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Genetic manipulation of microalgae for the production of bioproducts

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1. ABSTRACT

Microalgae have been used during the past four decades in the Bio-industries for the production of high added value products and development of useful approaches with environmental applications. The fast growing rate, simple growth requirements and using sunlight as the major source of energy are the key factors for usage of algae. In the past 15 years, a considerable progress has been made regarding the use of microalgae for production of proteins, nutraceuticals, food supplements, molecular tags for diagnostics and fixation of greenhouse gases. Nevertheless, genetic manipulation of microalgae still remains a fairly un-explored area which could boost the production of bioproducts. It is anticipated that in the near future use of microalgae will revolutionize its applications in diverse industries. The aim of this work is to present a critical review on potential of microalgae for the production of high-added value molecules, their practical applications, and the role of genetic engineering in its utilization as a unique niche in industry. In addition, current challenges within synthetic biology approaches are discussed.

2. INTRODUCTION

During the past five decades, biotechnology-derived researches have caused a deep impact in human society and natural habitats, generating novel products that aid current worldwide needs. Nowadays, one of the most active research and development areas is synthetic biology and its novel advances for production of high-added value products (e.g. heterologous proteins, metabolites, among others). The conception of recombinant proteins production employing the scientific bases of Stanley Cohen and Herbert Boyer was commercially

achieved by Genentech and Eli Lilly in 1982 when the first licensed recombinant pharmaceutical drug, insulin, was presented in the market (1). Being this the first step in the scientific and commercial path of biopharmaceuticals, an important niche was exposed which today offers more than 300 bioactive molecules with market sales exceeding $100 billion USD (2, 3).

Therapeutic proteins are responsible for the highest market shares of sales worldwide (20%) followed by specific growth factors and hormones (15%) (4). Nevertheless, current studies and trends position alternative high-added value products that also exert important bioactivity in living organisms or marked industrial potential such as nutraceutical metabolites (5), enzymes (6), virus-like particles (VLPs) (7), phycobiliproteins (8), biomass for biofuel production (9), biomaterials (e.g. silica particles) (10), fatty acids (11) and bioactive peptides (12). Even though advanced screening techniques have been developed for product characterization and mass production, one of the most important and still recurrent topics is the adequate selection of the expression system when a novel product or strategy is to be designed.

The most common biotechnological production platforms employ bacteria and yeast cells, and specific higher applications require filamentous fungi and more complex eukaryotic cells (e.g. CHO cells) (13). If a process is designed with scale-up feasibility, economic and qualitative aspects play a vital role in selection of the expression system since inexpensive culture media and efficient production/ recovery yields are strictly desired. A complete comparison of expression systems employed in the biotech industry today was recently presented elsewhere (13). Bacterial and yeast systems are the current workhorses in important segments of the industry since they require short production times, economically available media components and provide a wide array of GRAS strains. Nevertheless, codon bias, poor gene expression of specific higher molecules or metabolites and the lack of post-translational modifications motivate R&D departments and research groups to optimize production through employment of higher eukaryotic expression systems such as filamentous fungi, insect cells or mammalian cells. The latter provide excellent refolding and post-translational modifications for heterologous proteins which can improve a bioprocess in terms of quality assurance (i.e. product with adequate 3D structure and activity). The downside of these technologies is the complexity of the unit operations to maintain quality and low contamination levels, as well as the heavy economic burden imposed to the bioprocess at hand (i.e. culture media, disposable operations).

Considering this scenario, alternative expression systems that allow economic bioprocess scale-up and still offer molecular advantages such as filamentous fungi or higher eukaryotic cells, must be studied and further employed. In this context, microalgae pose an excellent starting point for novel bioprocesses in research and commercial applications for the mass production of high-added value compounds. Microalgae are photosynthetic microorganisms that inhabit all the ecosystems in the planet and can grow under harsh conditions due to their compact unicellular or multicellular structures (14). In addition, important advantages have been described for bioprocess engineering approaches such as easy culturing techniques involved, culture media is inexpensive since they can use water unsuitable for human consumption, nutrient requirement is relatively simple and few physical space is needed for biomass propagation (15). Since cell growth is based on photosynthesis, these systems can complete growth cycles in a couple of days with sunlight as the primary energy source, increasing biomass concentrations significantly with little effort and capital investments.

The last 15 years have witnessed an increase in research papers and patents regarding microalgae use for application of cell biomass to capture greenhouse gases (e.g. CO_2) and biofuels development (16). In addition, considering the metabolism and structure of the near 30,000 microalgae species known today (17), a vaster array of biomolecules can be offered to the biotech industry in the form of phycobiliproteins (e.g. B-Phycoerthrin, C-Phycocyanin) for molecular diagnostics (18), polyunsaturated fatty acids with nutraceutical applications validated by the Food and Drug Administration (FDA-United States of America) (e.g. Docosahexaenoic acid {DHA} and Eicosapentaenoic acid {EPA}) (19), carotenoids with important antioxidant activity (e.g. beta-carotene, astaxanthin) (20), drug-delivery silica particles (10), among others.

Albeit the marked potential of microalgae in the industry today, research experts have established that microalgae expression systems could provide a broader set of biotech-derived solutions to society if they are positively enhanced through molecular biology and genetic engineering techniques. León-Bañares *et al.* (21) were the first to thoroughly describe the genetic tools used 13 years ago to develop transgenic microalgae strains to enhance production of heterologous proteins. Nevertheless, new strategies, protocols and species have been engineered in the past decade, thus uncovering a stronger research and commercial niche for biotechnological applications which today remains poorly explored. This review paper aims to discuss the most recent genetic manipulation techniques of microalgae, considering the genetic tools and protocols involved, as well as the bioproducts or applications that are currently obtained with these techniques. In addition, perspectives and

future challenges of employing genetically modified microalgae in the biotechnology industry are presented.

3. SELECTED APPLICATIONS AND HIGH VALUE PRODUCTS EXPRESSED IN MICROALGAE

Microalgae were first employed as research models of photosynthesis, and traditionally, they have been employed as food source for humans, animals and aquatic organisms (22). Recently they have been exploited as cell factories, mainly because of their easy culturing techniques in a wide variety of conditions. In this respect, microalgae are grown under the biorefinery concept, which has the objective of isolating and recovering the major number of compounds from one batch of biomass (23). Products of particular interest such as lipids and proteins are employed to produce biofuels. However, there is a high quantity of industrially-important secondary metabolites that are generated from this biomass such as pigments, vitamins, antioxidants, among others. These products, have increased the repertoire of high valued molecules obtained from microalgae, generating incentives for many industries such as food supplements, cosmetics, health promoters, animal feeds and biofuels to cultivate and obtain these molecules from photosynthetic species. This section aims to present a non-exhaustive list of uses and selected products from microalgae to give an example of the vast array of biomolecules and products that can be obtained from microalgae.

3.1. Microalgae as source for functional ingredients

Microalgae pose relatively high growth rates and biomass productivity and are composed of a varying amount of lipids, polysaccharides, antioxidants, vitamins, and minerals beneficial for health. In this tenor, some characterized species are currently being used as foods or sources of functional ingredients (24-26). Microalgae are also an indispensable diet component of many aquatic species (27). It has been proposed that the future use of microalgae biomass in the food industry will be as a source of nutraceuticals for functional foods rather than the direct use of such biomass (28).

The main interest of microalgal lipids are the contained essential fatty acids absent from food crops that exhibit beneficial effects in human health. In addition, these molecules are the feedstock for biodiesel production in the presence of a catalyst and an alcohol. Today the main focus of the microalgal biofuel research is given in the area of optimization of conditions for the production of lipid-rich microalga biomass (23). There is an increasing market demand for the long-chain polyunsaturated fatty acids (LC-PUFAs) of omega-3 and omega-6 families due to their diverse health-beneficial effects (29, 30). The global demand of the omega-3, eicosapentaenoic (EPA) and docosahexanoic (DHA), fatty acids is increasing and competing with the aquaculture industry. Microalgae are a promising alternative source for these valuable resources. They are highly productive and a proven source of omega-3 fatty acids, and therefore regarded as the most promising and sustainable alternative to EPA and DHA-fish oil in the future (31). Microalgae are a renewable source of omega-6 LC-PUFA dihomo-γ-linolenic acid (GLA), which is the immediate precursor of arachidonic acid (AA). It offers potential for the treatment of many disorders such as atopic eczema, psoriasis, asthma, atherosclerosis, arthritis, and cancer (32-35). There is an increasing amount of efforts focusing on the construction of microalgae strains able to grow fast and synthesize large amounts of lipids with a suitable fatty acid composition, focusing on the construction of strains with an enhanced photosynthetic efficiency (36).

On the other hand, microalgae proteins provide higher nutrition quality comparable to other referenced food proteins, due to their good amino acid composition. However, total protein decreases significantly when lipids or carbohydrates are accumulated due to stress conditions and their expression pattern changes under nutrient starvation. Besides, conventional protein purification methods are time and cost consuming, situation that affects protein stability and activity, raising the question whether microalgal proteins are appropriate to be used as foods (23). Though, one specific type of proteins that has gained scientific and research interest in the past 5 years are antifreeze proteins (AFP). These molecules have a binding ability towards ice crystals, enabling organisms to survive in subfreezing environments. They were first found in fish, but have also been observed in many plants, insects and bacteria that inhabit cold locations. In microalgae, AFP have shown to cope with the environmental adaptation of diatoms, expressed by genes regulated in response to stress conditions (37, 38). These algae proteins have been produced in recombinant organisms or collected from spent growth medium derived from extracellular secretion of selected organisms (38, 39). AFP from microalgae has been recently directed towards their application as cryoprotective agents in the medical field and food industry (38).

Another important field within microalgae research and potential are biological pigments. The color of microalgae is owed to chemicals that are part of their photosynthetic system. These pigments, typically proteins or carotenoids, have been employed as natural colorants for food and cosmetics, as well as diagnostic probes for advanced molecular screening tests (40). Carotenoids are a group of molecules that play a significant role in the photosynthetic pathway of microalgae as they allow the organism to harvest

light in several regions of the visible spectrum where chlorophyll does not. Since these hydrophobic molecules have the capacity to hinder the formation of reactive oxygen species (ROS), they are excellent antioxidants that pose interesting opportunities for product development as nutraceutical supplements (41). Increased attention has been given to beta-carotene, astaxanthin and lutein. Some microalgae species produce up to 100-fold the amount of beta-carotene in carrots, situation that has been addressed by several mid-size biotech-derived companies to industrially produce this high-value molecule (41). Astaxanthin is also an important carotenoid obtained from diverse micro and macroalgae species but current challenges are focused in the development of cost-effective and scalable technologies for the efficient primary recovery and purification of this compound with greener technologies (41, 42).

In addition to low molecular weight pigments, phycobiliproteins constitute one of the highlights of microalgae characterized worldwide. These proteins are considered accessory molecules that aid the photosynthetic system of host cells by efficiently capturing light in specific regions of the electromagnetic spectrum and thus promote growth. There are four well studied classes of these proteins: allophycocyanin, phycocyanin, phycoerythrin and phycoerythrocyanin. In the past 10 years, numerous studies using *Spirulina maxima, Spirulina platensis, Porphyridium cruentum* and *Porphyridium purpureum* have presented diverse strategies to maximize biomass yield, efficient culturing techniques and optimization of purification techniques (43, 44). Phycoerythrins constitute some of the most used tags in molecular diagnostics testing, allowing the detection of low concentration metabolites or analytes of interest. In its purest form (> 4, $P = Abs_{545}/Abs_{280}$), B-phycoerythrin has commercial values up to $50 USD/mg and if properly functionalized with antibodies or gold nanoparticles for target capture and detection, price tags up to $300 USD/mg are available (18). Nevertheless, the major challenges discussed by authors include the low production yields of such molecules in addition to complex purification trains that normally increase manufacturing costs of large scale processes.

3.2. Microalgae in the energy and environmental sectors

The energetic and environmental sectors have been deeply studied for the past 15 years in terms of microalgae characterization and growth processes optimization. Since some microalgae have high growth rates, high liquid accumulation and no land requirement, they can be employed for nitrogen and carbon fixation, as well as phosphorous removal from wastewater, and production of high end biofuels (45). The production of biogas is one of the main applications of microalgae, since biomass waste after lipid extraction can be fermented and hydrolyzed to produce biomethane and carbon dioxide through methanogenesis. Bioreactor design, hydraulic retention time and temperature are operational conditions that affect the production of this biofuel, as well as the culture conditions which impact the total amount of residual components of the biomass and biogas produced (46, 47).

In addition, two of the most important biofuels produced nowadays are biodiesel and bioethanol. It has been determined that certain microalgae species can present up to 25 times more biomass conversion efficiency to biodiesel production when compared to conventional energy crops. These microorganisms are considered a third-generation feedstock and success in biodiesel production and commercialization depends on low-cost cultivation systems, efficient biomass harvesting, separation methods, suitable oil extraction techniques and efficiency of microalgal biomass production, highly influenced by environmental conditions such as light, temperature, CO_2 concentration, nutrient composition, salinities, and mixing conditions (48). In terms of bioethanol production, the absence of lignin in microalgae also makes the fermentation process much easier when compared with those used with lignocellulosic biomass (49).

3.3. Bioactive compounds and the importance of microalgae genetic modification

In addition to biomass components (e.g. lipids, carbohydrates, proteins), microalgae are recognized as excellent producers of secondary metabolites with important bioactivity in health applications. Antioxidant, anti-inflammatory, anti-diabetic, anti-proliferative, antibacterial, anti-tumor, antiviral, antiparasitic, anticoagulant and hypoglycemic effects have been described and documented (50). Even though primary uses of microalgae in the past 20 years have been focused on food/biofuels/bioenergy (Figure 1), an important trend towards the use of bioactive molecules as active components for healthcare in the pharmaceutical sectors is arising.

Considering the vast potential of microalgae to produce high-added value molecules (Figure 1), the importance of these microorganisms as cellular factories is imminent. The microalgae biorefinery concept constitutes an integration of the many uses these microorganisms could provide in sectors such as energy, human and animal nutraceuticals, pharmaceuticals, health, beauty, and others (51). Freshwater, fertilizer, harvesting and extraction costs, are challenges to scale-up microalgae biorefinery technologies at commercial scale applications (52, 53). In order to make more efficient the use of microalgae, most manipulation techniques involve adaptation strategies (e.g. application of stress conditions on light

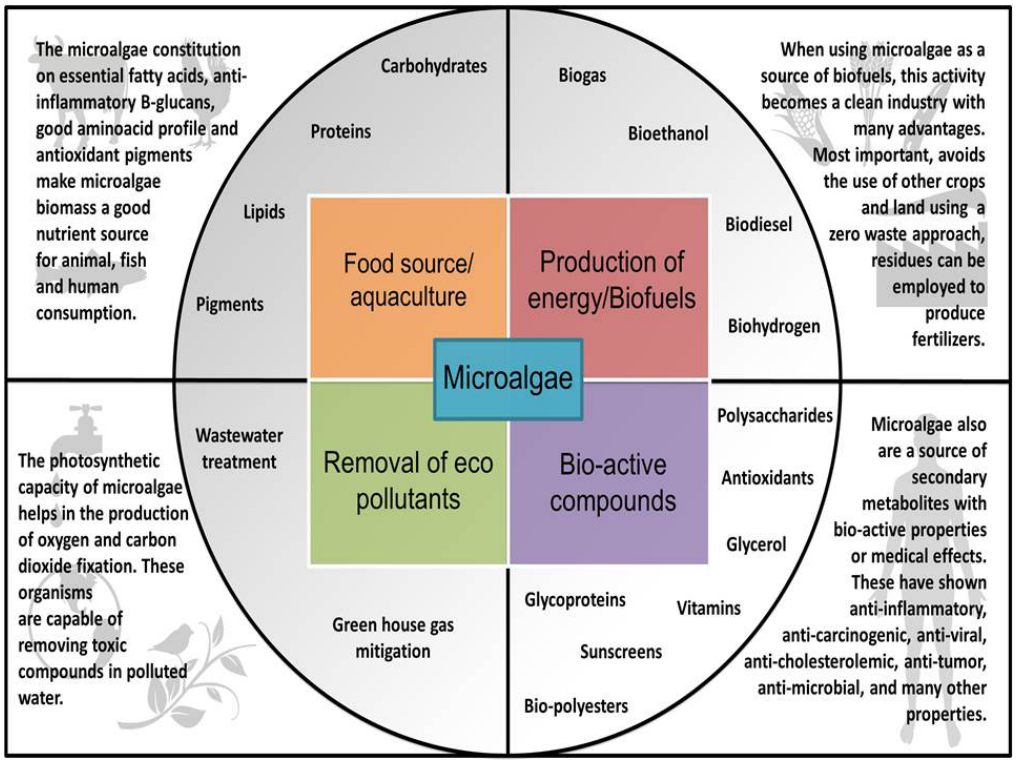

Figure 1. Net of high value products expressed in microalgae, including its usefulness in pollution control and biofuel production. Genetic engineering of microalgae strains favors the understanding of photosynthetic and other phenomena associated with the cost-effective use of microalgae in a biorefinery concept.

intensity, temperature, osmotic conditions, pH, nutrient limitations, cell density and age, among others), which in addition involve time, costs and effort. The ultimate goal of a microalgae-based bioprocess nowadays consists in reaching a desired productivity of bioactive compounds and biomass with a green or eco-friendly approach (54).

A cost-effective strategy for moving forward algal-based production technologies is its genetic manipulation. By studying and incorporating improved genes of a desired product, scientists will have to overcome the intracellular activities that hamper the broad industrial employment of microalgae as cell-factories. (54, 55). Understanding their complex metabolic pathways, together with genome data, the development of transformation protocols and genetic engineering to enhance natural and novel high-value products (e,g, antibodies, hormones, vaccines, and insecticidal proteins), are desired to use microalgae at economically viable levels (56).

Microalgae genetic manipulation could offer many advantages to the biotech industry. At a first glance, microalgae could be designed as a tool for synthetic biology and biotechnology. Important advantages for recombinant microalgae species can be envisioned. Selected species could present increased productivity with novel cultivation protocols, harvesting and downstream processing could be greatly enhanced, reduced energy inputs, increased stability and minimized extraction costs. Even the emergence of newly sequenced genomes might facilitate the application of different high-throughput approaches, like genomics, proteomics and metabolomics, for the identification of new genes and pathways that could serve as targets for genetic modification (56, 57).

Nowadays, the research on genetic engineering of microalgae is in route to expand the knowledge base for transgenic designs of photosynthetic organisms. The development of a single microalgae 'super-strain' to maximize production process profitability and the identification/isolation of prospective strains for successful commercialization of bioproducts are the main goals of microalgae strain research. In addition, genetically modified strains will provide additional avenues of sustainable microalgal-based products at competitive production costs and environmentally validated production processes (51, 53, 57, 58). The following section aims to describe the current state of the art regarding genetic-manipulation techniques of microalgae species and thus provide the current background and evolution of these protocols in the past 10-15 years in order to determine current success, challenges and trends.

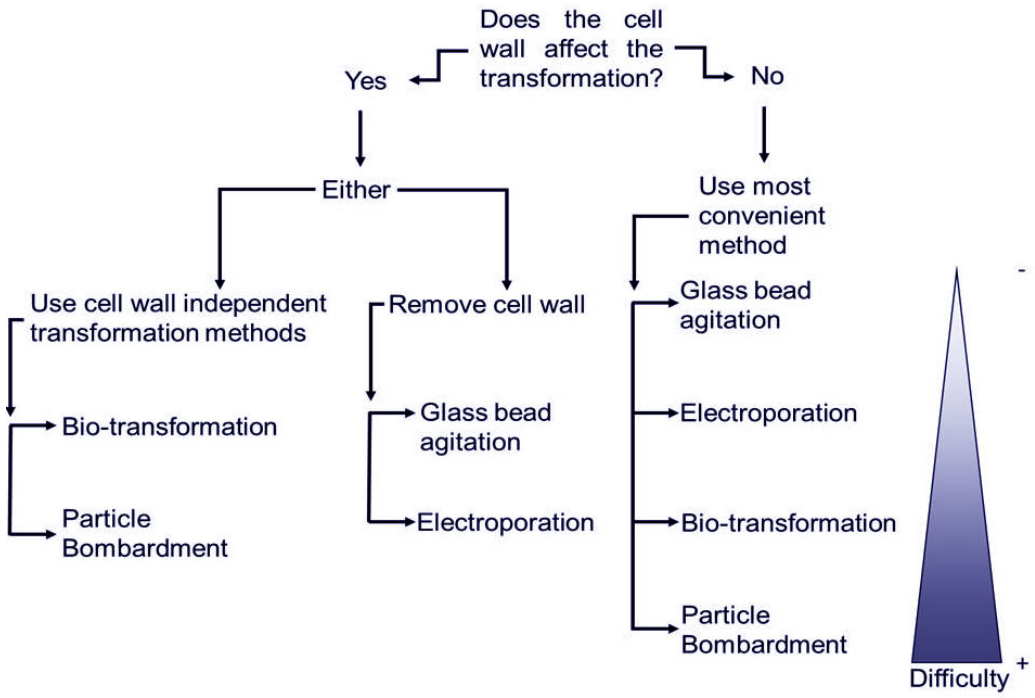

Figure 2. Microalgae transformation flow chart. If the cell wall affects the transformation outcome, it should be considered to be either removed or the use of a cell wall independent transformation method like Bio-transformation and Particle bombardment. If the cell wall is removed, Glass bead agitation or electroporation can be used. If the cell wall is not an issue any protocol can be used, the most simple method is glass bead agitation, followed by electroporation, bio-transformation and particle bombardment.

4. TRANSFORMATION METHODS FOR MICROALGAE SPECIES: CURRENT STRATEGIES

Methods for generation of recombinant microalgae cells have been developed in the past 15 years. Numerous challenges and novel protocols have been described to successfully transform these complex organisms and achieve high yield of bioproducts.

4.1. Cell walls in algae transformation

Cell walls of microalgae are rigid structures with a very diverse composition. Green algae walls are composed of polysaccharides, mainly cellulose that can be coated with glycoproteins arranged in one or multiple layers, scales, or fibrils (59). Red algae walls are composed of galactan heteropolymers of xylose, glucose, and galactose that contain sulfate residues (60). Diatoms have rigid silica cell walls called frustules (61). The cell walls are an extra barrier for transforming-DNA to reach its target within the cell. In some cases, it should be partially dissolved to facilitate transformation. Depending on the cell wall need to be removed or not the transformation method of choice can vary (Figure 2).

In *Chlamydomonas reinhardtii*, the cell wall can be removed by the addition of supernatant from mating cells in which the cell wall is enzymatically degraded to facilitate cell fusion (62). Cell wall deficient mutants have also been isolated, however these strains have a low survival in agar plates (63, 64). Commercially available enzymes can also be used to degrade cell walls prior to transformation. Combinations of cellulases, chitinase, driselase, hemicellulose, lysozyme and macerase have been used in *Chlorella* spp. and *Nannochloropsis* spp. (65-67).

4.2. Transformation methods

A method that requires little investment in equipment to transform microalgae consists of mixing cells with glass beads of typically 0.5 mm in diameter, and vortexing the mixture. *C. reinhardtii*, *Dunaliella salina*, *Tetraselmis sp*. are among microalgae with reported protocols for transformation with this method (68-70). Cell wall-deficient strains such as *C. reinhardtii* mutants or cells that do not possess a thick cell wall like *D. salina* have higher efficiencies of transformation than their wt-cell wall or thick cell wall relatives (71).

Electroporation is an efficient method to deliver DNA into microalgae cells. In *C. reinhardtii* cell wall deficient mutants transformed by this method had a 9 to 163 fold increase in transformation efficiency compared to glass-bead transformation (63). In some microalgae like *Monoraphidium neglectum* a

cell wall-weakening pretreatment with lithium acetate and dithiothreitol made the cells more efficient to transformation by electroporation than untreated cells (72). Whereas other algae like *Chlorella vulgaris, Neochloris oleoabundans, Nannochloropsis spp.* and *Phaeodactylum tricornutum* were transformed with no pretreatment (73-77).

Transformation by the assistance of bacteria such as *Agrobacterium tumefaciens* does not require cell wall removal. Diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* can be transformed by *Escherichia coli* mediated conjugation (78, 79). Marine microalgae species have also been transformed by *A. tumefaciens*. In species like *Dunaliella salina*, *Dunaliella bardawil*, *Parachlorella kessleri*, are among microalgae with reported protocols for Agrotransformation (80-82). When marine algae are *Agro*-transformed, a reduction of the growth medium salinity to 1-1.2% (w/v) allows the co-culture of *A. tumefaciens* with marine microalgae, increasing the salinity to >5% after the transformation eliminates the bacteria from the culture which is desired after the transformation (80, 81).

As with Agrobacterium mediated transformation, particle bombardment (also known as biolistics) can be used to transform microalgae cells with their native cell wall present. Variables affecting the efficiency of this method are the particle type, rupture disc pressure and the bombardment distance between the gun and cells. For microalgae like *Cyclotella críptica*, *Fistulifera* spp., only this transformation method is reported (83-85). Even though this method is efficient in overcoming microalgae cell walls, it is limited by the high capital cost of the equipment and the dedicated setting required for its use. If the chloroplast is to be transformed, this method is more efficient than electroporation and glass-bead agitation (71).

Although most microalgae have been transformed with the discussed methods, novel protocols are necessary for a robust generation of transformants, especially in algae that require cell wall removal. Among non-traditional methods, inexpensive and non-toxic positively charged nanoparticles and cell penetrating peptides have been used to transform *C. reinhardtii* (86, 87). A promising method to transform microalgae is with the use of shock-waves, as fungi with cell walls have been transformed with this method (88).

4.3. Selection of transformants

After foreign DNA is internalized into microalgae, transformed cells are selected usually by a positive selection where transformed cells grow and un-transformed cells do not. The gene expressed confers such growth or survival advantage is termed a selectable marker.

Recessive selection markers require a previous mutation or gene knockout to function. In general, auxotrophic mutants are transformed with the previously knocked-out or mutated gene. The main advantage is that no transgene is introduced into the microalgae and no chemical is required. In *C. reinhardtii*, markers used include the *ARG7*, *NIT1*, *NIC7* and *THI10* encoding argininosuccinate lyase, nitrate reductase, quinolinate synthetase and hydroxyethylthiazole kinase respectively (89-91).

Other than *C. reinhardtii*, there are few reports of auxotrophic selection in microalgae. In *Pseudochoricystis ellipsoidea* and *Phaeodactylum tricornutum* the *UMPS* gene coding uridine monophosphate synthetase and the nitrate reductase (*NR*) gene in *Chlorella vulgaris and Dunaliella viridis* are examples of selection markers used (92-95). The main disadvantage of auxotrophic selection is the need to generate the mutant, which is time consuming and may produce deleterious mutations.

Dominant selectable markers can potentially be used in any genetic context such as antibiotic and herbicide resistance genes. In *C. reinhardtii*, the *AADA*, *APHA-6*, *APH7*, *APHVIII*, *CAT*, *BLE, GAT, PDS*, *PROTOX* rs-3, and *TETX* conferring spectinomycin, kanamycin, hygromycin B, paromomycin, chloramphenicol, zeocin, oxyfluorfen, glyphosate, norflurazon and tetracycline resistance respectively (96–104). Similar to *C. reinhardtii,* species of the genus *Chlorella* have been selected with many of the same antibiotic resistance genes, including the *ble*, *kanR*, *hpt*, *nptII,* PDS and *CAT* genes (76, 105–109). Resistance markers (Table 1) reported for fresh water species include the *PDS* gene for the astaxanthin producing *Haematococcus pluvialis,* and the *APHVIII* for the hydrocarbon-producing *Botryococcus braunii* (110, 111).

Selection of marine microalgae transformants have an additional challenge compared to fresh-water species. High NaCl concentrations can reduce the effect of some antibiotics such as G418 and Hygromycin B. Lowering the NaCl concentration in selective plates allows for reduced concentration of antibiotics used in selection (81, 83). However, some antibiotics such as streptomycin and kanamycin, cannot be used for selection even in low salt concentrations (112–115). For Marine microalgae species, selectable markers/ resistance genes (Table 1) are *BAR*/phosphinothricin, *BLE*/zeocin, *CAT*/chloramphenicol, *PDS*(L504R)/ norflurazon, *NAT*/nourseothricin (70, 74, 116-118).

Methods and requirements for microalgae transformation are as diverse as the own algae. Even though the cell wall might compromise genetic transformation, enzymatic cell wall digestion and cell wall independent transformation methods are

Table 1. Dominant selection markers used in microalgae transformation

Selection marker/resistance	Usage[1]	Resistant Species[1]	References
AADA/spectinomycin, streptomycin	F	Ds, Dt	96, 113
APHA-6, KANR, NPTII /kanamycin, G418	F,M	Ds	83, 93, 103, 113
APH7, NPT/hygromycin B	F,M	Dt, Dv	80, 81, 82, 87, 98, 113
APHVIII/ paromomycin	F	Ds, Dt, Dv	97, 113
CAT/ chloramphenicol	F, M	ND	76,77, 113
BAR/phosphinothricin	M	Ds, Dt, Dv	70, 113, 114
BLE/zeocin, Phleomycin	F, M	ND	78, 99, 100
GAT/oxyfluorfen	F	ND	104
NAT/nourseothricin	M	ND	78
PDS/glyphosate	F	Cr	104, 110
protox rs-3/norflurazon	F	ND	104, 109
tetX/ tetracycline	F	ND	101

[1]Selection marker/ resistance genes and their use in freshwater (F) or marine (M) species. Examples of species that are resistant to the antibiotics *Chlamydomonas reinhardtii* (Cr), *Dunaliella salina* (Ds), *Dunaliella tertiolecta* (Dt), *Dunaliella viridis* (Dv) Not determined ND.

readily used. The choice of which selection marker to incorporate in the transformation will most likely be linked to the microalgae habitat. Regardless, there are selection markers that can be used for both fresh and marine species. The current biotechnological era is in a current constant growth in which industrially important microalgae are transformed and used to produce industrially relevant products. Although our current understanding of maximizing metabolic fluxes and transgene expression is growing, there are promising examples of genetically modified microalgae that could scale up to commercial application as presented in the following section.

5. SUCCESSFUL GENETIC MODIFICATION OF MICROALGAE

As presented before, genetic manipulation of microalgae is complicated and numerous factors need to be considered for a successful transformation protocol. The genetic toolbox is limited among different microalgae species. This has become one of the major drawbacks to exploit these organisms as a platform for the major, industrial scale, production of recombinant proteins or secondary metabolites. There are plenty techniques for the transformation of different organelles (nucleus, mitochondria or chloroplasts), each of them with several advantages and disadvantages. Also, each species has a different codon bias, even between their own organelles.

There are several reports of successful expression recombinant proteins or enhanced production of secondary metabolites in microalgae, however, there are some cases where a higher production has been achieved despite the already explained complications. This section of the present review will focus on the discussion of these success cases. The studies analyzed here will be divided in two sections; the first will cover recombinant protein production, while the second discusses metabolic engineering efforts for different applications (lipid and carotenoid production, generation of hydrogen gas and carbon dioxide fixation).

5.1. Recombinant protein production

Recombinant protein productivity is usually measured as a percentage from total soluble protein content (TSP). The proteins expressed in microalgae have different potential applications, as they can be used in protein therapies or vaccines (antigens or viral particles/fragments).

All of the studies analyzed have taken a couple of factors into account before any transformation takes place. The methodology to be followed changes depending on which organelle is going to be transformed. During the design of the transgene, codon frequency analysis is critical as expression in the chloroplasts has a strict codon frequency.

As an initial approach (119), *C. reinhardtii* was transformed to produce the foot-and-mouth disease virus VP1 protein fused with Cholera toxin B. The transgene contained the atpA promoter and the rbcL terminator, along the selection marker gene *AADA* for granting resistance to spectinomycin. The transformation method was through biolistics, using gold particles and incorporated into the chloroplast genome through homologous recombination using the chlL sequence. This combination resulted in the accumulation of up to 3% of TSP.

This strategy was followed by a series of studies that achieved a high level production.

Allophycocyanin A and B were produced using the same genetic toolbox and achieved a production between 2% and 3% of TSP (120). The structural protein E2 from the classical swine fever virus accumulated up to 2% of TSP (121). Both recombinant proteins were produced on *C. reinhardtii*.

The production of the bovine mammary-associated serum amyloid (M-SAA) on *C. reinhardtii* helped to develop an improved strategy for protein production (122). The first step was to test production using the psbD promoter in a wild type strain of *C. reinhardtii* which resulted in a production of 0.25% TSP. Afterwards, the expression of M-SAA using psbA promoter was analyzed. To increase production, the native *PSBA* gene was replaced by the *M-SAA* gene during homologous recombination. This resulted in an increase to 3% of TSP, suggesting the presence of a stronger promoter but also that a promoter, in this case the psbA promoter will rather work to produce proteins involved in the metabolism of the microalgae, when the *PSBA* gene was replaced the promoter preserved its intense activity but now for the production of M-SAA.

The main disadvantage of this strategy is the loss of photosynthetic activity of the cells. To compensate this the cells producing M-SAA were transformed with a transgene for the recovery of the *PSBA* gene driven by the psbD or psbA promoter. The microalgae that was regenerated containing the psbA promoter stopped producing any detectable M-SAA, strengthening the hypothesis already discussed. The cells that contained the *PSBA* gene driven by the psbD promoter increased the M-SAA production to 12.5% TSP. Suggesting that the psbA promoter can drive a strong expression but when it is not required for the production of other proteins (like the *PSBA* gene). Also, photosynthetic capacity is required to maximize expression, but enhances expression when it does not interfere with the synthesis of the product of interest.

Another study analyzed the effect of protein degradation mechanisms and antibiotic resistance (123). The authors discovered that protein accumulation is a result of an equilibrium between protein synthesis and its degradation. Proteases are powered by ATP, suggesting a possible silencing of this family of proteins. Furthermore, it is discussed that genetic transformation can change the phenotype of the target cell and change its metabolisms, particularly, the protein degradation was analyzed and resulted in a change in the degradation rate. This can also possibly occur by the effect of antibiotic resistance. It is suggested that hyper-resistant strains can produce more product of interest as the genetic region becomes more active. This has been seen on other organisms, like *Pichia pastoris*, to force multicopy transformants to achieve a higher production yield (124). Surzycki's study produced the protein VP28 of the white spot virus as a case study and achieved a production of 20.9% TSP by applying the previous strategies in addition to novel protocols developed and published in their research.

A more recent improvement to recombinant protein production is to fusion hard-to-express proteins to others that have yielded high titers (125). A successful case was that presented by Manuell *et al.,* which also confirmed that psbA is a stronger promoter compared to atpA (122).

As a summary, to maximize production of recombinant proteins in microalgae the strategies here presented should be considered. *C. reinhardtii* is the current workhorse strain of microalgae for recombinant production, particularly in its chloroplast, since it does not present a known silencing mechanism. First, a *PSBA*-deficient strain should be considered, this to maximize the force of the psbA promoter, but also should include the *PSBA* gene under the control of another promoter, such as psbD or atpA. Second, the protein of interest could be fused with a well-expressed protein, this could help to improve production by stabilizing the protein of interest. Additionally, hyper resistant strains could ensure increased protein production by activating genetic activation of the region where the transgene is located.

5.2. Genetic modifications for metabolic engineering

Microalgae are known to produce diverse secondary metabolites that can have potential applications in different areas. They are known to produce lipids, compounds in the family of carotenoids, sterols and phycobiliproteins (phycocyanin, phycoerythrin and allophycocyanin), among others (126). They are also known to perform beneficial metabolism toward the environment, like carbon dioxide fixation and hydrogen gas production, which can be harvested for its use as a fuel (127).

The production of secondary metabolites can be improved through genetic modification of the microalgae, although not having a direct effect as in recombinant protein production, still will boost metabolic pathways to intensify their productivity. In contrast with the previous section where a certain protein of interest is desired, the modification of metabolic pathways can have several factors affecting the final result. For example, even if a certain enzyme is overproduced, if there is not enough substrate the metabolic flux will remain constant. The strategy to be followed is also different depending which metabolic pathway needs to be modified. This section will cover success cases of lipid production, hydrogen gas generation, improvement to carotenoids synthesis and carbon dioxide fixation.

5.2.1. Lipid production

Lipids overproduction has been achieved by disrupting the metabolic equilibrium that the microalgae has. This has been done successfully by three methodologies. The first (128) overexpressed the Malic Enzyme in *Phaeodactyllum tricornutum*, this allowed the accumulation of pyruvate and NADH, which gave substrate and reducing power to the lipogenesis. This strategy resulted in an increase of 2.5.-fold in the total lipid accumulation without affecting growth rate.

A second strategy was inhibiting the lipid catabolism therefore shifting equilibrium towards lipid synthesis (129). To achieve this, a multifunctional lipase was silenced through RNAi. This resulted in a 3.3.-fold increase of total lipid content during exponential growth phase. This strategy had no apparent adverse effect in cell growth rate, compared to wild type strains.

The third strategy worked on causing a systemic level approach by genetic engineering transcription factors (130). *Chlorella ellipsoidea* was transformed to produce a transcription factor from soybean, which granted an enhanced production of 52% of total lipid content. This caused a differential expression of up to 1046 genes, including acetyl-CoA carboxylases, which commit a substrate to fatty acids metabolism.

Other strategies have been followed, usually in the line of recombinant protein expression to improve a certain step inside a metabolic pathway, generally with poor results. The methodologies presented here achieved better results by affecting not only a certain step but the complete metabolism by giving it enough substrate, energy (silencing of the multifunctional lipase), without consuming finished product (inhibiting lipid catabolism) and triggering a genetic switch to turn on the complete pathway (inclusion of transcription factors).

5.2.2. Hydrogen gas generation

The generation of hydrogen gas is highly attractive due to the nature of H_2 as a fuel (no carbon products). Microalgae from the *Chlamydomonas*, *Scenedesmus*, *Lobochalmys* and *Chlorella* genres have the potential to produce hydrogen gas through the use of (Fe-Fe)-hydrogenases (131). Hydrogen production cannot occur during photosynthesis, as it requires anoxic conditions for the (Fe-Fe)-hydrogenase to be active.

To enhance H_2 production similar strategies as for lipid synthesis have been undertaken with mixed results. The hydrogenase has been engineered to decrease its sensitivity with oxygen (132) and overexpressed to enhance production up to 10-fold (133). Another strategy is to interfere with the photosystem II. It has been inactivated by the application of RNAi against a sulfate transporter (134).

Other techniques rely on mutating D1 of the photosystem II to induce anaerobiosis, which has allowed an increase in H_2 by 10-fold (135). Another alternative is to capture the O_2 present in the medium, for example transformation of leghemoglobin from soy (136), which resulted in a 4-fold increase.

The generation of hydrogen gas is a complicated process as improving it enters in conflict with biomass generation by reducing its photosynthetic capacities. Approaches in this area should be carefully planned as they need to create a positive equilibrium to have abundant microalgae present that generate H_2. It is possible that a combination of an overexpression of hydrogenase and decreased sensitivity to oxygen may improve overall results as they would not interfere with the growing and reproducing capabilities of the microalgae.

5.2.3. Carotenoids synthesis

Carotenoids are highly valued in the nutraceutical market and microalgae are a potential platform for their mass production due to their easy cultivation, in contrast to plants. But very little efforts have been done to improve their synthesis and few research has been made presenting mixed results. Most of the work has been done with the first two enzymes of the metabolic pathway for carotenoid synthesis: Phytoene synthase (PSY) and phytoene desaturase (PDS) (127).

Overexpression of PSY in *C. reinhardtii* has only achieved an increase 2.6-fold (with *Dunaliella salina* gen) (137) and 2.2.-fold (with *Chlorella zofingiensis*) (138) in lutein production. Another successful study overexpressed PDS in *Chlorella zofingiensis* and achieved an increase of 32.1% in total carotenoids and a 54.1% for astaxanthin (139). Other strategies, such as RNAi have not been successful.

This area can achieve more improvements if other approaches are undertaken, for example the incorporation of transcription factors for systemic level activation. Also, to provide with enough initial substrate might open up the possibility of shifting the equilibrium toward carotenoids products. Additional studies are required to understand the effect of manipulating other critical steps in the carotenoid pathway, by overexpressing or silencing every enzyme involved.

5.2.4. Carbon dioxide fixation

For microalgae cells to reproduce, grow and generate secondary metabolites carbon dioxide is

required. It becomes available through its fixation by the RuBP carboxylase/oxygenase enzyme, or Rubisco. It is a process driven by light in the photosystems I and II (140), but it is inhibited when high temperatures or when intense light are present.

Rubisco has been engineered by using Rubisco-deficient strains and transform only the small subunits (141). Carbon fixation has been improved using gene shuffling through PCR, which has improved the carboxylation reaction. Additionally, the Calvin-Benson-Bassham cycle has been modified in another step to enhance the need of carbon, therefore moving the equilibrium of Rubisco toward carbon fixation (142).

As it has been presented in this section, to successfully perform genetic modification to microalgae, it is mandatory to perform different techniques compared to bacterial transformation. To produce large amount of recombinant protein in the chloroplast, it is required to use a strong promoter that does not have another copy present in the genome. Like the psbA promoter with the psbA gene driven by the psbD promoter, while having the product of interest fused with a well-produced protein. Additionally, hyper-resistant strains are likely to produce larger amounts of protein. In contrast, for metabolic engineering strategies involving protein overexpression, gene silencing and the use of transcription factors will move the equilibrium of metabolism to generate the production of a certain secondary metabolite. Genetic modifications in microalgae are a complex area that requires further studies to systematically obtain successful results.

6. PERSPECTIVES AND CONCLUDING REMARKS

The constant progresses in the field of next generation sequencing are overcoming the previous barriers for understanding the molecular genetics and functional genomics required to direct metabolic engineering efforts in microalgae (143). Although the production of recombinant proteins is still a challenge in terms of yield, the elucidation of metabolic pathways involved in the biosynthesis and catabolism of fatty acids, triacylglycerol, and starch among other compounds of industrial interest is a promising opportunity for the development of high valued products derived from microalgae.

Currently, the genome edition of living organisms has become very common, showing high success and efficiencies in almost any system that has been tried. From these technologies, CRISPR/Cas9 is by far the most powerful and widely used due to its simplicity, specificity, high efficiency, versatility and relatively low cost. This is the case of at least three species of microalgae that have already been successfully genome edited by this approach: the oleaginous microalgae *Nannochloropsis oceánica* (144), the marine diatom *Phaeodactylum tricornutum* (145) and the single-cell algae model, *Chlamydomonas reinhardtii* (146).

Although there are aspects of the technique still that might be improved, optimized or standardized. In all the cases, CRISPR/Cas9 has proven to be a reliable technology for genome edition of microalgae. It has shown high efficiency and specificity when used either with plasmid vectors or by direct delivery of the Cas9-sgRNA ribonucleprotein complex (147). These findings expand the reverse genetics toolbox of microalgae for industrial applications and introduce numerous possibilities in the areas of systems and synthetic biology. Representing huge potential for solutions to environmental problems as finding alternatives for fossil fuels or reducing the accumulation of carbon oxides by improving microalgae metabolism for augmented carbon fixing rate. Moreover, the elucidation of specific regulatory networks might also contribute to increase the levels of recombinant production of proteins by manipulation of additional *loci* which might contribute to protein folding, translation efficiency, extracellular secretion or protein degradation among other possibilities.

Together, the CRISPR/Cas9 technology with experience learned from previous success cases can substantially increase microalgae attractiveness by overcoming previous obstacles. The creation of superproductive or knock-out strains can be specifically guided to enhance productivity by including the psbA promoter, *PSBA* gene or hyper resistance to antibiotics for recombinant protein production. For metabolic engineering, CRISPR/Cas9 can affect specific steps on a pathway, for example affecting the Malic Enzyme or the multifunctional lipase to increase lipid production. This is an advantage as there are known targets that CRISPR/Cas9 can modify, instead of starting a search for them. Microalgae have been a difficult net of organisms to work with, but now there is a great data set for their use to move forward with research and achieve the potential that has been recognized in them in the past decades.

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Abbreviations: VLP, virus-like particles; GRAS, generally recognized as safe; R&D, research and development; CHO, Chinese hamster ovary cells; FDA, food and drug administration; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; AA, arachidonic acid; GLA, dihomo linolenic acid; ROS, reactive oxygen species

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