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Review

Biotechnological Potential of *Chlamydomonas reinhardtii*: Advances, Challenges, and Applications

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Abstract

The biotechnological potential of microalgae represents a uniquely versatile platform, owing to a combination of inherent biological traits that make these organisms highly promising for a wide range of applications. For several decades, industrial biotechnology has predominantly relied on the metabolic engineering of bacteria and yeasts. However, microalgae offer a compelling alternative: they retain the advantages of unicellular systems while also possessing the capacity for autotrophic growth. This dual nature enables the development of new, more economically favorable biotechnological processes within relatively short timeframes, building upon the extensive experience accumulated with traditional microbial hosts. Bioproduction in heterotrophic microorganisms often requires strict sterility and complex, costly media formulations. In contrast, microalgae provide a more economical route for large-scale production of many compounds, particularly when low-cost, high-volume output is desired. A central advantage of microalgae lies in their highly efficient photosynthesis and their ability to capture carbon dioxide, ensuring a continuous supply of both substrate and energy for biosynthesis. This review summarizes recent advances in the biotechnological use of *Chlamydomonas reinhardtii*, highlighting its physiological, genetic, and metabolic characteristics, state-of-the-art engineering tools, and applications in medicine, food, industrial production, and environmental remediation.

Keywords: *Chlamydomonas reinhardtii*; metabolic engineering; synthetic biology; biotechnology; microalgae; CRISPR/Cas9; biofuel; bioremediation

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1. Introduction

1.1. Advantages of the Biotechnological Use of Microalgae

The biotechnological use of microalgae represents a unique platform for a wide range of applications due to the combination of natural properties that make them promising for diverse technological solutions [1]. Over the past several decades, industrial biotechnological systems have been developed primarily through the metabolic engineering of bacteria and yeasts. In this context, the use of microalgae which combine most advantages of unicellular microorganisms with the capacity for autotrophic growth allows the rapid development of new, more cost-effective industrial biotechnological systems based on accumulated experience with bacterial and yeast platforms [2]. Bioproduction in heterotrophic organisms imposes stringent requirements on sterility and relies on relatively expensive cultivation media. Therefore, the production of compounds at a lower cost and at large scale is often more advantageous in microalgae. A key advantage is their photosynthetic efficiency and ability to sequester carbon dioxide, ensuring a stable supply of substrates and energy for bioproduction. Studies demonstrate that certain microalgal species can absorb CO_2 10–50 times more efficiently than terrestrial plants, making them a powerful tool for mitigating climate change and valorizing industrial emissions [3]. An important application of microalgae is wastewater treatment, where they exhibit high efficiency in removing nutrients such as nitrogen and phosphorus from municipal and agricultural effluents, preventing eutrophication of aquatic ecosystems [4]. This process –phycoremediation not only purifies water but also enables the recovery of valuable nutrients

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in the form of biomass, which can be used as biofertilizer [5], establishing a circular system in which the waste from one process becomes a resource for another [6]. Microalgae are also applied in agriculture as biostimulants and feed additives due to their rich composition, including proteins, amino acids, vitamins, and phytohormones, which enhance crop productivity and improve animal health [5]. In the field of food and human health, microalgae show great potential as sources of high-quality protein, omega-3 fatty acids, antioxidants, and micronutrients [7,8]. Their bioactive compounds, such as carotenoids and phycocyanin, are widely used in pharmaceutical and cosmetic industries [9]. The concept of algal biorefinery enables the full utilization of microalgal biomass by converting it into a broad range of products, from biofuels to food additives [6].

Advances in genetic engineering are opening new opportunities for expanding the potential of microalgae by enabling the creation of strains with tailored properties for specific applications [10]. The development of genetic modification tools, including CRISPR/Cas9 systems, has revolutionized approaches to metabolic engineering of microalgae [11]. One of the most intensively developed areas is engineering microalgae for biofuel production, where metabolic modifications significantly increase lipid productivity [10], and current research focuses on altering metabolic pathways to enhance triacylglycerol synthesis and modify fatty acid profiles to improve biofuel quality [12]. Significant progress has been made in producing high-value compounds, where genetic modification increases yields of carotenoids and polyunsaturated fatty acids critical for creating sustainable sources of omega-3 fatty acids [13–15]. A promising direction is the use of microalgae as producers of recombinant proteins and biopharmaceuticals, with genetic engineering enabling the production of complex protein molecules, including vaccines, antibodies, and therapeutic proteins [16,17]. Modern research is also focused on increasing photosynthetic efficiency and biomass productivity through genetic engineering, where optimization of light-harvesting complexes and enhancement of carboxysome function can significantly accelerate growth and improve culture yields [18,19]. Advances in synthetic biology enable the design of sophisticated genetic circuits to regulate metabolic pathways in response to environmental stimuli, opening possibilities for developing cultures capable of adapting their metabolism to fluctuating conditions [20].

Another important aspect is engineering microalgae to enhance tolerance to abiotic stresses [21], which expands cultivation possibilities and reduces operational costs. Genetic modification is also used to improve microalgal capabilities for bioremediation by creating strains with enhanced capacity to detoxify heavy metals and degrade persistent organic pollutants [22]. The integration of genetic engineering advances with traditional microalgal cultivation approaches creates a powerful platform for sustainable production of a wide range of products – from biofuels and bioplastics to pharmaceuticals and food additives, providing innovative solutions to global challenges in energy, food security, and environmental protection [6,23].

1.2. Advantages of Chlamydomonas reinhardtii

C. reinhardtii occupies a unique position in modern biotechnology, serving not only as a classical model organism for studying fundamental processes such as photosynthesis, flagellar function, and cellular signaling, but also as a highly promising platform for applied synthetic biology. This versatility is driven by a unique combination of features that make it an ideal system for metabolic pathway engineering and the industrial scaling of biotechnological processes [24]. A critically important advantage is the availability of a fully sequenced and comprehensively annotated genome, supplemented by extensive OMICS databases, including genomic, transcriptomic, proteomic, and metabolomic data, offering researchers unprecedented capabilities for *in silico* modeling and targeted design of metabolic pathways [25].

From a technological perspective, *C. reinhardtii* cultivation is simple and economically efficient: the organism exhibits exceptionally rapid growth in inexpensive mineral media without the need for complex nutrient supplements. It can be successfully cultivated both in highly controlled photobioreactors and, theoretically, in open pond systems—an essential factor for industrial-scale bioprocessing [26]. The toolbox for genetic manipulation of *C. reinhardtii* is of particular value: highly

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efficient transformation protocols have been developed for all three genetic compartments—namely the nuclear, chloroplast, and mitochondrial genomes. In addition, advanced CRISPR/Cas9-based genome-editing tools are under active development, enabling not only gene knockouts but also site-specific knock-ins and fine-tuned regulation of gene expression [11,24].

The metabolic potential of *C. reinhardtii* is characterized by exceptional diversity and plasticity. The organism possesses a highly efficient photosynthetic apparatus and a well-developed CO_2-concentrating mechanism, making it a promising platform for metabolic engineering aimed at optimizing carbon fixation, increasing biomass productivity, and redirecting carbon fluxes toward target products [18,19]. Particularly noteworthy is the unique ability of the *C. reinhardtii* chloroplast to perform complex post-translational modifications, including glycosylation, disulfide bond formation, and proper folding of complex proteins. These capabilities are essential for the production of functional eukaryotic biopharmaceuticals, such as monoclonal antibodies, subunit vaccines, and therapeutic enzymes, offering a significant advantage over prokaryotic expression systems [16,17].

The plasticity of primary and secondary metabolism allows *C. reinhardtii* to be used as a versatile producer of a wide range of valuable compounds—from triacylglycerols for biodiesel production [10] and molecular hydrogen under specific stress conditions [27] to high-value carotenoids such as astaxanthin, lutein, and β-carotene, as well as long-chain omega-3 polyunsaturated fatty acids. This highlights its potential for the food, feed, pharmaceutical, and cosmetic industries [13,14]. An important practical advantage is the relatively low risk of horizontal gene transfer to the environment, due to the presence of a robust multilayered cell wall and strict regulation of the sexual cycle. This simplifies biosafety procedures and facilitates regulatory approval for the use of genetically engineered strains in open cultivation systems—an essential argument supporting its commercial application [28].

1.3. Limitations and Challenges of Using Chlamydomonas reinhardtii in Biotechnology

Despite the remarkable potential of *C. reinhardtii* as a biotechnological platform, its commercialization and broad industrial application face several significant constraints and challenges that require further fundamental and applied research. One of the key issues remains the low productivity of biomass and target metabolites under large-scale cultivation. Although the organism demonstrates high growth rates under laboratory conditions [26], transitioning to industrial volumes introduces substantial difficulties associated with optimizing light distribution, CO_2 delivery, culture homogeneity, and contamination control, all of which markedly increase the final production cost [29]. An important technological barrier is the absence of standardized and cost-effective protocols for large-scale cultivation. A significant gap persists between optimized laboratory conditions and the requirements of industrial photobioreactors, particularly in terms of efficient mixing, gas exchange, and temperature control, which negatively affects overall productivity and process reproducibility [30].

A major constraint on metabolic engineering is the incomplete genome annotation and insufficient understanding of regulatory networks. Despite the availability of the sequenced genome [25], the functional roles of many genes remain unexplored, and our knowledge of the fine regulation of metabolic pathways and cellular signaling mechanisms is still fragmented. This severely limits the rational design of producer strains [31]. A particular challenge is the compartmentalization of metabolic pathways – redirecting carbon fluxes toward desired products often disrupts cellular homeostasis and induces metabolic stress, leading to reduced growth rates and necessitating complex trade-offs between productivity and cell viability [32].

Genetic engineering efforts also face the persistent challenge of low nuclear transgene expression and strong positional effects, caused by heterochromatin organization and complex transcriptional regulation [24]. Unlike chloroplast transformation, which enables high-level expression [16], nuclear transformation often suffers from gene silencing, unstable expression, and the need for labor-intensive selection of effective promoters and terminators for each specific application [33]. Although the CRISPR/Cas9 system has opened new opportunities for editing the nuclear genome [11], its

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efficiency in *C. reinhardtii* remains considerably lower compared to model higher plants and microorganisms, requiring further optimization of delivery methods and enhancement of homologous recombination efficiency [34].

A major limitation for biofuel production is the naturally low content of triacylglycerols in cells and the need for stress conditions—such as nitrogen deprivation or high light intensity—to induce lipogenesis. These stressors inevitably halt cell growth and division, creating a fundamental conflict between biomass biosynthesis and storage lipid accumulation [10,35]. Similar challenges arise during the induction of other high-value products; for example, molecular hydrogen production occurs only under strictly anaerobic conditions and is associated with a sharp decline in photosynthetic activity and light-energy conversion efficiency [27].

For medical applications, a critical limitation is the difficulty of achieving high yields of recombinant proteins and their subsequent purification. Despite the organism's capacity for proper folding of complex proteins [17], the overall yield of target products is often orders of magnitude lower than in traditional prokaryotic and eukaryotic expression systems. Furthermore, the extraction of target proteins from cell lysates is complicated by the presence of a robust cell wall and the need to develop specific and economically viable purification protocols [36,37].

Finally, regulatory barriers and biosafety concerns associated with the use of genetically modified microorganisms in open systems create additional obstacles to commercialization. Although strategies for biocontainment and genetic isolation are under development [28], their reliability requires further validation, and the approval process for genetically modified strains remains lengthy, costly, and regulatory-ambiguous in many jurisdictions. These factors hinder investment in large-scale projects involving *C. reinhardtii* [38].

2. Microbiological Aspects and Biotechnological Applications of *Chlamydomonas reinhardtii*

Effective biotechnological use of *C. reinhardtii* requires a deep understanding of its physiology and optimal cultivation conditions. Control over growth parameters enables the redirection of cellular metabolism toward the maximal accumulation of target products.

2.1. Growth Regimes

C. reinhardtii exhibits exceptional metabolic flexibility and is capable of photoautotrophic, heterotrophic, and mixotrophic growth [26]. Photoautotrophic growth under illumination with CO2 as the sole carbon source is the most cost-effective approach for large-scale biomass production [26]. However, for the synthesis of specific metabolites, mixotrophy – light combined with an organic carbon source or heterotrophy – use of organic carbon in the dark, are often employed, as these modes allow a substantial increase in biomass yield and induce lipid accumulation [26,39]. Recent studies indicate that two-stage cultivation – initial mixotrophic growth for rapid biomass accumulation followed by stress induction under photoautotrophic conditions, can significantly enhance overall process productivity [10].

2.2. Nutrient Media

The choice of nutrient medium is critically important for determining the metabolic pathway and productivity of *C. reinhardtii*. A key advantage of this organism is its ability to grow rapidly on inexpensive mineral media without the need for complex nutrient supplements, which is essential for reducing the cost of industrial cultivation [26].

- TAP medium (Tris-Acetate-Phosphate), developed by Gorman and Levine [40], is the standard medium for mixotrophic and heterotrophic cultivation. Acetate (17 mM) serves both as a carbon source and as a buffer. TAP is widely used for genetic transformations and mutant screening [24].

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- HSM (High-Salt Minimal) photoautotrophic medium, developed by Sueoka [41], contains no organic carbon, making it ideal for studies of photosynthesis and nutrient deprivation [35].
- 6xP medium, containing sixfold phosphate concentration, is optimized for high-density photoautotrophic cultivation in bioreactors and prevents phosphate-limited growth [42].
- T10 rich medium, with elevated acetate levels (40 mM) and yeast extract, is designed for heterotrophic cultivation aimed at maximal biomass yield [43].

2.3. Temperature

The optimal temperature range for *C. reinhardtii* growth is 20–25°C [26]. Deviation from this optimum acts as an abiotic stress factor. Increased temperature may be used to induce specific responses, such as the expression of chaperones for proper folding of recombinant proteins, recombinant protein production, and heat-shock responses [44]. Engineering temperature stress tolerance is an active area of metabolic engineering aimed at expanding the geographic zones suitable for cultivation [21].

2.4. Stress and Nutrient Deprivation

The deliberate induction of stress conditions is a key tool in biotechnology for redirecting cellular metabolism from growth toward the synthesis of storage compounds. The most widely used approach is nitrogen deprivation, which is a classical and powerful inducer of triacylglycerol (TAG) synthesis and accumulation [10,35,45]. However, this process creates a metabolic conflict that leads to the arrest of cell division [35]. Sulfur deprivation, in turn, is the standard protocol for inducing molecular hydrogen production under anaerobic conditions [27,46]. Phosphorus deprivation induces the accumulation of starch and lipids [47]. These stresses create a metabolic conflict between growth and product accumulation, necessitating optimization of induction timing [48].

2.5. Illumination

Light is the main energy source during photoautotrophic and mixotrophic cultivation. Light intensity, photoperiod, and spectral composition are critically important. Optimal parameters include: light intensity – 100–300 μmol photons m^{-} s^{-1} [49], photoperiod – 16:8 or 24:0 (light:dark) [26,42], spectrum – blue and red wavelengths are most effective for photosynthesis [50]. Insufficient illumination limits growth, whereas excessive illumination causes photoinhibition and photodamage to photosystems. Optimization of light conditions in photobioreactors e.g., through system design and mixing, is one of the main engineering challenges to ensure high photosynthetic efficiency and prevent photolimitation in dense cultures [29]. Genetic engineering efforts focus on optimizing light-harvesting complexes to improve light-use efficiency [18].

2.6. Aeration

Aeration in bioreactors serves two key functions: supplying CO_2 (a substrate for photosynthesis) [29] and ensuring mixing for culture homogenization [51], preventing cell sedimentation, and removing excess oxygen to avoid photooxidative damage [29]. Mixing also helps cells move in and out of illuminated zones uniformly [29]. However, excessive aeration may cause cellular damage due to shear stress. The supply of CO_2-enriched air (1–5%) is often necessary to maintain high growth rates in dense cultures because atmospheric CO2 concentrations are insufficient [29]. The optimal aeration rate is approximately 0.5–1.5 vvm (volume of gas per volume of medium per minute).

2.7. Carbon Sources

During heterotrophic and mixotrophic cultivation, acetate is the standard and preferred carbon source for *C. reinhardtii* because it is easily assimilated and metabolized [26]. However, alternative, more cost-effective substrates are being investigated to reduce production costs, such as glycerol [52], glucose, after strain adaptation [53], and even organic components of wastewater, integrating

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biomass production with wastewater treatment processes [26]. Lactic acid is also a promising carbon source for mixotrophic growth [54].

2.8. Nitrogen Sources

The most commonly used nitrogen sources are nitrate (NO_3^-), the standard form but one that is taken up relatively slowly [35], ammonium salts (NH_4^+), which are assimilated rapidly but acidify the medium [55], and urea, an efficient alternative nitrogen source [56]. *C. reinhardtii* is capable of utilizing all of these forms; however, their selection influences medium pH and cellular metabolism [35,55]. As noted above, nitrogen availability is the primary regulatory factor determining the transition between the growth phase and the storage lipid accumulation phase [35]. Nitrogen deprivation is a key tool in metabolic engineering for redirecting carbon flux into lipid synthesis [10].

2.9. Summary of the Section

The microbiological aspects of *C. reinhardtii* cultivation represent a complex optimization challenge. Standard growth modes and inexpensive nutrient media constitute the foundation for scalable cultivation [26]. However, modern biotechnological applications require specialized media such as 6xP, T10, and two-stage cultivation strategies [48]. Strategic manipulation of cultivation conditions—such as nutrient deprivation, particularly nitrogen [10,35], light regime [29] and aeration—serves as a powerful tool for inducing the synthesis of target products, including biofuels (lipids, hydrogen) [10,27] and high-value metabolites. Nevertheless, the fundamental conflict between maximizing biomass growth and maximizing product accumulation under stress remains the central challenge. Its solution lies in combining optimized cultivation conditions with metabolic engineering approaches [10,21,35]. Advances in understanding the physiology of *C. reinhardtii* [26] and the development of optimized cultivation protocols [42] open new possibilities for the industrial application of this versatile microalga.

Table 1. Cultivation parameters for biotechnological application of *C. reinhardtii*.

Parameter	Description	Application
Growth modes	Photoautotrophic, heterotrophic, mixotrophic	Photoautotrophy is economical for biomass scale-up; mixotrophy and heterotrophy are used for specific metabolite synthesis and lipid accumulation
Nutrient media	TAP, HSM, 6xP, T10	TAP is standard for mixotrophic and heterotrophic growth; HSM is for photosynthesis and nutrient deprivation studies; 6xP optimized for high-density photoautotrophic cultivation; T10 used for maximal biomass yield under heterotrophy
Temperature	20-25°C	Optimal for growth; increased temperature induces stress responses useful for metabolic engineering
Stress and deprivation	Nitrogen, sulfur, phosphorus deprivation	Induces storage compound accumulation such as triacylglycerols, starch, and molecular hydrogen production
Illumination	Intensity 100-300 μmol photons m^{-2} s^{-1}, photoperiod 16:8 or 24:0, blue and red light spectrum	Optimized for maximal photosynthetic efficiency

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Aeration	CO_2 enriched air (1-5%), mixing	Supplies substrate for photosynthesis, homogenizes culture, prevents cell damage
Additional carbon sources	Acetate, glycerol, glucose, lactic acid	Acetate is the standard; alternatives researched to reduce costs
Nitrogen sources	Nitrate (NO_3^-), ammonium salts (NH_4^+), urea	Affect pH and metabolism; nitrogen deprivation directs carbon towards lipid synthesis

3. Genetic Engineering of *Chlamydomonas reinhardtii*

In recent years, substantial progress has been made in both research and practical applications involving the microalga *C. reinhardtii* in biotechnology. The Chlamydomonas Resource Center (www.chlamycollection.org), the largest repository for acquiring, cataloging, storing, and distributing wild-type and mutant *C. reinhardtii* strains, currently contains nearly 4,000 characterized strains and more than 400 plasmids. Notably, the majority of these resources have been added within the past five years. The available set of selective markers and promoters for efficient gene expression has also expanded significantly. Combined with recent advances in enhancing phototrophic cultivation efficiency, described earlier, this makes *C. reinhardtii* a highly promising platform for biotechnological applications. In this section, we review modern strategies for genetic and metabolic engineering of *C. reinhardtii*. However, several existing limitations must first be addressed. First, efficient transgene expression is hindered by the fact that nuclear transformation most commonly occurs via non-homologous end joining (NHEJ). This greatly complicates the generation of stable transformant lines [57]. Integration via homologous recombination in the nucleus of *C. reinhardtii* is far less common [58]. The frequency of such events, relative to random integration, ranges from 1:1000 when using the glass bead method to 1:24 following bombardment with DNA-coated tungsten microparticles [59]. In contrast, homologous recombination in chloroplasts is highly active. However, due to the large number of chloroplast genome copies, transformation typically results in heteroplasmy, with a high risk of subsequent loss of the introduced transgene [60]. Second, the introduction of heterologous genes must be accompanied by codon optimization, since the genome of *C. reinhardtii* exhibits unusually high GC content (approximately 65%) [25]. Third, the use of the CRISPR/Cas9 system for site-directed mutagenesis appears to be hindered by the toxicity of Cas9 protein in *C. reinhardtii* [61]. Fourth, the genetic and metabolic regulatory mechanisms in this organism remain poorly characterized. Consequently, conditions for modulating gene expression and altering metabolic pathways generally must be selected empirically.

3.1. Transformation Methods

Currently, the most commonly used transformation methods for *C. reinhardtii* include glass bead agitation, electroporation, gene gun delivery, Agrobacterium-mediated transformation, and CRISPR/Cas9-based systems.

3.1.1. Glass Beads Method

This is the simplest and least expensive transformation method, originally described in 1990 [62]. It consists of vortexing *C. reinhardtii* cells with glass beads in the presence of target DNA and polyethylene glycol. The method requires cell-wall-deficient strains or enzymatic pre-treatment to remove the cell wall. For example, a recent study proposed using alcalase for rapid and efficient cell-wall removal [63]. This approach yields transformation efficiencies of approximately 10^3 transformants per μg DNA, which is lower than those achieved by other techniques. An alternative version of the method involves the use of silicon carbide whiskers instead of glass beads. This variation enables transformation of intact cells and provides an 80% increase in cell survival compared to the original technique, although with slightly lower transformation efficiency [64]. In

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addition, successful chloroplast transformation using the glass bead method has also been demonstrated [65].

3.1.2. Electroporation

Electroporation is the most efficient transformation method, achieving up to 10^5 transformants per µg DNA. It is based on exposing cells to short high-voltage pulses that transiently increase membrane permeability, allowing DNA to enter the cytoplasm. Traditional electroporation protocols use the following parameters: 250 µL of cell suspension at a density of 1–4 × 10^8 cells/mL in a 4-mm cuvette, 10 µg/mL of target DNA, 200 µg/mL of carrier DNA, exponential pulses with a field strength of 1900–2400 V/cm [66]. A more recent alternative involves microfluidic electroporation at 192 V [67], though this method has not yet gained widespread adoption. Another interesting variation is square-wave electroporation [68,69]. Experiments using intact cells (density 5.0 × 10^7), 4-mm cuvettes, 400 ng/mL DNA, square-wave pulses at 500 V, six pulses (4 ms each) with 100 ms intervals demonstrated a transformation efficiency of 2–3 × 10^3 transformants per µg DNA [68]. Despite its relatively modest efficiency, this method allows the use of intact cells, requires an order of magnitude less DNA and provides higher cell viability.

3.1.3. Gene Gun (Biolistic Delivery)

This biolistic method is most suitable for chloroplast transformation. The technique is based on shooting tungsten or gold microprojectiles coated with target DNA. Due to their high kinetic energy, the particles penetrate the cells, releasing DNA that subsequently integrates into the chloroplast genome via homologous recombination. The efficiency of this method can reach 2.5 × 10^3 transformants per µg DNA. Clear advantages include methodological simplicity and high transformation speed [70–72].

3.1.4. Agrobacterium-Mediated Transformation

This method relies on infecting plant cells with Agrobacterium carrying a Ti plasmid harboring the target gene, which becomes integrated into the host genome. The technique enables the use of intact cells and can achieve transformation efficiencies up to 5 × 10^4 transformants per µg DNA, but is associated with a high frequency of false-positive results, and the transformation process itself is time-consuming [73]. Additionally, its efficiency is lower than that of simpler and faster electroporation methods [74].

3.1.5. CRISPR/Cas9

The CRISPR/Cas9 system for site-directed genome editing requires separate discussion. Since its introduction in 2012, this technology has found widespread use due to its precision and broad potential for gene modification in many eukaryotic organisms. *C. reinhardtii* is no exception. However, early studies demonstrated that constitutively expressed Cas9 protein (even a nuclease-inactive Cas9 variant) appears to be toxic to *C. reinhardtii* [61]. The solution was to introduce pre-assembled CRISPR/Cas9 ribonucleoprotein complexes (RNPs) into the cells via electroporation [75–77].

This system is commonly used for gene knockout [78–81] or knock-in mutagenesis [82–84]. In recent years, with the increasing number of studies, the potential applications of CRISPR/Cas9 in *C. reinhardtii* have expanded substantially. For example, a recent study used CRISPR/Cas9 to analyze the effects of 11 genes, most of which are involved in histone post-translational modifications, on transgene silencing [85]. The work employed single, double, and triple knockouts – a scale of genetic manipulation that would be extremely difficult to achieve without CRISPR/Cas9. Another study proposed using cycles of deactivation and reactivation of the selectable marker Nit1 (nitrate reductase) via CRISPR insertion of a stop codon in the first cycle, followed by restoration of the native sequence in the next cycle [86]. This creates the possibility of performing sequential genetic

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modifications within a single cell line while using only one selectable marker. In another work, it was shown that co-expressing Cas9 with a nuclear localization signal (NLS) from Agrobacterium enhanced nuclear delivery and consequently increased genome editing efficiency [87]. It was also demonstrated that a 50 bp homology arm length is optimal for efficient and precise DNA integration via HDR (homology-directed repair) [88].

3.2. Selectable Markers

Selectable marker genes can be divided into two categories: endogenous (native to *C. reinhardtii*) and exogenous — genes originating from other biological species (Table 2).

3.2.1. Endogenous Selectable Markers

Among the endogenous selectable markers, the most commonly used genes include *ARG7*, encoding argininosuccinate lyase, which enables growth on medium lacking arginine [89], *NIT1*, encoding nitrate reductase, which supports growth on medium where nitrate is the sole nitrogen source [90] and *CRY1*, encoding the cytosolic ribosomal protein S14 with the CRY1-1 mutation, conferring resistance to the eukaryotic translation inhibitors cryptopleurine and emetine [91]. Herbicide resistance–based markers are also widely used, including *ALS* (acetolactate synthase), which provides resistance to sulfonylurea herbicides [92], *PDS* (phytoene desaturase), conferring resistance to norflurazon [93,94], *PPO* (protoporphyrinogen oxidase), which provides resistance to oxyfluorfen [93] and *GAT* (glyphosate acetyltransferase), which confers resistance to glyphosate. However, the latter requires very high herbicide concentrations to inhibit cell growth, limiting its applicability in biotechnology [93]. A recent study proposed a novel endogenous marker, *SPD1*, encoding spermidine synthase, an enzyme involved in polyamine biosynthesis [95].

3.2.2. Exogenous Selectable Markers

Among exogenous selectable markers, the most frequently used are genes conferring antibiotic resistance and genes enabling fluorescent detection. The former include *aph7"*, *aphVIII*, and *aadA* [96–98] – which encode aminoglycoside phosphotransferases (resistance to aminoglycoside antibiotics), *ble* [99,100], encoding the bleomycin-binding protein (resistance to zeocin and bleomycin); and *NptII* [101,102], encoding neomycin phosphotransferase (resistance to neomycin and kanamycin). Fluorescent markers include *GFP* (green fluorescent protein) [103] and *Luc* (luciferase) [104].

During the past decade, several new exogenous markers have been introduced. For example, the *BSR* gene (blasticidin S deaminase from *Bacillus cereus*) was proposed, conferring resistance to blasticidin S without affecting resistance to other common antibiotics [105]. The *tetX* gene (an NADPH-dependent oxidoreductase) was used for the first time in the nucleus of Chlamydomonas, conferring resistance to tetracycline antibiotics [106]. Another marker proposed for nuclear transformation is *NAT* (nourseothricin N-acetyltransferase), conferring resistance to nourseothricin – an antibiotic to which bacterial resistance is uncommon [107]. A particularly interesting marker for negative selection is *codA*, encoding cytosine deaminase from *Escherichia coli*, which renders Chlamydomonas sensitive to the antifungal agent 5-fluorocytosine [108]. Herbicide-resistance genes remain an attractive option as well. One example is *CRTIop*, a codon-optimized bacterial phytoene desaturase for Chlamydomonas, which appears less sensitive to norflurazon compared with plant or algal *PDS* homologs [109].

Overall, selectable markers based on antibiotic and herbicide resistance are widely used due to their high efficiency in selection and protection of cultures from contamination. However, the downside is the potential risk of horizontal gene transfer to other species, along with the spread of resistance. This concern is growing increasingly relevant in the context of engineering scalable biotechnological organisms such as *Chlamydomonas reinhardtii*. In this regard, a new selectable marker, *ptxD*, encoding bacterial phosphite oxidoreductase, has been proposed. It enables cells to

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oxidize phosphite to phosphate and thus grow on media where phosphite is the sole phosphorus source [110,111].

3.2.3. Marker-Free Transformation

Another interesting strategy that helps avoid the problem of spreading antibiotic resistance is marker-free transformation (Table 2). This approach is most commonly implemented by using a strain carrying a mutation in one of the key photosynthesis genes. Such a mutant strain is unable to grow on minimal media unless it receives the missing gene as part of the transformation expression cassette. Genes frequently used for this purpose include *psaA*, encoding one of the major subunits of Photosystem I [112], *psbA* [113] and *psbH* [114], from Photosystem II, and *rbcL*, which encodes the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), a central enzyme in photosynthesis [115].

Another variant of this strategy involves the use of a dual marker system, combining *AadA* — the *Escherichia coli* aminoglycoside adenyltransferase gene providing spectinomycin resistance – for initial positive selection of transformants, together with *CodA*, encoding *E. coli* cytosine deaminase, which renders cells sensitive to 5-fluorocytosine, thereby enabling strong negative selection against the marker cassette in later stages [116].

Finally, it is worth mentioning the Cre/lox recombination system from bacteriophage P1, which can be used to excise a selectable marker after successful selection. In this case, a marker gene such as *aphVIII* is flanked by lox recognition sites, which are recognized and removed by the Cre recombinase [117].

Table 2. Selectable markers for *C. reinhardtii*.

Categories of selectable markers	Name	Description	Property	Reference
Endogenous	*ARG7*	Argininosuccinate lyase	Growth on medium lacking arginine	[89]
	NIT1	Nitrate reductase	Growth on medium where nitrate is the sole nitrogen source	[90]
	CRY1	Cytosolic ribosomal protein S14 with the CRY1-1 mutation	Resistance to the eukaryotic translation inhibitors cryptopleurine and emetine	[91]
	ALS	Acetolactate synthase	Resistance to sulfonylurea herbicides	[92]
	PDS	Phytoene desaturase	Resistance to norflurazon	[93,94]
	PPO	Protoporphyrinogen oxidase	Resistance to oxyfluorfen	[94]
	GAT	Glyphosate acetyltransferase	Resistance to glyphosate	[94]
	SPD1	Spermidine synthase	Involved in polyamine biosynthesis	[95]
Exogenous	*aph7″/ aphVIII/ aadA*	Aminoglycoside phosphotransferases	Resistance to aminoglycoside antibiotics	[96–98]
	ble	Bleomycin-binding protein	Resistance to zeocin and bleomycin	[99,100]
	NptII	Neomycin phosphotransferase	Resistance to neomycin and kanamycin	[101,102]
	BSR	Blasticidin S deaminase	Rresistance to blasticidin S without affecting resistance to other common antibiotics	[105]
	tetX	NADPH-dependent oxidoreductase	Resistance to tetracycline antibiotics	[106]

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	NAT	Nourseothricin N-acetyltransferase	Resistance to nourseothricin – an antibiotic to which bacterial resistance is uncommon	[107]
	codA	Cytosine deaminase	Renders Chlamydomonas sensitive to the antifungal agent 5-fluorocytosine	[108]
	CRTIop	A codon-optimized bacterial phytoene desaturase	Less sensitive to norflurazon compared with plant or algal PDS homologs	[109]
	ptxD	Bacterial phosphite oxidoreductase,	Grow on media where phosphite is the sole phosphorus source	[110,111]
	GFP	Green fluorescent protein	Fluorescent reporter	[103]
	Luc	Luciferase	Bioluminescence reporter	[104]
Marker-Free	*psaA*	Subunits of Photosystem I	Growth on minimal media	[112]
	psbA/ psbH	Subunits of Photosystem I I	Growth on minimal media	[113,114]
	rbcL	Large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco)	Growth on minimal media	[115]
	AadA/ CodA	Aminoglycoside adenyltransferase and cytosine deaminase	Spectinomycin resistance – for initial positive selection and 5-fluorocytosine sensitivity, thereby enabling strong negative selection against the marker cassette in later stages	[116]

3.3. Additional Factors for Efficient Transgene Expression

One of the main obstacles to realizing the impressive biotechnological potential of *C. reinhardtii* is the relatively low level of transgene expression. Identifying effective strategies to overcome this limitation remains an important challenge for researchers. In recent years, substantial progress has been made in this direction: highly efficient constitutive and inducible promoters have been developed; intron insertion into transgene coding sequences has been proposed to improve export of mature mRNA from the nucleus into the cytoplasm; the addition of 5′ or 3′ untranslated regions (UTRs) has been shown to enhance expression; strategies have been introduced to suppress or bypass the strong endogenous transgene-silencing mechanisms; and codon optimization based on the GC-rich genome of *C. reinhardtii* has been widely adopted [118]. Moreover, targeted nuclear transformation using zinc-finger nucleases has been successfully applied in Chlamydomonas [119].

Several new high-efficiency promoters have been proposed, including NOS, the nopaline synthase promoter from Agrobacterium [120]; GA, a synthetic promoter composed of fragments from the promoters of GDH2 (glutamate dehydrogenase) and ACP2 (acyl carrier protein), which exhibits maximal sensitivity to blue light [121]; and CnAFP, a cold-inducible promoter from a cold-shock protein of the polar diatom *Chaetoceros neogracile* [122]. Another important advance in understanding the architecture of endogenous *C. reinhardtii* promoters is the identification of short regulatory DNA motifs that enhance transgene expression [123]. Studies in this area pave the way for creating new, highly efficient synthetic promoters.

A complementary strategy involves reducing transgene silencing by treating *C. reinhardtii* cells with histone deacetylase inhibitors [124]. Since histone acetylation decreases chromatin compaction and thereby enhances transcriptional activity, inhibition of deacetylases may become a broadly

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applicable tool for boosting transgene expression. Along similar lines, a recent study identified several additional genes associated with epigenetic regulation whose knockouts resulted in enhanced transgene expression. These include the histone lysine methyltransferases *HLM4*, *HLM6*, *HLM14*, and *HLM25*; the DNA methylase *DMC5*; histone deacetylases *SRTA*, *SRTB*, and *SRTC*; the serine/threonine protein kinase *MUT9*; the histone-methyltransferase-complex subunit *MUT11*; and the intron-associated gene ortholog Vasa *VIG1* [85]. Another study demonstrated increased expression when transgenes were supplemented with intercistronic expression elements and assembled into constructs containing a VOR element and the Rep protein, enabling formation of a mini-chromosome that remains stable and transcriptionally active in chloroplasts [125]. Given the strong transgene silencing characteristic of *C. reinhardtii* and the frequent separation of the target gene from the selectable marker during or after integration, researchers have proposed the use of viral 2A peptide sequences, which allow two or more genes fused in a single open reading frame to be translated into separate proteins through ribosomal reinitiation [126]. Successful application of this system in *C. reinhardtii* has been reported [126,127].

As molecular tools for gene cloning in *C. reinhardtii* continue to expand and improve, efforts are underway to standardize and modularize these resources. For example, a Golden Gate Modular Cloning (MoClo) toolkit has been developed for nuclear and chloroplast transformation, containing all major functional modules (promoters, terminators, selectable and reporter genes), each flanked by Type IIS restriction sites to allow rapid assembly of expression cassettes using Golden Gate cloning [128].

3.4. Summary of the Section

The expanding toolbox of genetic engineering methods undeniably strengthens the appeal of *C. reinhardtii* as a platform for solving biotechnological challenges. The accumulation of new strains and plasmids, refinement of high-efficiency transformation and integration protocols, and introduction of diverse selectable markers all simplify methodological workflows and broaden the community of researchers working with this organism – a crucial factor for the field's growth. However, the next major step – the development of fully optimized industrial strains of *C. reinhardtii* will require deeper research into the mechanisms of epigenetic regulation of transgene expression, along with methods to ensure stable and controllable production of target compounds.

4. Modern Biotechnological Applications of *C. reinhardtii*

The list of compounds successfully expressed in *C. reinhardtii* is extensive and continues to grow at an accelerating pace each year. Below, we highlight some of the most notable reports from recent years.

4.1. Medicine

In this field, most research efforts focus on producing antimicrobial compounds. Successful expression in *C. reinhardtii*, followed by isolation/excretion and effective activity against Gram-positive and/or Gram-negative bacteria, has been reported for laterosporulin [129], isoform 3 of the anti-lipopolysaccharide factor [130,131], enterocin RM6 [132], and even a multimeric form of amyloid β-peptide [133].

Additionally, efficient expression of the larvicidal toxin Cry11Ba in *C. reinhardtii* has been demonstrated for safe delivery to mosquito larvae that naturally consume this alga in aquatic environments [134]. This approach holds promise for enhancing control over mosquito-borne diseases.

C. reinhardtii is also capable of producing more complex biologically active compounds, including human proteins. For example, successful expression has been reported for the pro-inflammatory human C-reactive protein [135], the pro-angiogenic growth factor hVEGF-165 [136], the anti-tumor cytokine interleukin-29 [137], and the pro-apoptotic type II metacaspase enzyme [138].

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All of these molecules are involved in various human diseases, and therefore an efficient platform for heterologous expression facilitates the study of their biological effects and the mechanisms underlying their activation or inhibition.

4.2. Food Industry

C. reinhardtii also attracts growing interest due to its ability to produce food-related compounds for use in animal husbandry, agriculture (as biofertilizers), and even for human consumption. Genetic engineering allows this alga to be enriched with additional valuable nutritional molecules. Recently investigated examples include: bacterial phytase, which improves phosphorus assimilation in animals [139]; zeolin, a nutritious storage protein generated by fusing γ-zein and phaseolin, designed to optimize the amino acid profile of biomass [140]; the monoterpenoid alcohol geraniol, used in food and cosmetic industries [141]; highly phosphorylated and glycosylated osteopontin, a potential additive for infant formulas [142]; and N-acetylmuramoyl-L-alaninamidase and a lysozyme-like enzyme as feed supplements in poultry farming [143]. Additionally, strategies for enhancing the production of previously reported compounds are being refined. For example, the influence of light stress on the profile of synthesized carotenoids has been investigated [144].

4.3. Ecology

There are several promising directions for the use of *C. reinhardtii* in bioremediation. One of the most significant is the removal of microplastics from aquatic environments. It should be emphasized that microplastics are toxic to *C. reinhardtii*. Studies have shown that polystyrene microplastics, depending on their concentration and size, inhibit growth and induce oxidative stress, with the strongest effects observed for the smallest particles [145]. These effects likely stem from disruptions in metabolic pathways related to photosynthesis, oxidative phosphorylation, and secondary metabolite biosynthesis [146]. However, we believe this challenge reveals an intriguing opportunity: algae engineered to express microplastic-degrading enzymes may gain resistance to environments with high microplastic concentrations, receiving a strong selective advantage despite the organism's robust intrinsic transgene-silencing mechanisms. Such selective pressure could facilitate the emergence of strains capable of rapidly cleaning polluted environments. Indeed, successful expression of polyethylene terephthalate hydrolase (PETase) and polyester hydrolase PHL7, both catalyzing the degradation of PET – the most widely used plastic worldwide, has already been demonstrated in *C. reinhardtii* [114,147,148]. Further research in this direction is highly relevant and promising.

Microplastics are far from the only toxic contaminants found in water. Expression of a cyanobacterial cyanase in *C. reinhardtii* has been reported for cyanide bioremediation [149], and expression of cytochrome P450 BM3 MT35 enables degradation of the herbicide diuron, with potential applications for inactivating various pharmaceutical and herbicidal pollutants in wastewater [150].

Additionally, several studies describe the use of *C. reinhardtii* for the removal of metal contaminants such as arsenic [151,152], cadmium [153–156], chromium [157], mercury [158], and zinc [153].

4.4. Industrially Significant Compounds and Biofuels

C. reinhardtii has received particular attention as a promising producer of industrially relevant compounds, and this interest has been growing rapidly in recent years. This expansion is driven by discoveries that enhance the overall biosynthetic capacity of the alga. For example, increased expression of certain nuclear genes has been shown to improve inorganic carbon fixation – the primary substrate for bioproduction [159]. Another study identified parameters for light-dependent accumulation of triacylglycerols (TAGs), an industrially important storage lipid, under overexpression of diacylglycerol acyltransferase [160]. The influence of cullins (structural

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components of ubiquitin ligases) on lipid metabolism under nutrient-deprivation conditions has also been characterized [161]. Overexpression of chloroplast glyceraldehyde-3-phosphate dehydrogenase substantially increased lipid production and biomass accumulation during nitrogen starvation [162]. α-Lipoic acid was shown to enhance lipid biosynthesis and mitigate the effects of salt stress [163]. Overexpression of the transcription factor MYB1 boosted expression of key genes involved in TAG, fatty acid, and starch biosynthesis, enabling cells to accumulate high levels of these metabolites as well as proteins, ultimately leading to elevated biomass productivity [164–166]. Fluorescent probes have been developed as a strategy for selecting algal cells with superior production properties, including higher growth rates and improved photosynthetic activity [167]. Another particularly interesting approach involves overexpressing fatty acid transporters – FAX1 and FAX2 in the chloroplast and ABCA2 in the endoplasmic reticulum, resulting in a twofold increase in TAG content [168]. A novel strategy for enhancing TAG production via membrane lipid remodeling, especially of the abundant mono- and digalactosyldiacylglycerol species, has also been described [169,170].

More targeted work has demonstrated the production of high-value compounds. Researchers have engineered and introduced into *C. reinhardtii* a metabolic pathway for synthesizing β-caryophyllene, a promising candidate for aviation biofuel [171].

Another study proposed an enzyme mixture – cellobiohydrolase CBM3GH5, β-glucosidase celB, endoglucanase B, and endoxylanase XynA, for converting *C. reinhardtii* cell walls into glucose. However, the current efficiency remains relatively low, reaching only about 17% of glucose from the initial lignocellulose mass [172]. For industrial hydrogen production, a new chimeric hydrogenase, consisting of segments from the hydrogenases of *Chlamydomonas reinhardtii* and *Scenedesmus obliquus*, has been proposed [173].

5. Conclusions

Overall, it can be stated that *C. reinhardtii* is becoming increasingly attractive for biotechnological applications, and this trend is accelerating. The number of publications reporting promising developments over the past five years has grown compared with earlier periods. Nevertheless, several significant limiting factors remain. Despite substantial progress in improving the microbiological aspects of *C. reinhardtii* cultivation, the challenge of maintaining high productivity and standardizing cultivation protocols during scale-up remains unresolved. Overcoming this barrier is essential for bridging the gap between well-established laboratory cultivation and efficient large-scale production.

At present, the most significant advances are occurring in the field of genetic and metabolic engineering of *C. reinhardtii*. Optimization of transformation protocols, development of new selectable markers, and creation of robust promoters certainly contribute to this progress. However, in our view, the most transformative developments bringing *C. reinhardtii* closer to large-scale biotechnological deployment include the application of CRISPR/Cas9 for precise genome editing, the discovery of epigenetic regulatory mechanisms influencing gene expression, strategies to circumvent strong intrinsic transgene silencing, and the establishment of marker-free selection systems.

Indeed, thanks to these advancements, the number of reports describing the production of valuable compounds in *C. reinhardtii* for use in medicine, cosmetics, the food and fuel industries, as well as in environmental bioremediation continues to grow. We are confident that in the coming years such reports will become even more numerous, and the biotechnological potential of *C. reinhardtii*, long discussed in the scientific community, will finally translate into real large-scale industrial applications.

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