), mixotrophic (0.1 M glycerol)	[113]

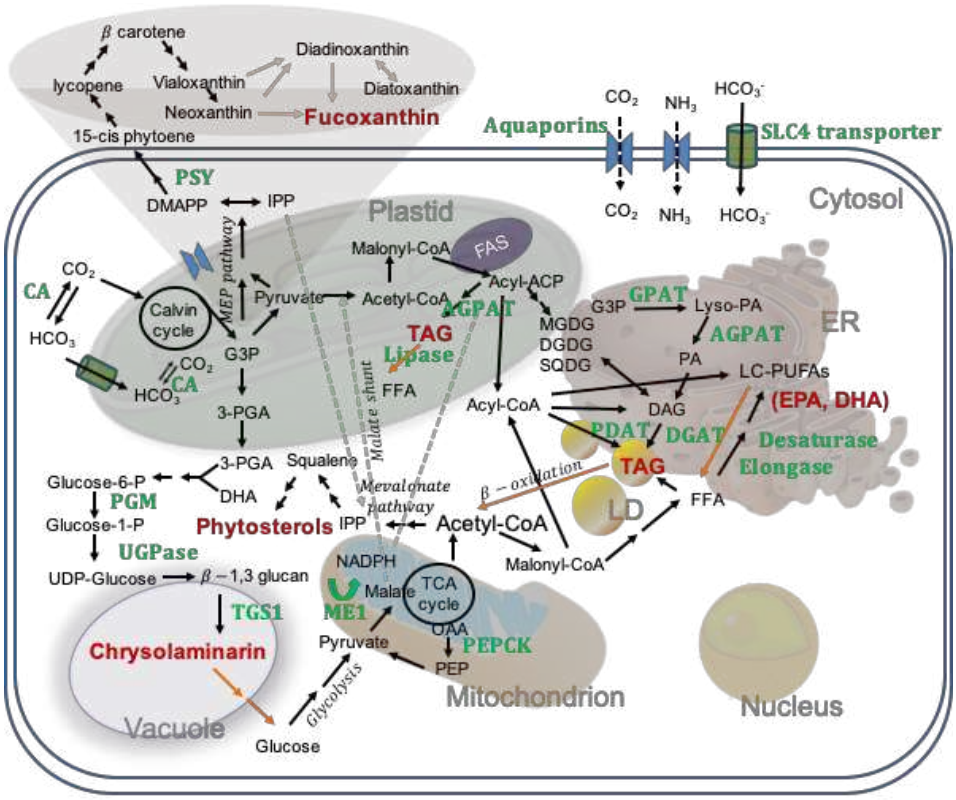

Figure 1: Biochemical knowledge of synthesis and accumulation of key products (in red font) within key organelles in *P. tricornutum,* resulting from investigations in recent years. Relevant enzymes or proteins are in green font. Black arrows indicate synthesis routes and red arrows indicate degradation routes. Grey arrows indicate hypothesized routes. Dotted arrows indicate transfer between organelles; DMAP – Dimethylalyl diphosphate; IPP – Isopentyl diphosphate; G3P – Glyceraldehyde-3-phosphate; 3-PGA- 3-phosphoglycerate; FFA – Free fatty acid; PEP – Phosphoenol pyruvate; OAA – Oxaloacetate; MGDG – Monogalactosyl diacylglycerol; DGDG – Digalactosyl diacylglycerol: SQDG – Sulphoquinovosyl diacylglycerol; PA - ; DAG – diacylglycerol; TAG - triacylglycerol; GPAT - Glycerol-3-phosphate acyltransferase; AGPAT -1-acyl-glycerol-3-phosphate acyltransferase; ; PDAT – Phospholipid diacylglycerol acyltransferase; DGAT – Diacylglycerol acyltransferase;

TGS1 – 1,6-β-transglycosylase; PGM - Phosphoglucomutase; UGPase – UDP glucose pyrophosphorylase; ME1 – Malic enzyme 1; PEPCK – Phosphoenolpyruvate carboxykinase; PSY – Phytoene synthase; CA – carbonic anhydrase.

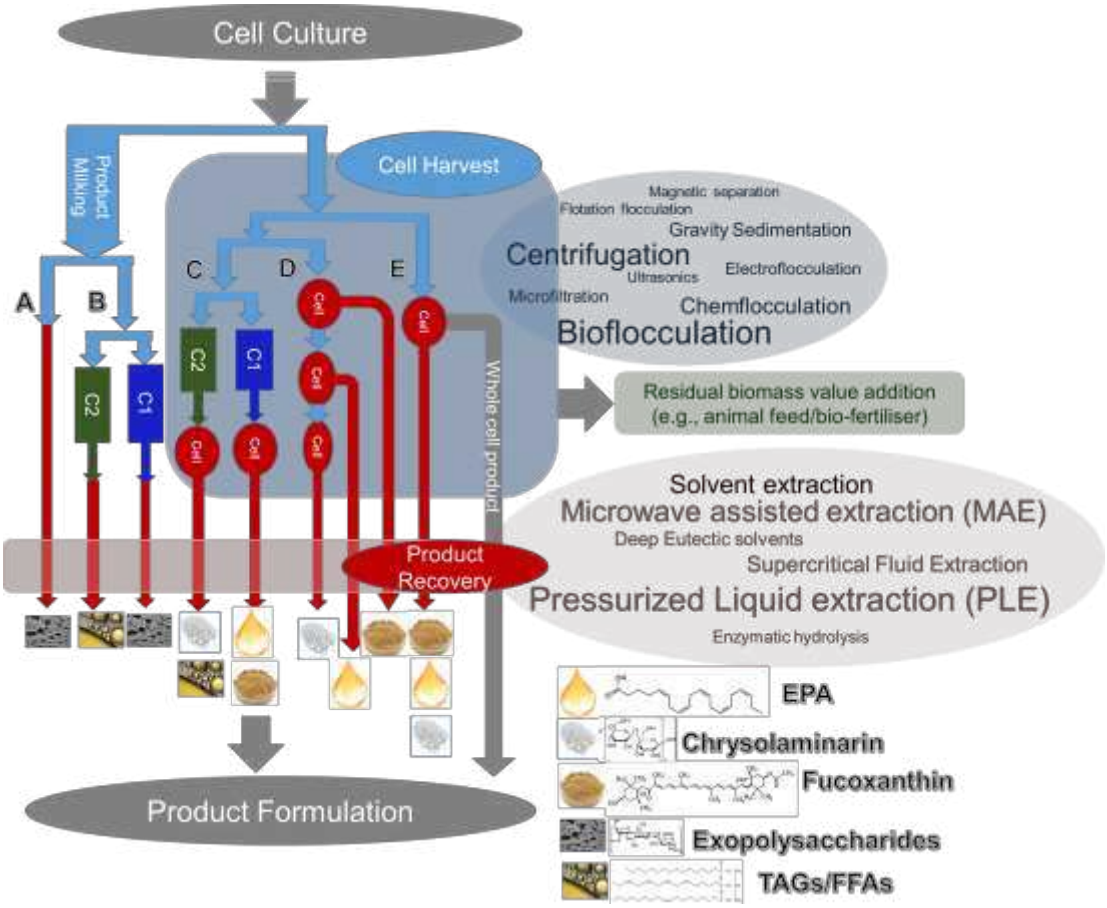

Figure 2: Options for the development of a biorefinery approach with *P. tricornutum* cultivations. (A) Direct milking of products from culture; (B) Alternating product centered operations (C1 and C2 are different operational conditions), followed by direct milking of products; (C) Alternating product centered operation (C1 and C2 are different operational conditions), followed by cell harvest and product recovery; (D) Sequential extraction of products following temporally separated harvesting of cells; (E) Simultaneous or sequential recovery of products following cell harvest (for e.g., [13]). A and B do not involve separation of cells from the culture, whilst C, D and E involve cell harvesting. Five products (EPA, chrysolaminarin, fucoxanthin, exopolysaccharides and triacylglycerides (TAGs) or free fatty acids

(FFAs)) are shown as example cases. C1 and C2 could be N replete, low light conditions and N deplete, high light conditions, respectively, as exemplar conditions for the chosen products in option C. Combination of options could also be envisaged. The key downstream processing stages of cell harvest and product recovery (extractions) from the cells that have been explored for *P. tricornutum* are highlighted on the right. Sizing of the letters indicates relative effectiveness (larger size – more effective) considering factors including, performance, suitability to commercial application, cost effectiveness, toxicity, etc. Green extraction techniques are highlighted in green. A preferred environmentally sustainable option could be bioflocculation followed by pressurized liquid extraction of products. Products accumulated in whole cells could also be marketed. The residual biomass can be used to generate additional value chains, for e.g., as animal feed or bio-fertiliser.

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