Microalgae as expression systems for recombinant protein production

Microalgas como sistemas de expresión para la producción de proteínas recombinantes

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Keywords

Genetic transformation; genetic engineering; recombinant protein; microalgae.

Abstract

In the field of biotechnology, recombinant proteins have revolutionized many industries, including pharmaceuticals, agriculture, and bioenergy. By producing high-value proteins in heterologous hosts, cell factories may offer a more efficient, cost-effective, scalable, and environmentally friendly solution to traditional protein production and extraction methods, which can be highly laborious and resource intensive. Microalgae have emerged as attractive hosts due to their Generally Recognized as Safe (GRAS) status, versatile metabolism, genetic diversity between species, ease of cultivation and scale-up, and general cost-effectiveness. For genetic engineering, their capability for complex protein synthesis and post-translational modifications and ease of transformation in comparison with chasses outside of their category make microalgae an advantageous solution on many fronts. Microalgae can be transformed to enable efficient protein expression, most commonly in the nucleus and the chloroplast, each with their specific advantages and limitations. The present literature review compiles some of the techniques, features, and latest advances related to recombinant protein production in microalgae, exploring different genetic transformation techniques and their limitations. Recombinant protein production is only one of the many processes that can become more sustainable and efficient by using microalgae as a platform.

Palabras clave

Transformación genética; ingeniería genética; proteína recombinante; microalgas.

Resumen

En el campo de la biotecnología, las proteínas recombinantes han revolucionado muchas industrias, incluyendo la farmacéutica, la agricultura y la bioenergía. Al producir proteínas de alto valor en huéspedes heterólogos, las fábricas celulares pueden ofrecer una solución más eficiente, rentable, escalable y amigable con el ambiente, en comparación con la producción de proteínas y métodos de extracción tradicionales, que pueden exigir muchos recursos y mano de obra. Las microalgas han emergido como huéspedes atractivos debido a su estatus como organismos Generalmente Reconocidos como Seguros (GRAS), su metabolismo versátil, diversidad genética entre especies, facilidad de cultivo y escalabilidad y su rentabilidad general. Para la ingeniería genética, su capacidad para síntesis compleja de proteínas y modificaciones post-traduccionales y su relativa facilidad de transformación hacen a las microalgas una solución ventajosa en muchos aspectos. Las microalgas pueden ser transformadas para permitir la expresión eficiente de proteína, más comúnmente en el núcleo y el cloroplasto, cada uno con sus ventajas y limitaciones. La presente revisión de literatura compila algunas de las técnicas, características y últimos avances relacionados con la producción de proteínas recombinantes en microalgas, explorando diferentes técnicas de transformación genética y sus limitaciones. La producción recombinante de proteínas es solo uno de los muchos procesos que se pueden convertir más sostenibles y eficientes al usar microalgas como una plataforma.

Introduction

Prior to the advent of recombinant proteins, proteins of interest were extracted from their natural sources through expensive processes and poor yields. With the arrival and development of this technology, proteins can now be expressed in a variety of hosts, making the yields much higher and the costs much lower, thus allowing the production of these proteins on an industrial scale [1], [2]. The production of these proteins has a wide spectrum of uses in the pharmaceutical industry; recombinant approaches have been used to produce monoclonal antibodies, antigens, therapeutic proteins, and enzymes [3]. They are also implemented in the nutritional, environmental, industrial, bioenergetics, and biomaterials sectors [4].

Amongst the most popular hosts for recombinant protein expression are bacteria, yeasts, insect cell lines, plants, mammalian cell lines, and transgenic animals, each with their strengths and limitations [5]. Some of these hosts are limited by lacking the ability to generate complex molecules, their high costs, or their difficult scalability; as a result, in recent years, microalgae have emerged as an alternative with valuable capabilities to produce complex proteins due to their great genetic diversity [6], genetic modification possibilities, and ease of management, including biomedical applications in tissues [7].

Microalgae are unicellular organisms characterized by their versatile metabolism, representing efficient and economic platforms to gather organic compounds like proteins, lipids, pigments, sterols, and carbohydrates for several commercial applications such as nutraceuticals, pharmaceuticals, and biofuels [5]. They have a short doubling time when compared with other microorganisms, are inexpensive to grow due to their basic nutrient requirements and have the potential to express high value recombinant proteins using synthetic biology [8], [9]. Furthermore, proteins of microalgal origin tend to be more biocompatible with humans; this is because, unlike bacterial expression systems, eukaryotic microalgae can carry out complex protein folding, glycosylation, and extensive post-translational modifications (PTMs). In fact, many eukaryotic algal species produce proteins with minimal changes to the universal core glycosylation pattern also present in human proteins and are generally considered safe, as they do not share any common pathogens with humans [8], [10].

Through an updated review of literature, this work aims to analyze microalgae as versatile, efficient, and sustainable biofactories, highlighting their usefulness to produce complex proteins of recombinant origin.

Methodology

The sources used for this review were selected to highlight the use of microalgal platforms to achieve efficient and complete production of recombinant proteins. The search was based on a criterion of no more than five years since the publication of the submission to showcase current trends, apart from standout older case studies. When using databases, only indexed publications were selected using search terms such as “microalgae”, “cyanobacteria”, “protein expression”, “chloroplast”, “genetic transformation techniques”, “recombinant proteins”, and “heterologous gene expression”.

Heterologous protein production systems

Recombinant protein technology is based on the expression of modified genes, originally from a different species, in a host cell [10]. Due to their extensive applications in various fields, there is a demand for large-scale and cost-effective production [11]. One of the most important decisions is an appropriate expression system [10]. This decision must consider factors like the physicochemical and structural properties of a protein, the structural need for glycosylations

and post-translational modifications, projected costs of production, yield and time to market, biosafety, plasticity of the organism, and the effect of cellular degradative processes such as proteolysis and autophagy [4], [10], [12], [13]. Limitations in various expression systems include the inability to carry on certain molecular modifications, lack of genetic tools and techniques, and toxic by-products [11]. If an organism lacks the required protein processing machinery, the stability and functionality of the resulting protein will be negatively affected [4].

Recombinant proteins can be expressed in a wide variety of hosts, including bacteria, yeast, insect cells, mammalian cells, plants, and microalgae [10]. In general terms, prokaryotic expression systems are less expensive and have short processing times and overall relative genetic and physiological simplicity; however, these systems are unable to produce extensive post-transcriptional and post-translational modifications, such as intron splicing, multimeric protein assembly and glycosylation [10], [14], [15]. On the other hand, eukaryotic systems can produce complex proteins, but they are generally more expensive, complex, highly sensitive to their environment, and have lower expression levels [10]. Biopharmaceuticals production is commonly developed in mammalian Chinese hamster ovary (CHO) cells, followed by expression systems such as *Escherichia coli, Saccharomyces cerevisiae,* and *Pichia pastoris* [4].

Characteristics of non-microalgae recombinant protein expression systems

Bacteria

Common bacterial species used as expression systems include *E. coli*, *Lactobacillus sp.*, and *Bacillus subtilis. E. coli* is a widely used model organism in genetic engineering [10]. Usually, bacteria have low production costs, low protein quality, medium recombinant product yield, high immunogenicity, and short production times [10], [16]. They are characterized by the lack of specific molecular machinery to perform PTMs such as N-glycosylation during protein biosynthesis [14]. Other drawbacks of choosing bacteria cells as hosts include the unintended production of inclusion bodies as insoluble aggregated forms of recombinant protein, toxic accumulation of protein in the periplasm, and general inability to assemble multidomain, complex proteins [4], [10], [16].

Yeast

The most used yeast species in genetic engineering and industrial biotechnology include *S. cerevisiae* and *P. pastoris* [10]. These eukaryotic microorganisms are characterized by low production cost, low protein quality, high recombinant product yield, high immunogenicity, and short-to-medium production times [10], [16]. Yeast presents distinct advantages such as the secretion of proteins to culture media, cellular machinery to perform PTMs and assemble complex proteins, and general cost-effectiveness [16]. However, some drawbacks of using these expression systems include their preference for highly glycosylated recombinant proteins, which are immunogenic to humans [4], [10], [16], limited enzymatic reactions to support proper folding [10], and occasionally incorrect PTMs [4].

Insect cells

Insect cells are characterized by low production cost, low protein quality, medium-to-high recombinant product yield, high immunogenicity, and varying production times [10], [16]. Baculovirus-infected insect cells are an often-used expression system for recombinant proteins [16], [17], [18], [19]. These cells allow for the assembly of oligomeric, complex proteins, as well as various PTMs like glycosylation, phosphorylation, formation of disulfide bonds, and

myristylation [16]. Some drawbacks of using insect cells include their high sensitivity to stress, their non-status as GRAS organisms, and potential degradation of proteins as a result of cell lysis by late stages of baculovirus infection [10], [16], [19].

Mammalian cells

Mammalian cells are preferred expression systems due to the resemblance of their processing machinery to human cells. Currently, CHO cells are a reliable and relatively well-established technology for biopharmaceutical production in the global market [10]. Mammalian cells are characterized by high production cost, high protein quality, medium-to-high recombinant product yield, low immunogenicity, and long production times [10], [16]. Some of the drawbacks of these expression systems include low levels of expression, highly immunogenic murine glycosylation, difficulty to scale-up, sensitivity to contamination by prions and human viruses, general instability due to being highly sensitive, and overall lengthy and laborious downstream processing [4], [10], [16], [20], [21].

Plant cells

Like microalgae, plants hold an advantage regarding cultivation and medium costs. Both organisms are photosynthetic and have lower initial investment costs, contrary to bacteria and mammalian cells [4]. They are characterized by an overall high production cost, high protein quality, high recombinant product yield, high immunogenicity, and very long production times [10], [16]. The main limitations of these expression systems include concerns for allergic reactions, slow growth cycles, less uniform production due to the presence of functional parts, and significative differences in N-glycosylation processes [4], [11], [16], [22].

Given the disadvantages of the previously discussed expression systems, unicellular eukaryotes constitute a well-founded alternative that combines the high productivity and simplicity of the bacteria and yeast with the advanced cellular machinery mechanisms of insect, mammalian, and plant cells [10].

Characteristics of microalgae as recombinant protein expression systems

Microalgae are unicellular photosynthetic microorganisms that inhabit saline, freshwater, and terrestrial ecosystems [4], [16], [23]. These microorganisms constitute an emerging platform to produce recombinant proteins [4], [10], [11], [13], [24].

In general terms, microalgae are known for their cost-effectiveness [4], [10], [11], [12], [16]. The previously discussed expression systems are mostly heterotrophic naturally and have higher cultivation costs; meanwhile, microalgae can generate biomass on carbon dioxide and light, lowering the carbon footprint in commercial processes [4]. Lower production costs are also associated with their rapid photoautotrophic growth rates [4], [11], [25]; easy maintenance and cultivation requirements, such as the lack of need for growth regulators or complex media [4], [11], [23], [26]; and reduced investment in downstream processing protocols such as isolation and purification [10].

Normally, downstream processing costs can account for up to 80% of total costs in industrial bioprocesses [10]. The reduction of downstream processing costs is related to microalgae's general biocompatibility, given their classification as GRAS by the Food and Drug Administration (FDA); this means they are safe for human consumption as they are free of pathogens, endotoxins, human viruses, and prion-like contaminants [4], [10], [11], [12], [23]. Additionally, microalgae have a large-scale growing capacity and are easily grown in isolated bioreactors and large areas; these characteristics facilitate the implementation of good manufacturing practices (GMP) and protect the environment from the flow of transgenes [4], [11], [23], [26]. Compared to plants,

they lack functional parts such as roots and therefore allow for uniform batch production and aren't restricted by seasonal-environmental conditions [4], [10]. On the other hand, compared to mammalian cells, they are easily adapted to more extreme environments and are not as sensitive to stress factors [12], [25].

Eukaryotic microalgae are known to offer distinctive advantages in comparison with traditional expression systems. Recently, they have gained relevance due to their potential commercialization in the biomedical industry [10]. In principle, they can perform extensive PTMs and complex protein synthesis and maturation, including the capability of glycosylation and disulfide bond formation [10], [11], [12], [23], [26]. Compared to mammalian cells, this can be advantageous as microalgae are more easily scalable at lower costs [10].

Despite the promising prospects of large-scale microalgae production, there are still relevant bottlenecks to defeat for advancing their proper commercialization. Limitations are currently tested by a series of genetic engineering and scaling techniques [24]. The main challenges are associated with insufficient yields and physiobiological barriers, such as thick cell walls; additional cellular membranes; inconsistent glycosylation patterns in different eukaryotic microalgae; interference by immune responses at mucosal surfaces; and gaps in knowledge regarding microalgae genetics [4], [10], [11]. It is worth noting that microalgae protein-based drugs have yet to pass Phase III clinical trials due to deficiencies in yield [10], [27]. A relevant bottleneck reported is the drying stage, for which spray drying has been proposed as an alternative to developing microalgae-based oral vaccines [26].

If these issues are solved and research gaps are filled, microalgae hold as potential cell bio factories to produce different bioactive compounds, including protein-based drugs in the pharmaceutical industry. This could potentially address issues with the high consumer prices that are associated with biopharmaceutical proteins [10].

Chlamydomonas reinhardtii as a model organism for protein production

The microalgal species *Chlamydomonas reinhardtii* is a model organism for genetic engineering and to produce different recombinant proteins and metabolites. It has been employed in metabolic engineering methods for over 100 recombinant proteins [28]. Its characteristics favor its extensive use, such as its unicellular morphology; growth in simple media; autotropic, heterotrophic, and mixotrophic growth ability; haploid genome; and sexual mating for genetic complexity [13]. *C. reinhardtii* is commonly used for recombinant protein production due to its high protein content with minimal nutrient requirements [11]. It can be easily cultivated in bioreactors, minimizing contamination risk in laboratory settings and facilitating its biocontainment, ensuring the security of transgenes from the environment [11]. The species is also among the first engineered algal species to be studied in commercial settings, which allows academic and industrial researchers to understand the challenges involved in scale-up and product recovery using genetically modified microalgae for commercial-scale production [29].

C. reinhardtii was the first microalgal species with complete sequencing available for all three genomes: nuclear, mitochondrial, and chloroplastic (ctDNA), all of which are amenable to multiple transformation methods [30]. It has a prominent molecular toolkit for plastome engineering among photoautotrophs, serving as a convenient chassis for recombinant protein expression [31]. Another unique advantage is the availability of an easily accessible, near-complete mutant library for *C. reinhardtii* [28].

The *C. reinhardtii* chloroplast genome was the first ctDNA to be manipulated by transgenesis [32]. The number of resources available for the *C. reinhardtii* manipulation has increased exponentially since then. These include well-defined protocols for growth, sexual propagation, and mutagenesis, as well as numerous published biochemical, analytical and reporter assays.

Supporting the *C. reinhardtii* research are important collections such as the *Chlamydomonas* Resource Center, a collection by the University of Minnesota with stock centers, and practical protocols; a collection of photosynthesis mutants is provided by Chlamy Station, hosted by the National Centre for Scientific Research with the Sorbonne University; and The Chlamy Sequence Optimizer is another publicly available tool for sequence optimization in *C. reinhardtii* chloroplasts [33]. Resources for *C. reinhardtii* use are under consistent growth and development, which promotes the development of new studies related to heterologous engineering in *C. reinhardtii* [34], [35].

Genetic transformation methods in microalgae

In the past two decades, genetic transformation methods have been reported for over 40 different microalgae species [23], [36]. Common subjects of study include *C. reinhardtii*, *Dunaliella salina*, *Volvox cartieri*, and *Haematococcus pluvialis* [23]. The method of choice can depend on factors such as the target organelle; for instance, electroporation is often used for nuclear transformation, while biolistics is preferred when transforming the chloroplast genome [13]. Table 1 summarizes some reported microalgae transformation methods.

Table 1. Summary of reported cases of microalgae transformation.

Transformation method	Microalgae species	Reported efficiency	Expression site	Reference
Microparticle bombardment	*Chlamydomonas reinhardtii*	10-20 TF/µg DNA	Chloroplast	[37]
	Haematococcus pluvialis	8.5-9.5 TF/µg DNA	Chloroplast	[38]
	Porphyridium purpureum	NR	Chloroplast	[39]
	Volvox carteri	2.5×10^{-5}	Nucleus	[40]
	Phaeodactylum tricornutum	NR		[41]
Agrobacterium-mediated transformation	*Dunaliella salina*	4.0×10^{-5} TF/cells	Nucleus	[42]
	Dictyosphaerium pulchellum	6.5×10^{4} TF/mL	Nucleus	[43]
	Parachlorella kessleri	2.5×10^{-5} TF/cells	Nucleus	[44]
E. coli- mediated transformation	*Phaeodactylum tricornutum*	1.0×10^{-5} TF/cells	Nucleus	[45]
Electroporation	*Neochloris oleoabundans*	5.2×10^{-4} TF/µg DNA	Nucleus	[46]
	Schizochytrium sp. TIO1101	NR	Nucleus	[47]
	Dunaliella salina	$0.7\text{-}19.1 \times 10^{-4}$	Nucleus	[48]
	Nannochloropsis limnetica	$10\text{-}11 \times 10^{-6}$	Nucleus	[49]
	Acutodesmus obliquus	125 TF/µg DNA	Nucleus	[50]
Glass beads	*Chlamydomonas reinhardtii*	NR	Chloroplast	[26]
	Platymonas subcordiformis	1×10^{-5}	Nucleus	[51]

TF = transformants (transformed colony forming units), NR = Not reported.

Microparticle bombardment

Microparticle bombardment (or biolistics, short for "biological ballistics") consists of using DNA-coated particles as delivery systems for exogenous genetic material; these particles pierce through the cell wall and algal membrane at high speeds and are usually made of gold or tungsten [10], [11], [23]. Tungsten binds to DNA more efficiently, but it can inhibit cell growth and damage DNA integrity [52]. This method is said to be effective for nuclear and chloroplast genome transformation [13]. It has been widely adopted for microalgae transformation due to its simplicity and efficiency [24]. The success of this method is determined by the type of microparticle used, distance from target cells, release pressure, and microalgae species [13]. It is also reported to be highly reproducible and does not significantly damage the cell wall [23].

This method is particularly effective for DNA insertion into the chloroplast because it can pierce through the chloroplast membrane, even after penetrating the cell wall and membrane, due to its ballistic motion and speed [13], [24]. The earliest report of particle bombardment for chloroplast genome editing was done in order to restore photosynthetic capacity in *C. reinhardtii* mutants [37]. Some examples of this method include the transformation of *H. pluvialis* to increase production of astaxanthin, a commercially valuable carotenoid [38]; the introduction of the CRISPR-Cas9 ribonucleoprotein (RNP) delivery system into *Tetraselmis* sp. to enhance lipid productivity through a gene knockout [53]; and the transformation of *Porphyridium purpureum* for the chloroplast-expression of antimicrobial peptides [39]. Other species that have been transformed through biolistics include *Chlorella vulgaris* [24], *Dunaliella salina* [54], *Volvox carteri* [40], and *Cylindrotheca fusiformis* [55].

Bacteria-mediated transformation

Agrobacterium tumefaciens is a phytopathogenic bacterium used to transfer DNA into plant cells [10], [13]. Through modification, *A. tumefaciens* has also been used to infect microalgae for transferring exogenous DNA [10], [11]. This method is known to be fast and non-laborious [23]. One disadvantage is the inability to formulate oral products based on microalgae transformed through *Agrobacterium*; this drawback is mainly due to the possible presence of residual bacteria [23]. Some examples of *Agrobacterium*-mediated transformation in microalgae include the insertion of a β-carotene hydroxylase gene into *D. salina* to enhance the production of violaxanthin and zeaxanthin [42], and the modification of *Dictyosphaerium pulchellum* to produce erythropoietin, a therapeutic protein for anemia-related disorders [43]. Other species that have been transformed through this method include *Parachlorella kessleri* [44], *Dunaliella bardawil* [56], *Dunaliella tertiolecta* [57], *Euglena gracilis* [58], and *Scenedesmus almeriensis* [59].

Additionally, there have been at least three reports of genetic modification through bacterial conjugation using *E. coli* [13], [45], [60], [61]. In the case of *Phaeodactylum tricornutum,* conjugation through *E. coli* was reported to be significantly more efficient than microparticle bombardment transformation [45].

Electroporation

Electroporation consists of applying electric pulses of high intensity to allow exogenous DNA to enter the cell through the creation of temporary micropores in the membrane [10], [13], [16], [23]. An optimized electroporation protocol balances the membrane permeability and cell survival rate [13], [16]. Successful electroporation protocols are usually established empirically, and their effectiveness can vary depending on factors such as growth phase, electroporation conditions, pretreatment of cells, membrane and cell wall composition, organism size, and microalgae species [13], [23]. However, it remains a routine method utilized for microalgae and

microalgal nuclear transformation [10], [16], [23]. Some cases of successful electroporation protocols include the modification of *Neochloris oleoabundans* to enhance its lipid content and usage as diesel feedstock [46], the transformation of *Chlorella* sp. DT to enhance hydrogen production [62], and the transformation of *Schizochytrium* sp. TIO1101 to improve growth rates previously limited by the accumulation of acetate content [47]. Other species that have been transformed through this method include *Nannochloropsis salina* [63], *Dunaliella salina* [48], *Nannochloropsis limnetica* [49], and *Scenedesmus obliquus* [64].

Other notable transformation methods

Other less common transformation methods have been reported for microalgae. For example, glass bead disruption is a fast and practical method for transgenesis, where beads induce cell permeabilization and DNA transfer through agitation [10], [23]. This method was used in *C. reinhardtii* to produce a vaccine against salmonoid alphavirus (SAV) [26]. The agitation with glass beads is limited due to microalgae's rigid cell wall, and low survival rates are associated with this method [16]. On the other hand, the use of viral vectors to insert genetic material is a promising technology but is not yet widely used in microalgae [10]. Common viruses used for eukaryotic microalgae infection include *Chlorella* viruses (PBCV-1, OSy-NE5, ATCV-1); *Chaetoceros* viruses (CdebDNAV and ClorDNAV); and *Tetraselmis* viruses (TetV and Tsv-N1) [65]. Other methods that are used in microalgal genetic engineering include chemically induced transformation [66], [67] and lipid-based transfection methods [68].

Limitations for the genetic transformation of microalgae

A very significant limitation when it comes to the genetic transformation of microalgae is the cell wall. This component consists of a thick, complex, and highly recalcitrant structure that usually ensures cell viability and stability [11], [13], [69]. The algal cell wall composition depends on the genus, species, and strain of microalgae; it is usually a heteropolymer composed of a series of carbohydrates, lipids, proteins, and hydrocarbons [13], [69], [70]. As an example, the cell wall of *Chlorella* sp. is composed of irregular stereo-polysaccharides, chitin, chitin-like polysaccharides, rhamnose, and galactose [71]. Even so, complete cell wall disruption is not needed for transformation; instead, this structure needs to remain in a damaged but recoverable state to produce viable cells [13].

Another limitation associated with microalgae is genomic ploidy. When there is more than one copy of the genome, there is a higher chance of unintended repair of transgenes due to the presence of a repair template; this has been the case for diploid and polyploid microalgae species [13].

Thirdly, some microalgae are particularly resistant to antibiotics. This can influence the growth of untransformed microalgae in selection plates. Therefore, antibiotics must be used in very high concentrations [13].

Other limitations that are linked to low levels of expression in microalgae for heterologous proteins include nuclease activity, codon bias, silencing of transformants, requirement for special equipment (e.g., for microparticle bombardment), and low efficiencies related to temperature and consistent light source [16], [23].

Organelle-directed protein expression in microalgae

The demand for high-value molecules has been the driving force behind the engineering of eukaryotic microbial platforms, whether in their nuclear, mitochondrial, or chloroplastic genome. All three systems can be transformed for organelle-directed protein expression in microalgae [72], each with their unique advantages and disadvantages, which will be explored further.

Nuclear protein expression

In microalgae, modifications in the nuclear genome are more common than chloroplast-directed engineering efforts [73]. One advantage of nucleus-directed expression is the protein availability for PTMs and intracellular targeting, such as the endoplasmic reticulum or the Golgi apparatus for export and localization to the cell membrane [10], [16]. In the case of chloroplast expression, the proteins are retained within the organelle, limiting PTMs [74]. Although chloroplast expression may permit other bioactivity-dependent modifications such as the formation of disulfide bonds [73], PTMs like N-glycosylations are necessary in many proteins to ensure stability, correct folding, protein localization, and may impact functions such as immunogenicity, pharmacokinetics, and catalytic activity [75].

However, compared to chloroplast-targeted expression, relatively few methods, regulatory elements, and transformation vectors have been identified for the nucleus. Significant efforts are being made to expand a molecular toolbox, allowing an efficient and robust expression of transgenes from microalgal nuclear genomes [30], [76]. Another limitation of nucleus-directed heterologous protein expression is the consistently lower yields compared to protein accumulation in the chloroplast; in fact, gene expression in the nucleus can occupy up to 9% of the total soluble protein content, whereas chloroplasts can reach up to 21% of the total soluble protein content [10]. This phenomenon is likely due to nuclear silencing mechanisms, which have been a challenge to engineer out of the system because it is hypothesized that they evolved as a protective measure against intracellular pathogens or viruses [77]. These mechanisms can cause excising, truncating, or rearrangement of the gene of interest, and random integration in the nucleus can result in variable expression among clones [13]. Avoiding gene silencing will represent a principal barrier before recombinant proteins can be expressed in the nucleus at commercially viable levels [24].

New methods have helped overcome this problem, such as the development of strains with impaired transgene silencing by using UV mutagenesis and selection and media that permit higher antibiotic tolerance proportional to higher expression of the exogenous product for selecting strains with improved protein accumulation [78], [79]. The development of CRISPR-Cas9 systems in microalgae may also offer a solution for targeting transgenes into specific sites in the nuclear genome, which can help avoid random integration events [32].

Protein expression in the chloroplast

In the model organism *C. reinhardtii*, plastid genes are arranged on a circular chromosome with high polyploidy, around 83 copies, which is uniparentally inherited [73]. Algal plastomes are displayed in a quadripartite structure with small and large single-copy regions, divided by two large, inverted repeat regions [73]. The high copy number in chloroplast expression can enable efficient expression of recombinant proteins, and chloroplasts have their own ribosomes and translation elements for this process [24], [32]. Another advantage is that the proteins synthesized and reserved within the chloroplast, come to show higher effectiveness of cell transformation and consequently greater yields than those proteins released to the extracellular

medium from the nucleus [4]. In the case of *C. reinhardtii*, there is a single large chloroplast, which allows it to accumulate high amounts of expressed proteins with high homogeneity [16], [73].

The use of chloroplast transformation can integrate exogenous genes at specific sites in the chloroplast genome via homologous recombination [72]. One significant advantage of chloroplast transformation is that transgenes can be easily directed to integrate via homologous recombination, whereas nuclear transformation of microalgae usually results in random integration events [80]. Homologous recombination has been successfully accomplished in the *C. vulgaris* chloroplast genome using the 16S-*tml/tmA*-23S as the flanking fragments in the inverted repeat region [24].

Many methods have been well established for *C. reinhardtiii* chloroplast transformation, and over 100 different proteins have been successfully produced [81]. In almost all reported cases, the complexity of the genetic engineering is low and typically involves introduction into the plastome of just a single transgene together with a selectable marker [81]. Chloroplast transformation systems have been established for various microalgae species, such as *C. reinhardtii, H. pluvialis,* and *Tetraselmis subcordiformis* to express industrially valuable biomaterials and recombinant therapeutic proteins as oral vaccines [24], [82].

Protein expression in the mitochondria

Targeted expression in the mitochondria is the least common of all three transformation systems. Due to each cell possessing multiple mitochondria, the maintenance of genetic engineering stability is limited [13]. However, mitochondria could be an attractive target for modification towards energy, biomass production, and manipulation of oxidative stress [83]. Few cases have been reported of successful recombinant protein expression in mitochondria, however, *C. reinhardtii* mitochondria have been transformed to revert a mutation involved in the biosynthesis of cytochrome b, via biolistics and homologous recombination [84].

Conclusions

The use of microalgae for the biomanufacturing of therapeutic recombinant proteins has great potential, even though its development has not been sufficient. The microalgae-based expression also offers real potential for cost savings, thus making it an alternative to increase production yields. Moreover, different transformation techniques have been exploited in microalgae, and some of them must be selected according to each species' characteristics to guarantee a successful transformation. Microalgal platforms consist of promising sources for treatments, industrial products, and services. Therefore, it is important to focus efforts on research and development for optimized protocols to enhance recombinant protein production in microalgae. At the Biotechnology Research Center of the Costa Rica Institute of Technology, researchers such as *Dr.rer.nat.* Montserrat Jarquín-Cordero and Master Luis Barboza-Fallas conduct studies in this field, such as the expression of human pro-angiogenic growth factors [7] and the production of a bioherbicide in microalgae.

Author Contributions

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Conflicts of interest

The authors declare no competing interests. Luis Barboza Fallas is the CEO of Hemoalgae, a startup company using microalgae as technological platforms to produce high-value compounds.

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