# Exploring potential genetic routes for enhancing lipid accumulation in microalgae for biofuel application

**A thesis submitted for the degree of**

**Doctor of Philosophy in Energy Science and Engineering**

**By**

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## STATEMENT

I do hereby declare that the matter embodied in this thesis entitled **"Exploring potential genetic routes for enhancing lipid accumulation in microalgae for biofuel application"** is the result of investigations carried out by me in the School of Energy Science and Engineering, Indian Institute of Technology Guwahati, India, under the guidance of **Prof. Lingaraj Sahoo** and **Prof. Vaibhav V Goud.**

In keeping with the general practice of reporting scientific observations, due acknowledgement have been made wherever the work described is based on the findings of other investigators.

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## CERTIFICATE

**It is certified that the work described in this thesis, entitled "Exploring potential genetic routes for enhancing lipid accumulation in microalgae for biofuel application",** done by **Mr. Prabin Kumar Sharma** [Roll No. 156151006] for the award of degree of Doctor of Philosophy is an authentic record of the results obteined from the research work carried out under my supervision in the School of Energy Science and Engineering, Indian Institute of Technology Guwahati, India, and this work has not been submitted elsewhere for a degree.

August, 2021

**Prof. Lingaraj Sahoo**
(Thesis Supervisor)

**Prof. Vaibhav V Goud**
(Co-Supervisor)

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***Dedicated to my beloved parents***

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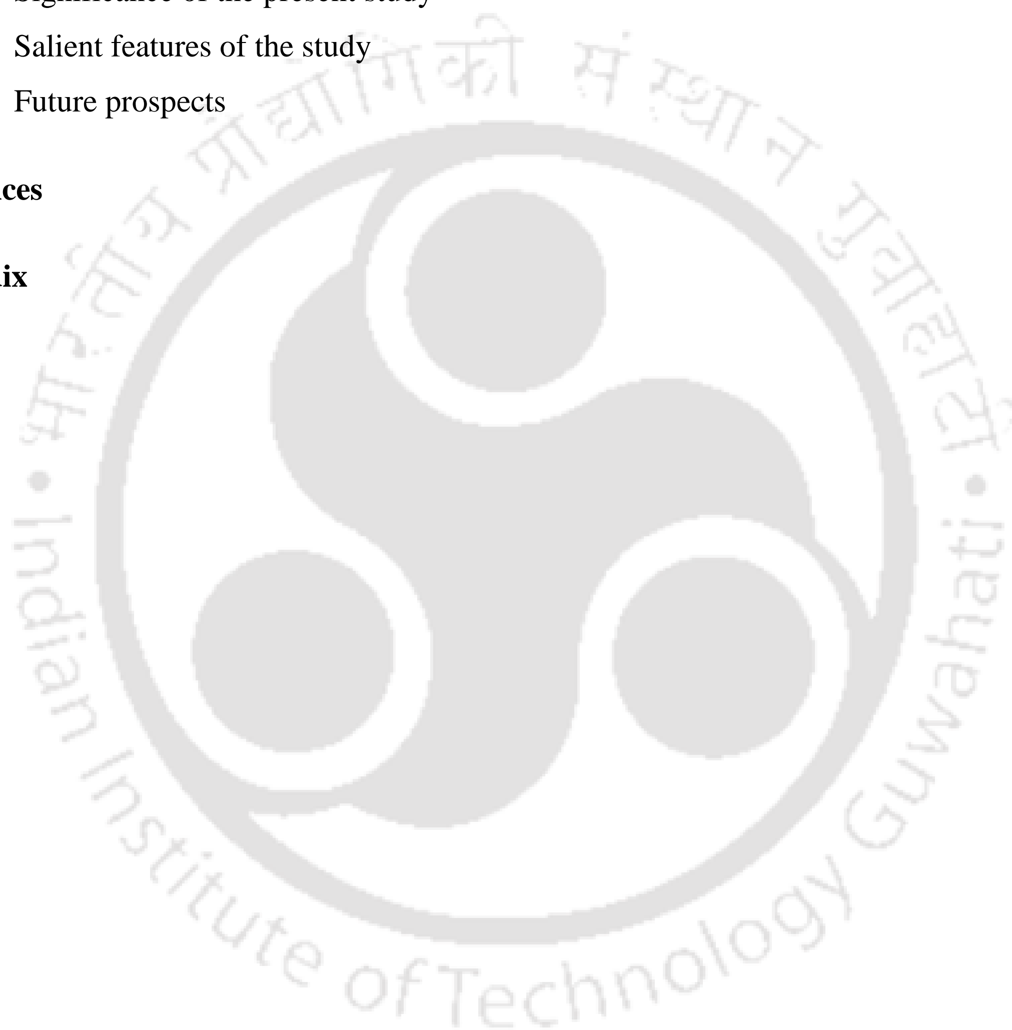

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# ABSTRACT

Depleting fossil fuels, soaring international crude oil prices, the energy crisis, and alarming global warming reports have upsurged global interest in alternative renewable energy sources. Biofuels made from photosynthetic organism-based feedstocks, including land plants and aquatic microalgae, provide enormous opportunities to meet the global energy demand, satisfying carbon-neutral solutions and enabling carbon dioxide ($CO_2$) sequestration from the atmosphere. Oil-rich microalgae are fast emerging as an alternative source of lipids/triacylglycerols (TAGs) for large-scale biodiesel production due to their ability to produce large biomass and lipids cells without competing for arable land. Despite having numerous advantages in biofuel and biorefinery production, the inherent low growth rate of oleaginous microalgae poses the biggest obstacle in its use as biofuel feedstock. Finding high biomass-producing algal strains with metabolic routes for carbon allocation to TAGs is essential to sustainable biofuel production. Improving lipid content in the wild strains of microalgae through genetic regulation of TAG biosynthesis could boost these strains' potential use for biofuel production. The lipogenic TAG biosynthetic pathway's targeted engineering has been considered the most straightforward strategy to increase lipid content in microalgal strains without compromising cellular physiological characteristics. The TAG biosynthetic pathway in microalgae is considered to be similar to that of higher plants. In the de novo TAG biosynthetic pathway, diacylglycerol acyltransferase (DGAT; EC 2.3.1.20) catalyzes the process's final and rate-limiting step. The DGAT enzyme is thought to be universal among all organisms in de novo TAG biosynthesis. Type 1 and type 2 DGAT enzymes are the two most studied classes of these enzymes. Although both forms of DGATs perform similar roles, their sequences and preferences have developed separately. Both forms of DGATs occur in plants, with DGAT1 being a significant contributor to TAG accumulation in seeds. In previous studies, ectopic



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expression of plant DGAT1 resulted in an increase in seed oil content and a shift in fatty acid composition, demonstrating the pivotal role of DGAT in TAG synthesis. Therefore, manipulating plant *DGAT1* gene expression could be beneficial for microalgae's metabolic engineering for increased oil content and customized fatty acid (FA) composition. Apart from that, in microalgae it was shown that simultaneous expression of five acyltransferases (phosphatidic acid phosphatase, LPAAT, glycerol-3-phosphate dehydrogenase, GPAT, and DGAT) resulted in a two-fold increase in lipid content. These co-expressing multiple enzymes exemplify the effectiveness of system-level control of metabolic flux toward lipid overproduction. Transcriptional regulation can have a similar effect on the systemic metabolomic flux as transcription factors can target multiple regulatory points in a metabolic pathway. Overexpression of genes encoding transcription factors targeting the up-regulation of downstream lipid biosynthesis genes can increase oil content. In this realm, WRINKLED 1 (WRI1) can be a suitable candidate to enhance the lipid content in microalgae as WRI1 is a master regulator of lipid accumulation in the seed of *Arabidopsis*. WRI1 is a member of the APETALA2-ethylene-responsive element-binding protein (AP2-EREBP) family and is controlled by LEAFY COTYLEDON2 (LEC2). WRI1, in turn, regulates fatty acid biosynthetic genes with promoters containing an AW-box sequence [CnTnG(n)7CG], such as *PKp-ß1, BCCP2,* and *KAS1*, in *Arabidopsis*.

In the present study, transgenic *C. sorokiniana* strains were developed that showed enhanced TAG accumulation properties by the overexpression of two genes namely, *DGAT1* from Jatropha and *WRI1* from *Arabidopsis*. A transformation protocol was also developed to generate transgenic *C. sorokiniana*. In *C. sorokiniana*, the lack of a reliable and efficient *Agrobacterium*-mediated gene transfer method limits its potential uses in commercial-scale utilization. In the present work, we described an efficient *A. tumefaciens*-mediated genetic transformation in *C. sorokiniana*. For the



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first time in *C. sorokiniana*, the transformation method highlighted the reliable detection of stable transgene integration and expression, which opens up limitless possibilities in biofuel production and other commercially valuable commodities. Furthermore, considering the crucial role of *Agrobacterium vir* gene induction on effective T-DNA transfer, we optimized crucial cocultivation conditions that influence efficient T-DNA transfer to *C. sorokiniana* cells. We found cocultivation of *C. sorokiniana* cells ($OD_{680}$=1.0), on BG11 agar medium with pH 5.6, supplemented with 100 µM of acetosyringone, for three days at 25 ± 2 °C in the dark, with *Agrobacterium* at a cell density of $OD_{600}$ = 0.6 resulted in the efficient transformation of *C. sorokiniana*. Our finding opens up new possibilities for microalga with commercial potentials such as *C. sorokiniana* through genetic manipulation for specific target genes associated with diverse cellular pathways.

The second phase of investigation involved heterologous expression of the *DGAT1* gene from Jatropha in *C. sorokiniana* to enhance the lipid content. Transgene integration and expression in hygromycin selected cells were confirmed by PCR, Southern blot, and spectro-fluorometric analysis of Nile red-stained cells. The transformed microalgal cells showed a 1.2-fold increase in total lipid content than that of untransformed control. An increase in mono and poly-unsaturated fatty acids, primarily linolenic acid content up to a 1.16-fold in the transformed cells than WT, makes the transgenic microalgae-derived oils ideal for combustion of its biofuel. The enhanced lipid content and unsaturated fatty acids did not significantly impair growth and photosynthetic rate in transformed lines. The results offer a valuable strategy for enhancing oil production and might facilitate a platform strain with industrial potential.

The third phase of investigation involves heterologous expression of Arabidopsis *WRI1* gene in *C. sorokiniana* to increase lipid production. *AtWRI1* seems to affect TAG synthesis and total lipid contents in *C. sorokiniana*. Total lipid content in the transformants was increased up to 1.23-fold



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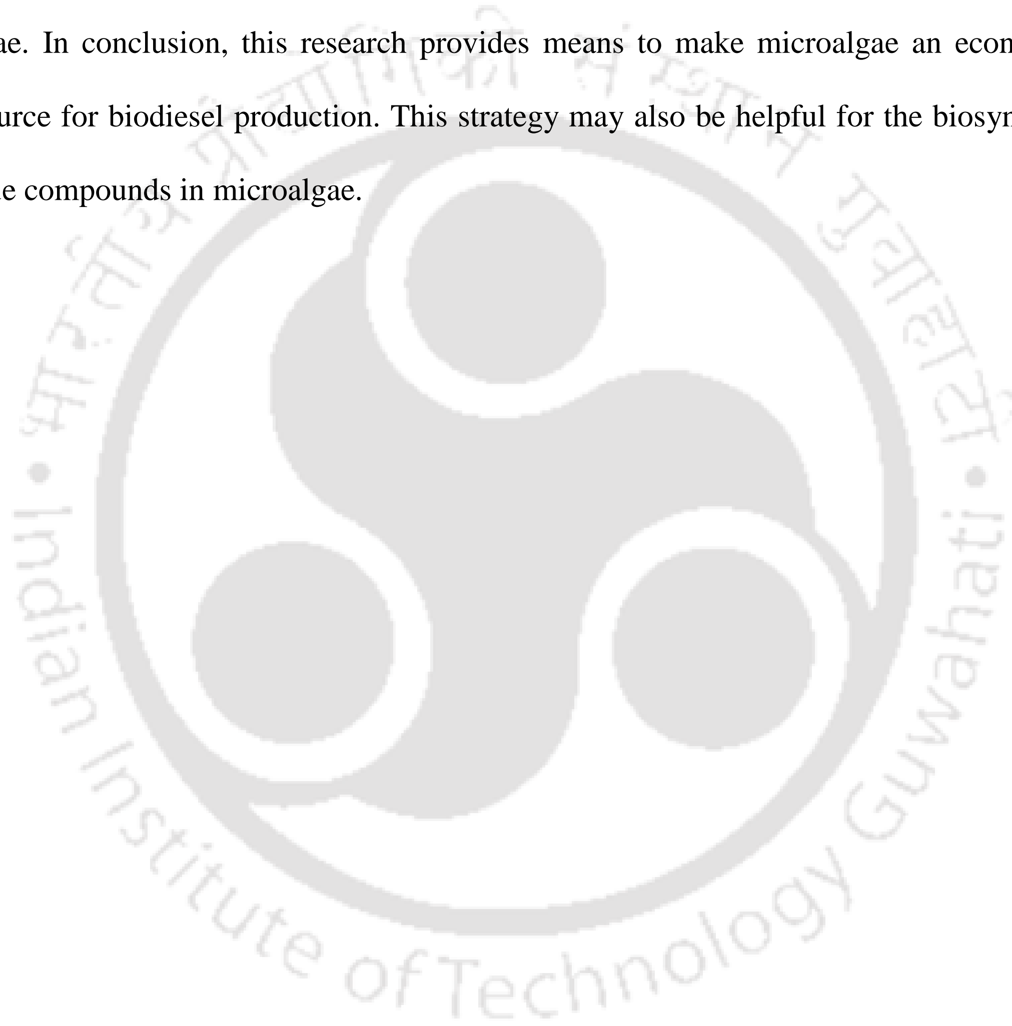
from that of control including significant increase in unsaturated fatty acids. Enhanced lipid content and rise in unsaturated fatty acids content did slightly impair growth in the mid-exponential phase in transformed cells, however the photosynthetic rate remained unaffected in the transformed cells. The results provided an excellent proof of concept for heterologous expression of key regulatory transcription factors from plants to improve lipid production in industrial microalgae. In conclusion, this research provides means to make microalgae an economically viable source for biodiesel production. This strategy may also be helpful for the biosynthesis of high-value compounds in microalgae.



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# ABBREVIATIONS

| | |
|---|---|
| g | Gram |
| mg | Milligram |
| µg | Microgram |
| DNA | Deoxyribonucleic acid |
| RNA | Ribonucleic acid |
| PCR | Polymerase chain reaction |
| qRT-PCR | Quantitative real-time PCR |
| TAG | Triacylglycerol |
| dNTP | Deoxyribonucleotide triphosphate |
| cDNA | Complementary DNA |
| T-DNA | Transferred DNA |
| gDNA | Genomic DNA |
| bp | Base pair |
| kb | Kilobase pair |
| L | Liter |
| mL | Milliliter |
| M | Molar |
| mM | Millimolar |
| nm | Nanometer |
| mg/L | Milligram per liter |
| v/v | Volume /volume |
| w/v | Weight/volume |
| hptII | Hygromycin phosphotransferase |
| GUS | Β-glucoronidase |
| DIG | Digoxygenin |
| CTAB | Cetylrmethyl ammonium bromide |
| CaMV | Cauliflower mosaic virus |
| OD | Optical density |
| SSC | Sodium chloride and sodium citrate buffer |
| FAME | Fatty acid methyl esters |
| FA | Fatty acid |
| TLC | Thin layer chromatography |
| GC-MS | Gas chromatography mass spectroscopy |
| FT | Furrier transform |
| WT | Wild-type |
| DGAT | Diacylglycerol acyltransferase |



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| | |
|---|---|
| WRI | Wrinkled |
| EST | Expressed sequenced tag |
| rRNA | Ribsomal-ribonucleic acid |
| LC-PUFA | Long-chain polyunsaturated fatty acid |
| $CO_2$ | Carbon dioxide |
| CRISPR | Clustered regularly interspaced palindromic sequences |
| Cas9 | CRISPR-associated protein 9 |
| TAP | Tris-acetate–phosphate |
| PAGE | Polyacrylamide gel electrophoresis |
| DCW | Dry cell weight |
| NMR | Nuclear magnetic resonance |
| kDa | Kilo Dalton |
| PSII | Photosystem II |
| VirG | Virulence G gene |
| NPQ | Non-photochemical quenching |

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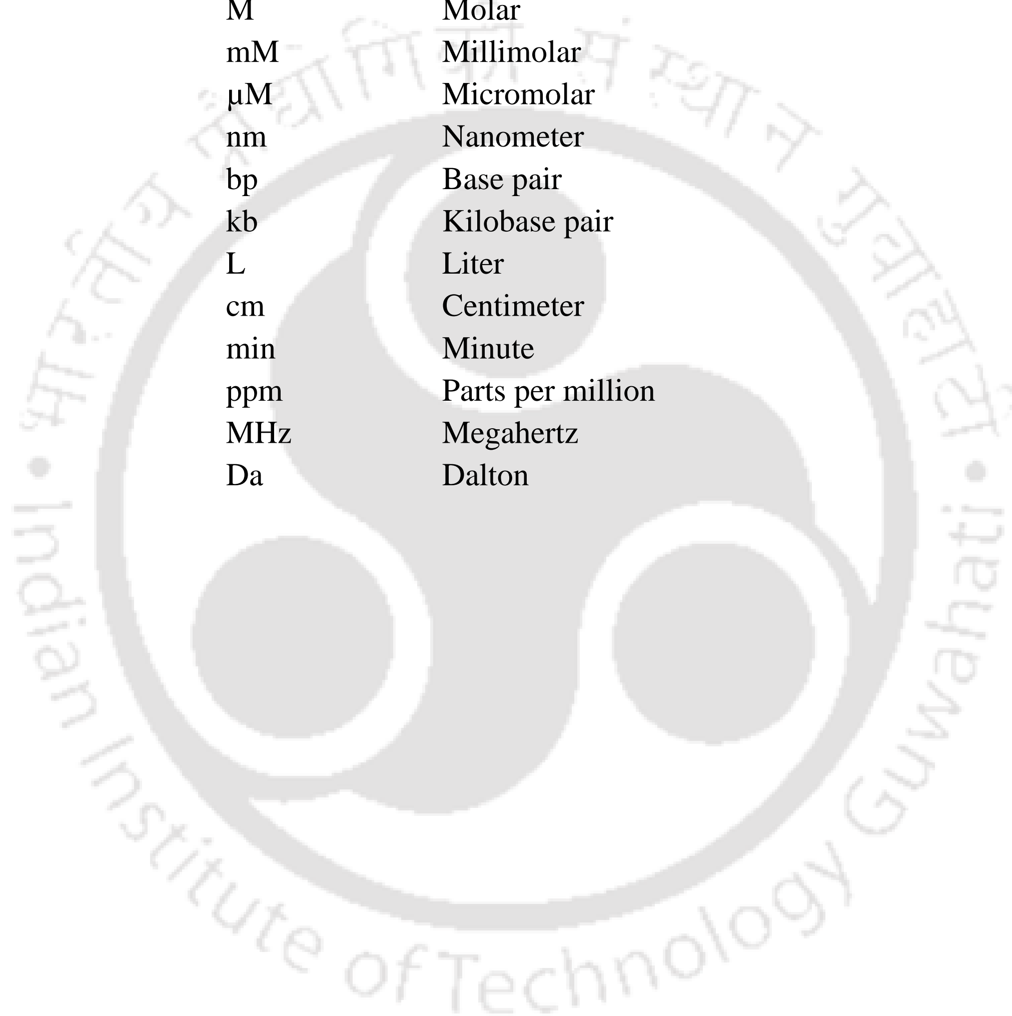
# UNITS

| | |
|---|---|
| g | Gram |
| mg | Milligram |
| µg | Microgram |
| °C | Degree celsius |
| µL | Microliter |
| M | Molar |
| mM | Millimolar |
| µM | Micromolar |
| nm | Nanometer |
| bp | Base pair |
| kb | Kilobase pair |
| L | Liter |
| cm | Centimeter |
| min | Minute |
| ppm | Parts per million |
| MHz | Megahertz |
| Da | Dalton |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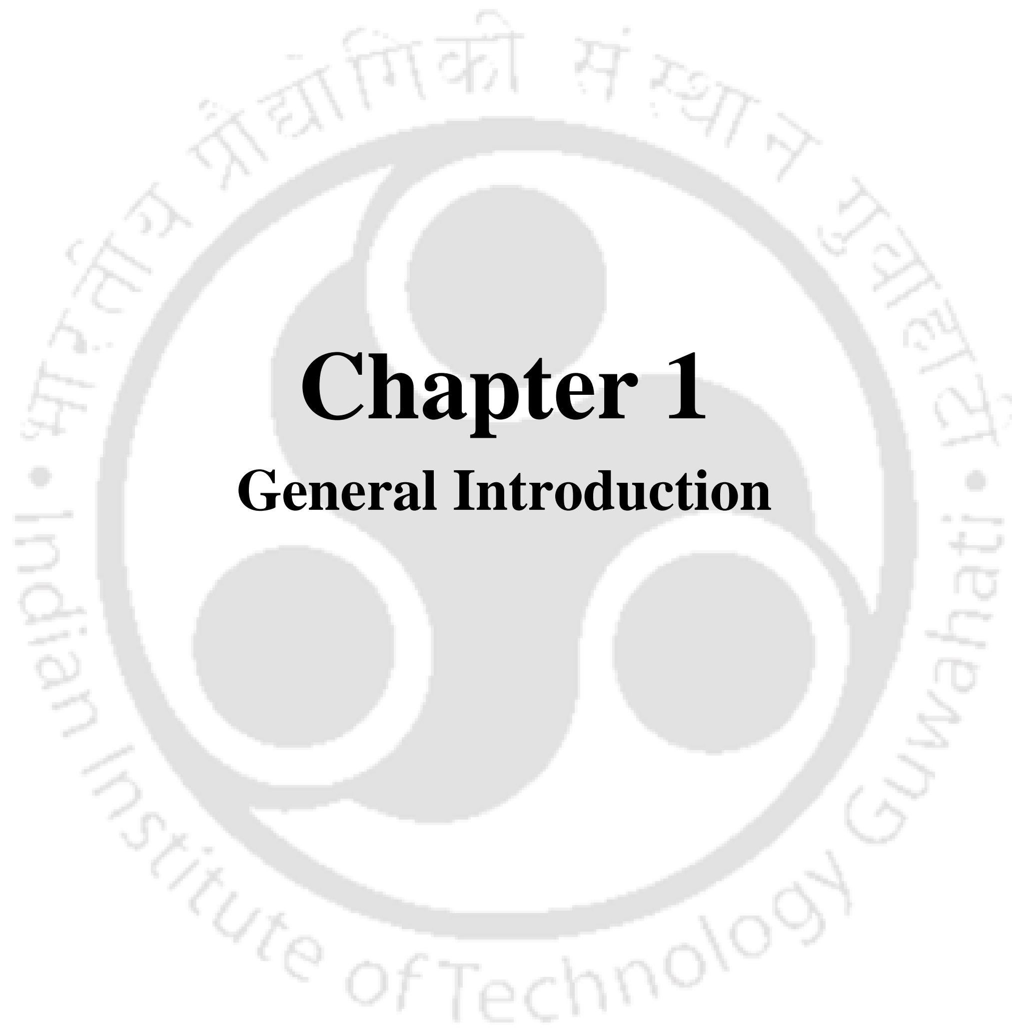


# Chapter 1

## General Introduction

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## 1.1 Introduction

Depleting fossil fuels, soaring international crude oil prices, the energy crisis, and alarming global warming reports have upsurged global interest in alternative renewable energy sources (Behera et al., 2015). Biofuels made from land plants and aquatic microalgae-based feedstocks provide enormous opportunities to meet the global energy demand, satisfying carbon-neutral needs and enabling carbon dioxide ($CO_2$) sequestration from the atmosphere (Stephenson et al., 2011; Ravindran et al., 2017). Oil-rich microalgae are fast emerging as an alternative source of lipids/triacylglycerols (TAGs) for large-scale biodiesel production due to their ability to produce large biomass and lipids cells without competing for arable land (Zienkiewicz et al. 2016). Their capacity to recycle atmospheric $CO_2$ through high photosynthetic efficiency, sequester greenhouse gases, absorb nutrients from wastewater, store significant amounts of starch for effective utilization in ethanol production and biosynthesis of high-value bioactive metabolites have attracted increased attention (Shaikh et al. 2019). Microalgae withstand adverse environmental conditions owing to TAGs, the typical lipids with a storage function in the cells (Sharma et al. 2012). Despite having numerous advantages in biofuel and biorefinery production, the low growth rate inherent of oleaginous microalgae poses the biggest obstacle in its use as biofuel feedstock (Pribyl et al. 2012). Nutrient availability (Courchesne et al. 2009), light- and temperature-dependent growth (Randhawa et al. 2017), and stress-inducible lipid accumulation (Shaikh et al. 2019) in microalgal cells impose additional constraints in their utilization for commercial-scale production of biofuel and bio-commodities. Developing high biomass-producing algal strains with metabolic routes for carbon allocation to TAGs hold the key to sustainable biofuel production



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(Pribyl et al. 2012). Improving lipid content in the wild strains of microalgae through genetic regulation of TAG biosynthesis could provide the genetic means to boost their use in biofuel production (Zhu et al. 2016).

The oleaginous microalgae, *Chlorella sorokiniana*, is a highly productive species among the most studied microalgae genus, *Chlorella*, stated by various consortiums (Rosenberg et al. 2014). The species of the *Chlorella* genus of green algae (Chlorophyceae), with both photoautotrophic and heterotrophic mode of growth, are widely utilized for production of nutritional supplements (Cordero et al. 2011), bioremediation (Naidoo et al. 2019), and as model organism for studying basic microalgal physiology and metabolism (Rosenberg et al. 2014). Among various *Chlorella* species, the whole genome of *C. sorokiniana* is fully sequenced, annotated, and publicly available allowing comparative genomics study for biofuel applications (https://greenhouse.lanl.gov/greenhouse/organisms). Genome sequencing and annotation projects have enabled the reconstruction of metabolic pathways, and widespread use of RNA-Seq methods has allowed a more accurate prediction of microalgal metabolisms (Merchant et al. 2012).

Genetic engineering over the years has emerged as practical means to complement conventional screening for microalgae strains with commercially valuable traits. It is now well documented that overexpression of enzymes involved in lipogenesis can significantly increase the oil productivity in microalgae (Li et al., 2016; Chen et al., 2016; Zhu et al., 2018). Particularly, overexpression of diacylglycerol acyltransferase 1 (DGAT1), rate limiting enzyme for TAG biosynthesis significantly increased TAG accumulation in microalgae (Úbeda-Mínguez et al., 2017; Zulu et al., 2018). Simultaneous expression of five acyltransferases (phosphatidic acid phosphatase, LPAAT,



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glycerol-3-phosphate dehydrogenase, GPAT, and DGAT) resulted in a two-fold increase in lipid content (Hsieh et al., 2012). The co-expression of these enzymes indicated the effectiveness of system-level control of metabolic flux toward lipid overproduction. Transcriptional regulation can have a similar effect on the systemic metabolomic flux as transcription factors can target multiple regulatory points in a metabolic pathway. Overexpression of genes encoding transcription factors targeting the up-regulation of downstream lipid biosynthesis genes can increase oil content. In this context, WRINKLED 1 (*WRI1*) identified as master regulator of lipid accumulation in the seed of *Arabidopsis*, has emerged as a suitable candidate TF to enhance the lipid content in microalgae. Previously, heterologous expression of Arabidopsis *WRI1* gene shown to enhance lipid accumulation in microalgae (Kang et al. 2017). Advances in our understanding of genetic regulation of TAG biogenesis creates opportunities for genetic manipulation of oil biosynthesis for enhanced oil accumulation in microalgae. This may help in surpassing existing limitations in screening high-oil synthesizing microalgae strains and minimize the time required to generate a commercially valuable microalgae strain.

The functional characterization of genes involved in TAG biosynthesis and photosynthesis (Courchesne et al. 2009), generation of expressed sequenced tag (EST) databases, prediction of de novo chloroplast TAG biosynthesis (Zienkiewicz et al. 2016), and identification of transcription factors involved in oil metabolisms have brought new opportunities to harness the benefits of algal biotechnology (Bajhaiya et al. 2017). However, development of efficient DNA transfer method stably integrating transgenes in microalgae is a prerequisite for improvement of wild strains, understanding basic metabolic processes underpinning growth, photosynthesis, and oil



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biosynthesis, and the production of high-value bioactive metabolites and recombinant proteins (Ng et al. 2017). Except for a few model algae species (Mini et al. 2018) and diatoms (Han et al. 2020), the method of introducing DNA into eukaryotic microalgae is still far from routine (Sharma et al. 2018).

Therefore, the present study was undertaken to establish an efficient gene transfer method based on stable DNA integration through *Agrobacterium*-mediated transformation in *C. sorokiniana*. The crucial parameters known to influence efficient T-DNA transfer, including the type of cocultivation medium, acetosyringone concentration, and co-culture duration were optimized.

The method developed was employed to overexpress Jatropha *DGAT1* and *Arabidopsis WRI1* genes under constitutive CaMV35S promoter to develop transgenic *C. sorokiniana* for enhanced accumulation of TAG.

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## 1.2 OBJECTIVES

The present investigation was undertaken with the objectives to develope transgenic *Chlorella sorokiniana* overexpressing *Jatropha curcus diacyl glycerol acyl transferase 1* (*JcDGAT1*) and *Arabidopsis thaliana wrinkled 1* (*AtWRI1*). The salient objectives outlined as:

- Development of *Agrobacterium*-mediated gene transfer system for *C. sorokiniana* CG12.
- Overexpression of the rate limiting enzyme of oil biosynthesis for improving lipid production in *C. sorokiniana* CG12.
- Transcriptional regulation of oil biosynthesis pathway for enhanced lipid production in *C. sorokiniana* CG12.



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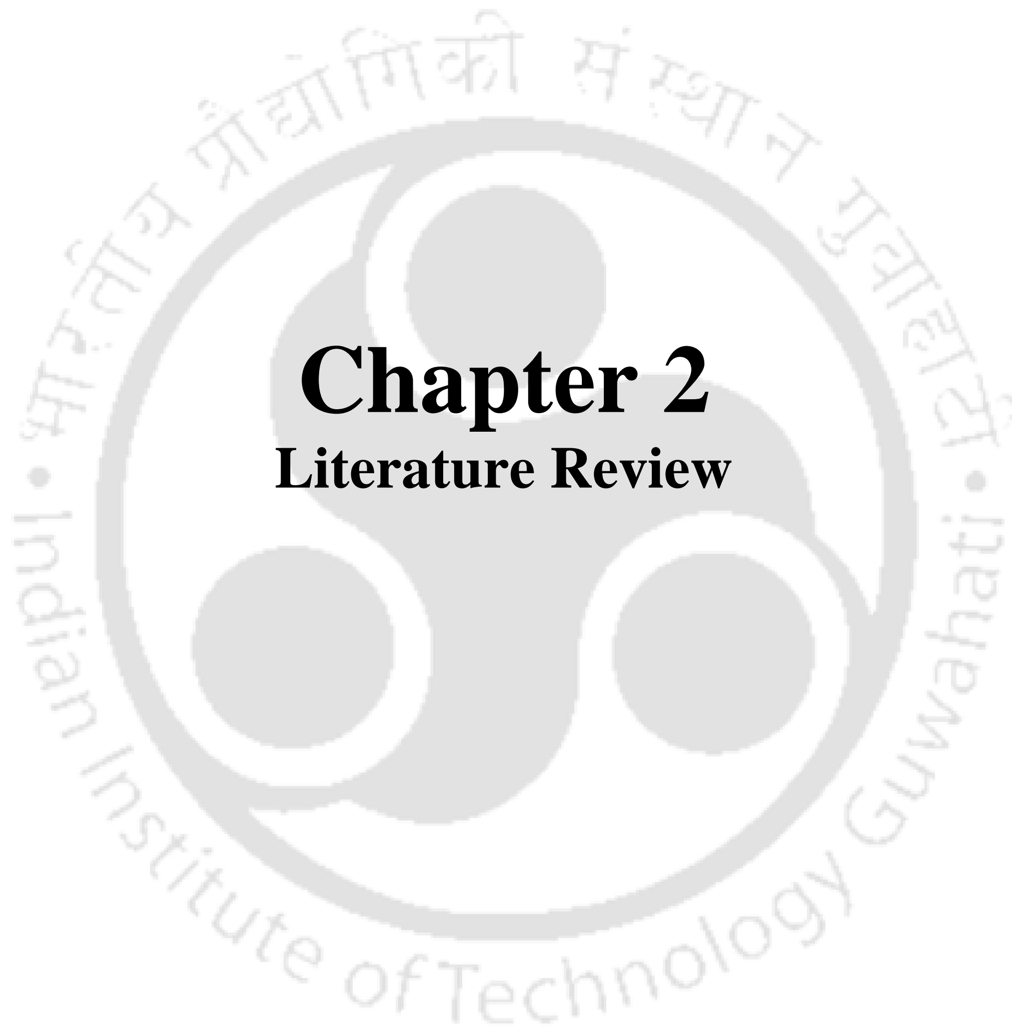
# Chapter 2
## Literature Review



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Biodiesel is a proven fuel that has replaced fossil fuels in few instances and has emerged as the most viable alternative that can mitigate global energy crisis. It offers unique opportunities in energy generation due to its sustainable, eco-friendly and renewable nature. The renewed interest in biodiesel production has resulted in increased demand for quality feedstock materials. Biodiesel is usually produced from oil crops, waste cooking oil and animal fats, but realistically these feedstocks cannot satisfy the current demand as it would require unsustainably large land areas for oil crops cultivation. Microalgae being an aquatic organism dramatically improves the scenario, if they are used as a feedstock. In reference to **Table 2.1**, microalgae appears to have the potential to completely replace petroleum diesel.

**Table 2.1** Comparison of some sources of biodiesel (Chisti, 2007)

| Crop | Oil yield (L/Ha) | Land area needed (M ha) [a] | Percentage of existing US cropping area [a] |
|---|---|---|---|
| Corn | 172 | 1540 | 846 |
| Soybean | 446 | 594 | 326 |
| Canola | 1190 | 223 | 122 |
| Jatropha | 1892 | 140 | 77 |
| Coconut | 2689 | 99 | 54 |
| Oil palm | 5950 | 45 | 24 |
| Microalgae[b] | 136,900 | 2 | 1.1 |
| Microalgae[c] | 58,700 | 4.5 | 2.5 |

[a] For meeting 50% of all transport fuel needs of the U.S.
[b] 70% oil (by wt) in biomass.
[c] 30% oil (by wt) in biomass.



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## 2.1 Microalgae

Microalgae are a polyphyletic diverse group of aquatic and autotrophic microorganisms (**Figure 2.1**) that are known as primary producers in aquatic ecosystems and also fixes atmospheric carbon dioxide. The vast diversity in microalgal species results in a wide diversity in metabolism and metabolites that can be tapped for biosynthesis of valueble products.

The evolutionary origin of microalgae is thought to be around 3 billion years ago with the emergence of first prokaryotic cyanobacteria ancestors and then around 1 to 1.5 billion years ago the eukaryotic microalgae evolved. The well-accepted theory of endosymbiosis is thought to be the reason for the origin of vast diversity in microalgae.

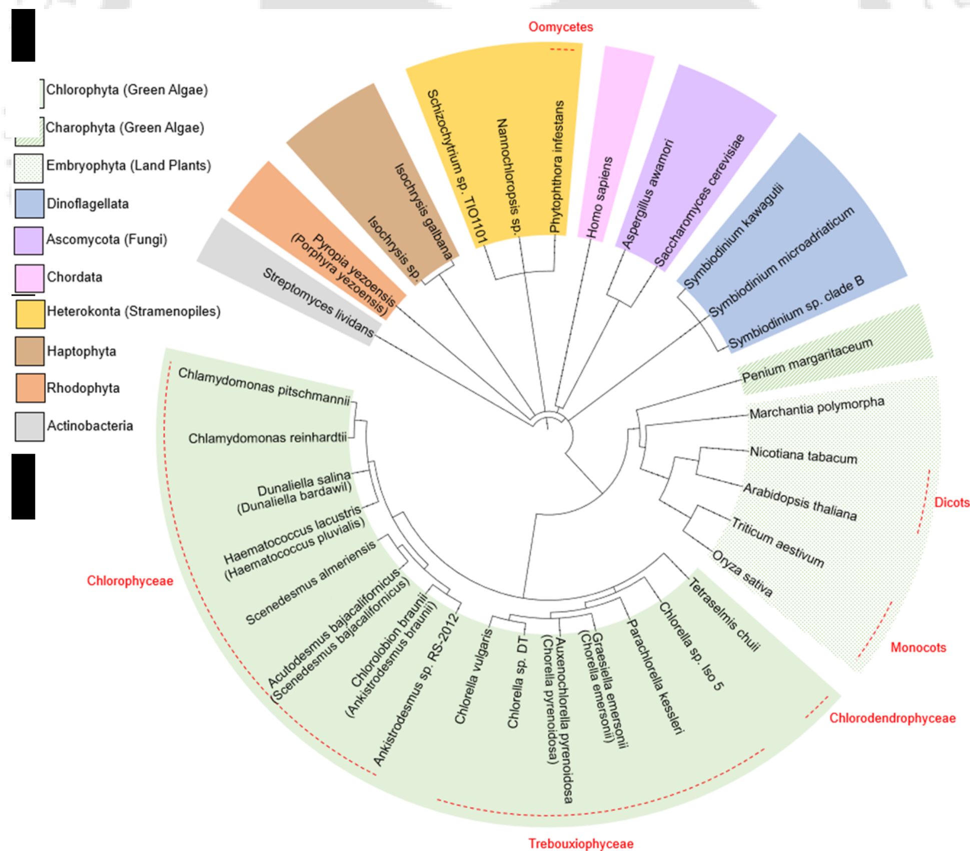

**Figure 2.1** Microalgal diversity on the eukaryotic tree of life. Examples across common different groups are shown. Tree information was generated from NCBI taxonomy using the tool phyloT (available at http://phylot.biobyte.de/index.html) database version 2017.7 (biobyte solutions GmbH). Tree output was visualized using iTOL (Interactive tree of life, available at http://itol.embl.de/ (Letunic and Bork, 2016)).

The present estimates of the total microalgae species are between 200,000 and 800,000 of which approximately 35,000 species have been identified and that is only a fraction of the true number of microalgal species present in various ecosystems (Ebenezer et al., 2012). The vast diversity in microalgae aided in survival under diverse niches, such as snow algae, acidophiles, marine, and freshwater ecosystems. Microalgae are a significant contributor to Earth's net primary production (Falkowski et al., 2004) which signifies their ecological importance.

Previously, taxonomic classification of microalgae was much challenging as it was largely relied on pigmentation and cellular morphology. Those phenotypes can be shared between two distantly related microalgae and also may change depending on the growth conditions. With the availability of molecular phylogenic techniques such as barcoding using the ITS-2 regions of rRNA has cleared the boundaries between 'strain' and 'species'. However, evolutionary forces may act in a different way than in higher organisms, thereby microalgae with similar genetic makeup may have different physiology depending upon the environment of the culture collection site (Brand et al., 2013).

### 2.1.1 Commercial applications of microalgae

Microalgae have found numerous commercial applications (summarized in **Figure 2.2**) owing to their property of high productivity compared to other terrestrial systems (Sayre, 2010). Potential of microalgae as a cell factory has been realized recently, as they can effectively convert sunlight



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into valuable products without competing for arable land. Apart from that, microalgae can also facilitates a platform for expression of foreign proteins.

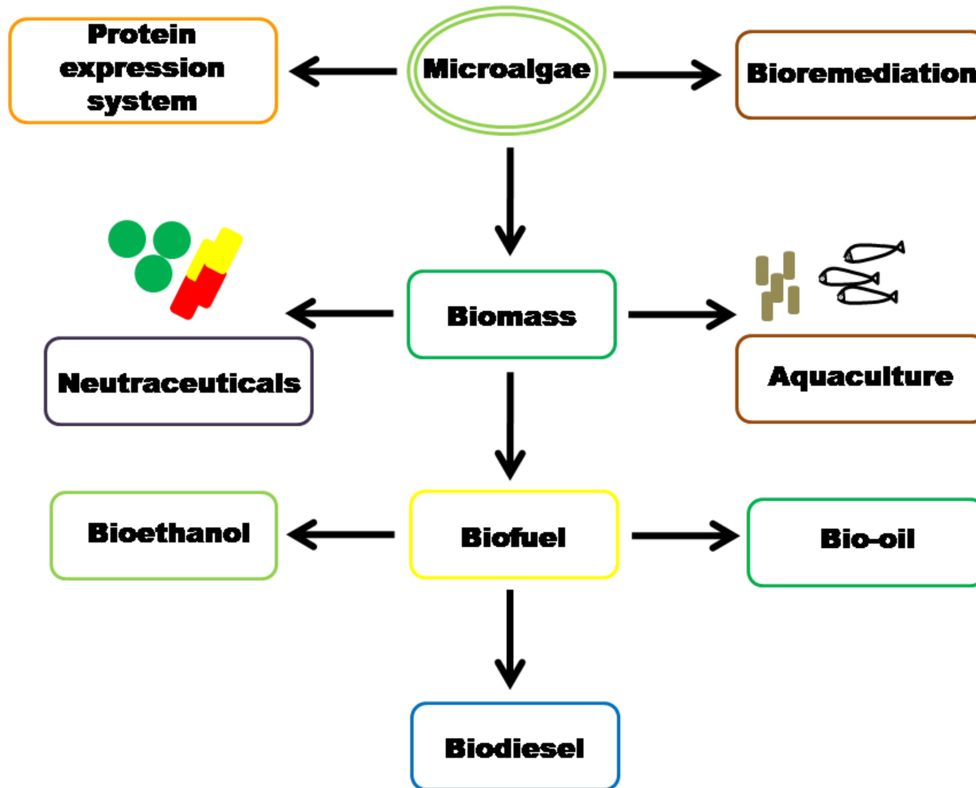


**Figure 2.2** Key biotechnological application of microalgae with a focus on valuable bioproducts.

### 2.1.2 *Chlorella sorokiniana*

The oleaginous microalgae, *Chlorella sorokiniana*, is a highly productive species among the most studied microalgae genus, *Chlorella* (Rosenberg et al. 2014; Barry et al. 2015; de Andrade and de Andrade 2017). This species of the *Chlorella* genus of green algae (Chlorophyceae), is a non-flagellated spherical microalgae with both photoautotrophic and heterotrophic mode of nutrition,



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and are widely utilized for production of nutritional supplements (Cordero et al. 2011), bioremediation (Naidoo et al. 2019), and as a model organism for studying basic knowledge on microalgal physiology and metabolism (Rosenberg et al. 2014). **Figure 2.3** presents a photograph of *C. sorokiniana CG12* strain taken using an optical microscope [Leica].

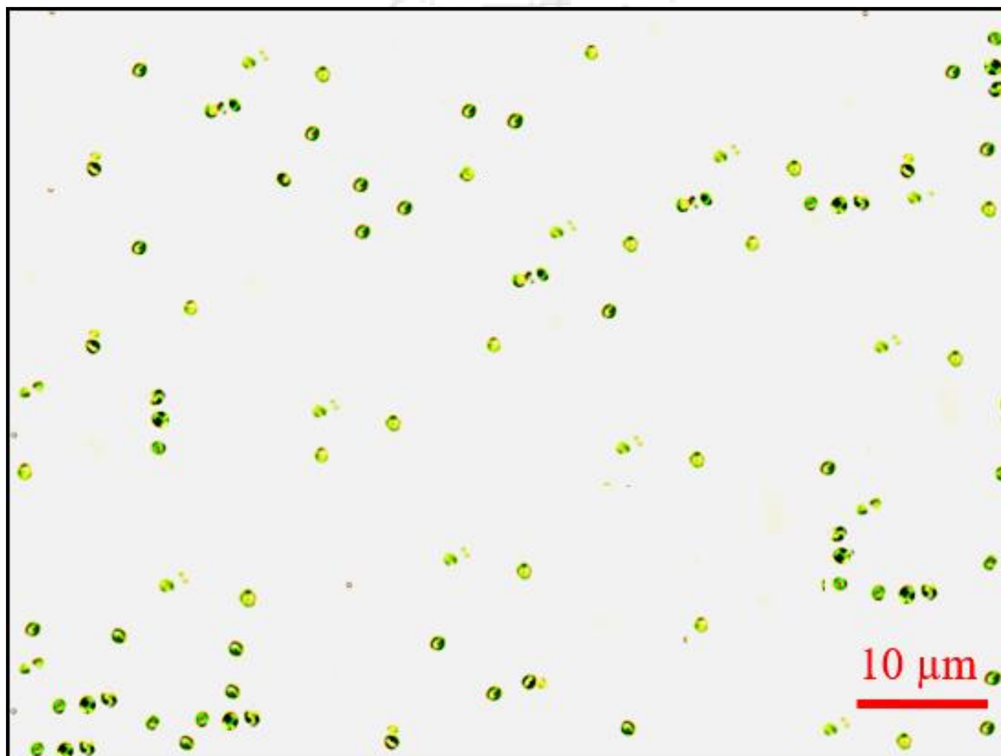


**Figure 2.3** A culture of *Chlorella sorokiniana* strain CG12

Among various *Chlorella* species, the whole genome of *C. sorokiniana* is fully sequenced, annotated, and publicly available allowing comparative genomics study for biofuel applications (https://greenhouse.lanl.gov/greenhouse/organisms).

For the first time in 1953, *Chlorella sorokiniana* was characterized by Sorokin and Myers as a putative *Chlorella pyrenoidosa* strain. Since its characterization, this species of *Chlorella* genus



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has been falsely described as a thermophilic mutant of *Chlorella pyrenoidosa* (Kunz, 1972) until DNA analysis officially established the strain of *Chlorella* as a separate species (Dorr and Huss, 1990; Kessler, 1985). Lack of trademark morphological features along with asexual reproduction mode has made conventional taxonomic classification of *Chlorella* very difficult and error prone. In order to remove ambiguities, modern molecular taxonomic approaches are followed to correctly identify different species of the *Chlorella* genus. The taxonomic classification for *C. sorokiniana* is described in **Figure 2.4**. Currently, the most efficient identification method is to analyze the differences in conserved and variable regions within the 16S rRNA and 18S rRNA gene (Burja et al., 2001; Volker Huss, 1999).

Apart from pharmaceutical and industrial applications for bioproducts, recently *C. sorokiniana* has gained interest as a potential feedstock for biofuel due to its high lipid content which is around 18-22% of its dry biomass (Gouveia and Oliveira, 2009). This ratio can be modified through changing growth parameters as shown by Feng and Johns (1991).



**Figure 2.4** Taxonomic classification of *Chlorella sorokiniana*.



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## 2.2 Microalgae as a biofuel source

Triacylglycerol is the primary form of energy storage in microalgal cells, which comprises 60–70% of the dry cell weight (Hu et al., 2008; Scott et al., 2010). Each TAG molecule consists of a glycerol backbone to which three fatty acid (FA) moieties are anchored (**Figure 2.5**). Each FA molecule is classified, depending on the degree of unsaturation, as either saturated FA (SFA), monosaturated FA (MUFA), or polyunsaturated FA (PUFA). Therefore, relative abundance of these diverse FAs in TAG decides the utility of TAG molecules for specific applications, including their use as transportation fuel, high value nutrient supplements, emulsifiers, and industrial polymers. Microalgae-derived biomass can supply a wide range of biofuels such as biodiesel, bioethanol, biohydrogen, biomethane, and bioelectricity. Among all projected applications, microalgae derived oils are the most promising for the production of biofuel (Shuba and Kifleb, 2018). Therefore, current microalgal studies worldwide primarily focus on enhancing lipid accumulation in microalgae under diverse growth conditions for higher oil production.

### 2.2.1 Biofuels from microalgae

Some species of microalgae are known to be rich in lipids (stored as TAGs) and lipids derived from these microalgae are used for production of biofuel, known as 3$^{rd}$ generation of biofuels. The first and second generations of biofuels were derived from edible (eg. palm and canola) and non-edible crops (eg. Jatropha) which have their own merits and demerits. The advantages of microalgal biofuels over its counterparts are their higher productivity, shorter growing seasons, minimum requirement for arable land, and ability to integrate into waste-water bioremediation systems (Chen et al., 2015; Kiran et al., 2014; Maity et al., 2014; Rawat et al., 2013).



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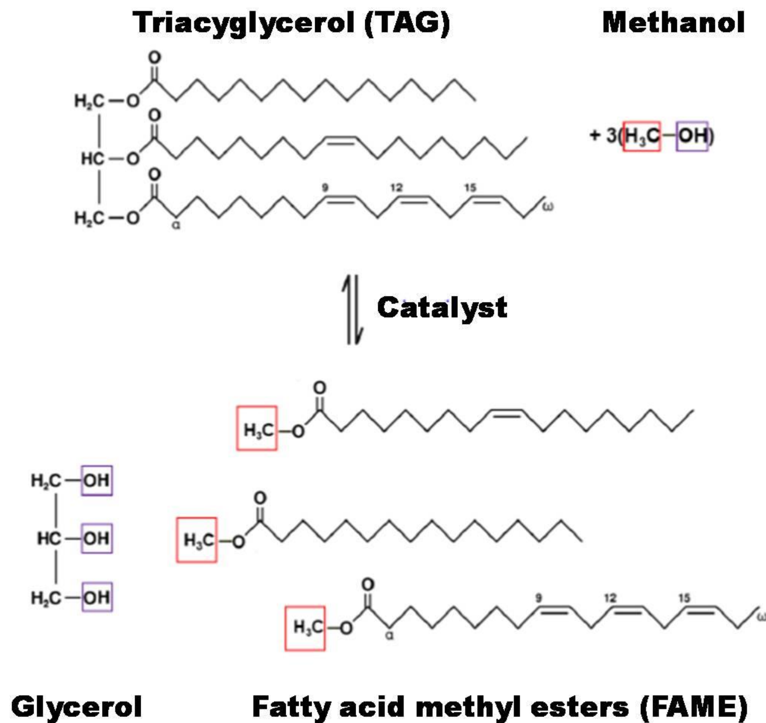


**Figure 2.5** Transesterification of TAG to FAME

Apart from that, the carbohydrate content in microalgal biomass ranges from 5-80% and consists mainly of polysaccharides and starch which are biofuel production friendly than the lignocellulosic contaminants present in feedstocks derived from plants (Tang et al., 2016).

### 2.2.2 Economic consideration of microalgal biofuel

Around the world there are multiple commercial biofuel pilot projects running successfully (Su et al., 2017), despite these commercialization of microalgae biofuel is still economically unviable



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owing to its high production cost. One reason for high production cost is the difficulty in maintaining high operational density during culture conditions due to reduced light penetration and problems with maintaining optimal pH. These issues results in three to eight times reduced biomass yield than that of the theoretically predicted yield (Show et al., 2017). Higher cell densities of microalgae can be achieved through mixotrophic and heterotrophic growth strategies, however that will impact the cost-effectiveness of the system. Current research on developing or screening of a microalgae strain aims on using cheap and waste carbon sources such as glycerol (Paranjape et al., 2016). However, such strategies can results in bacterial or fungal contamination in open-pond setup (Chew et al., 2018). Use of bioreactors can avoid such problems but the input and operational costs of bioreactor setups are high.

Another reason for high production cost is due to unavailability of an ideal microalgae strain with required features for industrial application, such as high salt-stress resistance, high productivities, and accumulation of high-value products and presence of auto-flocculation property that aid in harvesting process (**Figure 2.6**). In nature, all these characteristics are present in different microalgae species rather than in a single species making the process economically unviable (Lim and Schenk, 2017).

It is a general consensus that implementation of biorefinery approach in microalgae biofuel production may reduce the operational cost through combining a low value product like biofuel with a high value product like lutein. Further, in order to be competitive with conventional fossil fuel, integration of waste-water treatment and carbon sequestration technology with the microalgae biofuel process is necessary (Chen et al., 2015; Zhu, 2015). Due to these the current research interests have shifted towards diversification of products from microalgae with commercial value



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and this seems to be essential for microalgal biofuels to remain economically viable (Su et al., 2017).

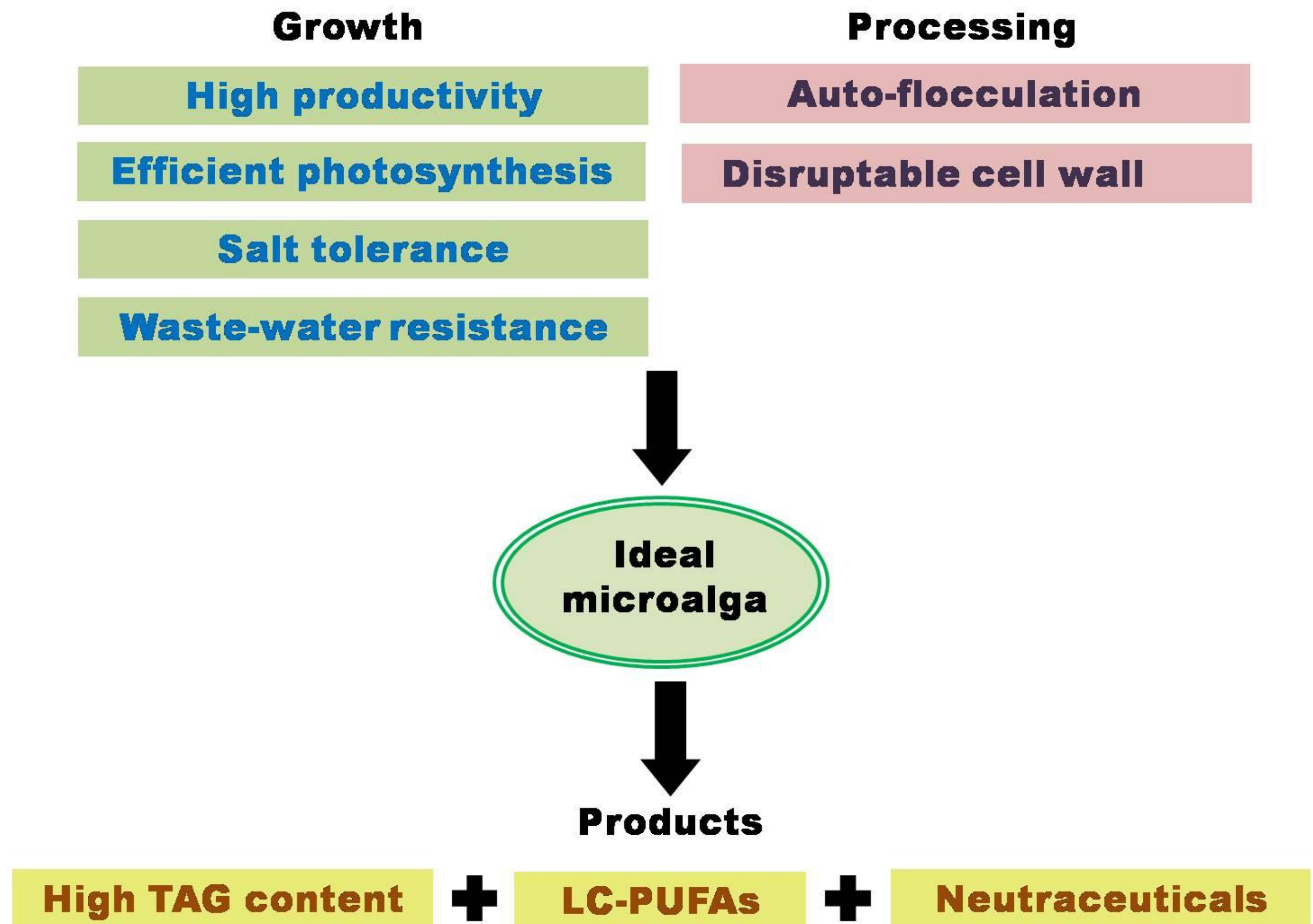


**Figure 2.6** Characteristics of an ideal microalga for production of bioproducts.

### 2.2.3 Nutritional and high-value products from microalgae

Microalgae world has a diverse range in biomass composition, for some species such as *Chlorella* sp. can contain upto 70% of dry weight as protein including all the essential amino acids (Wells et



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al., 2017). Apart from use in food supplements, microalgae such as *Chlorella vulguris* are shown to have anti-cancer and immune-boosting properties (Safi et al., 2014).

The use microalgal biomass is not only limited to human food supplements, in aquaculture industries microalgae are used as animal feed. However, the value of microalgal food supplements far exceeds that of aquaculture feeds (Sathasivam et al., 2019). In **Table 2.2**, few of the high-value products extracted from different microalgae species are mentioned. As most of these products are in research and development stage, lack of a regulatory framework may results in marketing of these products by poorly-regulated companies which ultimately can undermine the ethics of microalgal research.

**Table 2.2** Prominent microalgae species for production of high-value products.

| Sl no. | Microalgae species | Products | Status of R&D |
|---|---|---|---|
| 1 | *Haematococcus pluvialis* | Nutraceutical Astaxanthin | Commercially successful |
| 2 | *Parietochloris incisa* | Arachidonic acid | Physiology and genetic studies done |
| 3 | *Chlorella zofingiensis* | Astaxanthin, lutein, PUFA | Small and pilot scale production |
| 4 | *Scenedesmus* sp. | Lutein, carotenoids, PUFA | Pilot scale production |
| 5 | *Phaeodactylum tricornutum* | EPA, fucoxanthin | Physiology and genetic studies done |
| 6 | *Odontella aurita* | Fucoxanthin, LC-PUFA | Marketed as nutraceutical |
| 7 | *Haslea ostrearia* | Fucoxanthin, LC-PUFA | Physiology and genetic studies done |
| 8 | *Nannochloropsis* sp. | EPA, aquaculture feed | Pilot scale production |
| 9 | *Trachydiscus minutus* | EPA | Model organism or EPA production |
| 10 | *Pavlova lutheri* | EPA and DHA | Marketed as nutraceutical |
| 11 | *Isochrysis galbana* | Fucoxanthin, DHA | Pilot scale production |
| 12 | *Dunaliella* sp. | Aquaculture feed | Marketed as Aquaculture feed |
| 13 | *Chaetoceros* sp. | Aquaculture feed | Marketed as Aquaculture feed |



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## 2.3 Overview of oil accumulation in microalgae

The biosynthesis of lipid molecules in photosynthetic microalgae is an interconnected network of multiple metabolic pathways. It begins in the chloroplast of microalgal cells where the photosynthetic machinery utilizes atmospheric carbon to yield starch, which is later catabolized through glycolysis to form the building blocks of FAs and TAGs (**Figure 2.7**). Incorporation of these precursors in the form of acetyl-CoA to synthesize malonyl-CoA by acetyl-CoA carboxylase (ACC) initiates FA biosynthesis. Conversion of malonyl-CoA to malonyl-ACP marks the beginning of the elongation phase of FA biosynthesis, catalyzed by a prokaryotic type-II FA synthase (FAS II) localized in the stroma (Blatti et al., 2013; Shtaida et al., 2015). The process, however, is interrupted intermittently by fatty-ACP thioesterases (FATs) and as a consequence, the newly synthesized FAs escape from the acyl-ACP complex (Blatti et al., 2013). The generated free FA pool is assimilated while synthesizing various cellular lipids.

The synthesis of storage lipids, in particular, follows a set of reactions as part of the Kennedy pathway, which involves the incorporation of FAs into a glycerol backbone to form TAG. In the Kennedy pathway, acyl-CoA or acyl-ACP acts as an acyl donor while microalgae can follow an alternate pathway for sourcing acyl groups for TAG synthesis, which uses phospholipids as acyl donors (Li-Beisson et al., 2015). The rapid stride in the manipulation of oil biosynthesis in plants for oil enhancement provides clues for engineering the microalgal lipid metabolism, providing opportunities to understand the differences in the lipid metabolism process between microalgae and plants.



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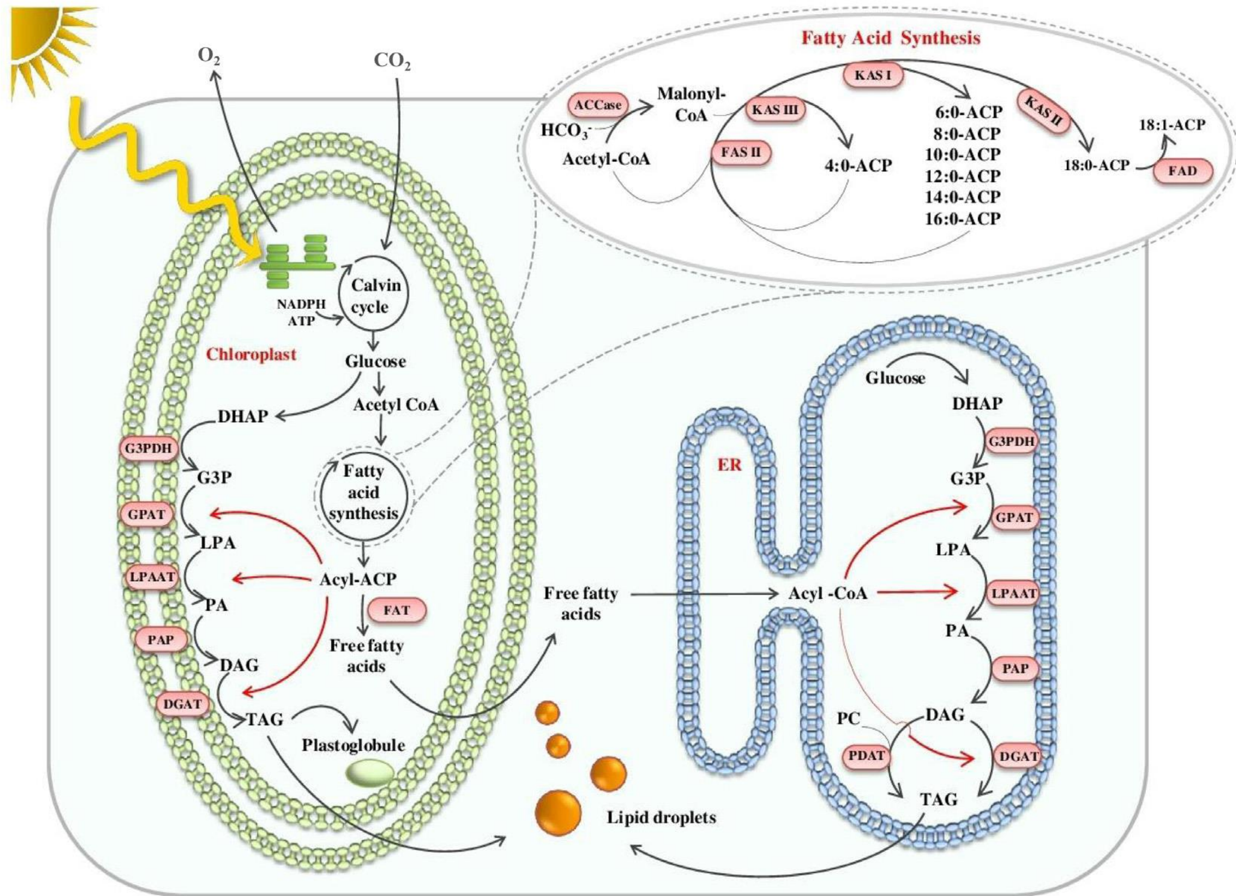


**Figure 2.7** Schematic illustration of TAG synthesis in microalgae. NADPH; Nicotinamide adenine dinucleotide phosphate; ATP, Adenosine Triphosphate; DHAP, dihydroxyacetone phosphate; G3P/G3pDH, Glyceraldehyde 3-phosphate / G3P dehydrogenase; GPAT, Glycerol 3-phosphate acyltransferase; PA/LPA/LPAAT/PAP, Phosphatidic acid/Lyso-PA/LPA acyltransferase/PA phosphatase; DAG/DGAT, di-Acylglycerol/ DAG acyltransferase; FAT, Fatty acyl-ACP thioesterase; ACP, Acyl-carrier protein; ER, Endoplasmic reticulum; PC, Phosphatidylcholine; PDAT, Phospholipid:DGAT; ACCase, Acetyl-CoA carboxylase; FAS, Fatty acid synthase; KAS, 3-ketoacyl-ACP synthase; FAD, Flavin adenine dinucleotide.



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Understanding of the model plant's lipid metabolism laid the foundation for the identification of key genes of TAG biosynthesis in microalgae. Microalgal omics has enabled the prediction and accurate annotation of lipid metabolism genes. Most of the predicted genes involved in FA biosynthesis are present as a single copy in the *Chlamydomonas* genome, suggesting that their encoded enzymes operate both in the chloroplast and mitochondria, in contrast to higher plants where compartment-specific enzymes increase the complexity of FA biosynthesis. Many copies of genes encoding acyl-CoA: diacylglycerol acyltransferases (DGATs) were predicted in the *Chlamydomonas* genome, compared with the fewer copies observed in higher plants, suggesting a crucial role of TAG in microalgal cell physiology (Liu and Benning, 2013). The model microalgae, *Chlamydomonas*, accumulates starch as the primary form for energy storage; however, stress redirects it to TAG formation. This carbon shifting is possibly due to adaptation for maintaining membrane integrity. Triacylglycerol molecules are catabolized back to release FAs upon stress reversal and used for membrane synthesis. Besides this, TAG molecules also act as a sink for channeling excess energy and reductive equivalents, which otherwise risk cellular metabolisms of microalgae (Sharma et al., 2012; Shtaida et al., 2015). Another marked difference is the presence of unique lipids such as betaines, which offer an advantage to microalgae in adapting to nutrient-limiting conditions. On the contrary, higher plants exclusively use phosphate-associated lipids that play a central role in maintaining the membrane integrity (Van Mooy et al., 2009; Liu and Benning, 2013).

In contrast to plants, microalgae contain distinct acyl groups in storage lipids (Garay et al., 2014). In plants, a majority of TAGs are assembled in the endoplasmic reticulum (ER), whereas in microalgae, a major fraction of TAGs are assembled de novo in the chloroplast (Giroud et al.,



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1988; Fan et al., 2011). The key difference between these two organelle pathways is the presence of a 16-carbon acyl group in the sn-2 position of the glycerol backbone in lipids derived from the plastid pathway while an 18-carbon acyl group occupies the same position in ER-generated lipids (Xu et al., 2016). However, the recent identification of an ER-localized acyltransferase enzyme having specificity toward C-16 acyl-donor suggests that the difference in the prokaryotic and eukaryotic pathways may not be due to spatial separation but due to complex systemic control (Kim et al., 2018).

The triggers for TAG accumulation also appear to differ in plants and microalgae. In plants, TAGs are accumulated in the developing seeds controlled through developmental signaling, whereas stress conditions trigger lipid accumulation, but the underlying mechanism is not fully established (Liu and Benning, 2013; Garay et al., 2014), presumably because of the coordinated sequential consequence of cell cycle arrest. Cornell et al. (1977) found that, at least in some cell culture types, lipid synthesis is controlled at certain checkpoints in the cycle. As stressors often obstruct cell cycle progression, it led to a speculation that they effectively trigger lipid accumulation (Kwok and Wong, 2005). However, this type of induction process requires validation. Changes in buoyancy due to lipid accumulation, assisting in motility and protection of microalgae, are possibly an adaptation in aquatic environments. Although several external agents initiate lipid accumulation, metabolic regulators such as nitrogen response regulator 1 (NRR1) and phosphorus starvation response 1 (PSR1) are core to this process as they sense the

changes in the cytoplasmic environment and activate various pathways associated with TAG biosynthesis (Gargouri et al., 2015).



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## 2.4 Strategies for enhancing lipid content in microalgae

An ideal microalgae strain for biofuel production must have the ability to accumulate large amount of lipids. Besides bioprospecting microalgae strains for a desired lipid profile, improving the inherent lipid production capacity of known microalgae strains through genetic means have attracted more attention. The goal of these two strategies were to enhance the total neutral lipid content and achieve an ideal fatty acid profile suitable for biofuel production. The strategies used to improve TAG content can be broadly categorized into two groups namely, conventional approach by altering the microalgae culture conditions and the other one being genetic or metabolic engineering to alter the regulation of key enzymes in the lipogenic pathways.

### 2.4.1 Conventional means for improving oil production

Cultural manipulations such as subjecting cells to stressors, like nutrient depletion, variable light intensity, temperature, salinity, and pH, are conventionally used to enhance lipid accumulation within the cells' biological limits (Bartley et al., 2014; Chu et al., 2015; Suyono et al., 2015). Among these stressors, nitrogen starvation is the most potent for lipid enhancement (Belotti et al., 2013). Dual-stage cultivation (Doan and Obbard, 2014) and coculture techniques with chemical additives (Singh S.K. et al., 2016) facilitate the enhancement of lipid production in culture systems. However, the genetic modification of microalgae offers more avenues for the precise control of target mechanisms leading to enhanced cellular lipid accumulation under normal growth conditions (Xue et al., 2015; Lim and Schenk, 2017). Genome sequence databases and pathway databases (KEGG, dEMBF, and MetaCyc) are now valuable resources for implementing targeted



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genetic manipulation for higher lipid biosynthesis in microalgae (Ogata et al., 1999; Caspi et al., 2014; Misra et al., 2016).

To date, successful nuclear transformation has been reported in more than 40 microalgal species, and considering the challenges posed by the enormous physiological and genetic diversities existing among these microalga species, this number appears significant (Gangl et al., 2015; Gimpel et al., 2015). In recent years, different tools such as metabolic selection markers and techniques like 'CHYSEL' have been developed to target both plastids and nuclear genomes, allowing for the expression of target genes (Specht et al., 2010; Rasala et al., 2014). With these rapid strides in microalgal biotechnology, 'algomics' and integrated system-biology modeling have deepened the understandings of interconnections between genes, proteins, and metabolites (Jamers et al., 2009; Koussa et al., 2014; Benmoussa, 2016). Such integrated multidisciplinary studies can provide a clear picture of oil and high-value metabolite biosynthesis pathways, thereby accelerating strain improvement for the commercialization of microalgal biofuel (Lauersen et al., 2015; Barahimipour et al., 2016).

### 2.4.2 Genetic engineering strategies for lipid enhancement

Enhancing oil synthesis in microalgae primarily depends on the manipulation of enzymes involved in lipid biosynthesis or other competitive parallel pathways aimed to divert the carbon and reductive equivalents flux toward lipid biosynthesis (**Figure 2.8**). The most widely used technique is the manipulation of individual genes encoding various steps of a metabolic pathway; however, owing to the multi-factorial regulation of lipid biosynthesis in microalgae, this strategy has seen mixed success (Bajhaiya et al., 2017). Recently, the transcriptional regulation of oil biosynthesis



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has brought widespread interests to control the activity or expression of multiple components of a metabolic pathway simultaneously (Courchesne et al., 2009).

Additionally, attempts to manipulate various other targets, such as improving light use efficiency, controlling cell quiescence, and improving carbon sequestration, etc., which indirectly influence the lipid content by altering cell growth characteristics, have gained attention.

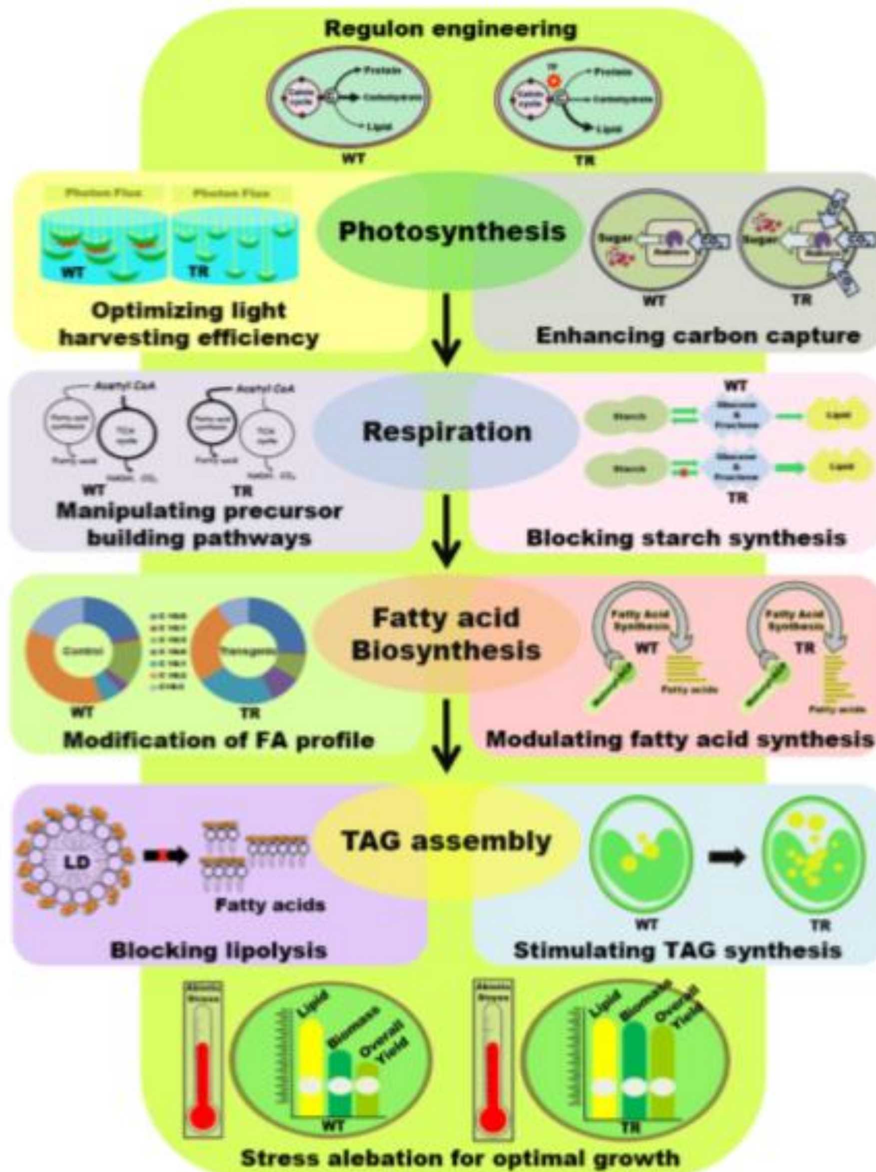

**Figure 2.8** Schematic illustration of different genetic engineering strategies applied in microalgae for biodiesel application. WT, Wild type cells; TR, Transgenic cells; TF, Transcription factor; TCA, Tricarboxylic acid cycle; NADH, Nicotinamide adenine dinucleotide; FA, Fatty acid; LD, Lipid droplet.

#### 2.4.2.1 Manipulation of the oil biosynthesis pathway

In contrast to subjecting microalgae to growth limiting stress conditions, efforts targeting enhanced lipid accumulation during the exponential growth phase are more practical means. According to Ohlrogge and Jaworski (1997), the FA supply regulates the lipid biosynthesis process, and therefore, some of the earliest attempts have been made to increase the expression of key enzymes involved in FA biosynthesis. Microalgal metabolic engineering aimed at increasing FA supply to lipid synthesis was first attempted by over expressing the acetyl-CoA carboxylase gene (ACCase) in *Cyclotella cryptica*, which codes for the enzyme that carboxylates acetyl-CoA to malonyl-CoA, the first committed step in FA synthesis (Dunahay et al., 1996). Although the transformed microalgae showed a two- to three-fold increase in ACCase activity, it was not accompanied with an increase in FA content (Sheehan et al., 1998). This clearly indicated that the up regulation of ACCase had no direct impact on lipid biosynthesis. However, the simultaneous overexpression of a subunit of ACCase (accD) along with malic enzyme (ME) *Dunaliella salina*, responsible for the conversion of malate to pyruvate, was successful in elevating the total lipid content in microalgae (Talebi et al., 2014). Therefore, it appears that ACC is not the sole rate-limiting step in lipid biosynthesis, indicating the existence of a secondary rate-limiting step apart from ACC. Limited availability of precursors for the whole lipid synthesis process (acetyl-CoA and glucose-6-phosphate) could be the secondary bottleneck in case ACC is overexpressed. Various studies attempted to elevate the intracellular concentration



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of lipogenic precursors, by tailoring the enzymes involved in the generation of reducing potential (NADH) and in carbon metabolism (Gimpel et al., 2015).

Manipulation in the expression of several enzymes like pyruvate dehydrogenase, phosphoenol pyruvate carboxylase, acetyl-CoA synthase, NAD(H) kinase, and glycerol kinase has significantly enhanced the lipid content in different microalgal species without adversely affecting cell growth (**Table 2.3**).

Another possible strategy to increase the intracellular lipid content is by blocking the metabolic pathways that are competitive to lipogenesis, for example, starch synthesis and lipid catabolism. Some strains of microalgae specially the lineage of green microalgae use starch as the primary storage metabolite, and suppressing starch synthesis can funnel the carbon flow toward lipid biosynthesis (Ravindran et al., 2017). Knockdown of key genes involved in starch synthesis showed elevated lipid accumulation by redirecting carbon pool toward lipogenesis (**Table 2.3**). Since accumulation of starch as an energy storage molecule is not universal in microalgae (León-Saiki et al., 2017), the suppression of lipid catabolism is a more legitimate option to enhance the lipid content irrespective of the microalgal strains. For instance, inhibiting the expression of a multifunctional lipase/phospholipase/acyltransferase enzyme in *Thalassiosira pseudonana* resulted in increased lipid yields without affecting the growth (Trentacoste et al., 2013). The mutant strain showed a 2.4 to 3.3-fold higher lipid accumulation in comparison with the control, when subjected to silicon starvation. However, blocking these vital metabolic pathways (lipid catabolism and starch synthesis) may result in reduced microalgal growth and lipid yield (Radakovits et al., 2010; Chu, 2017). One way to overcome this is by using RNAi mediated gene silencing under the control of



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inducible promoters. Upon attaining high cell-density, the mechanism can be activated to suppress the expression of key genes involved in starch synthesis and lipid catabolism.

**Table 2.3** Summary of genes manipulated for oil enhancement

| Gene | Mode of action | Species | Observation | Ref. |
|---|---|---|---|---|
| **Enhancing Fatty acid biosynthesis:** | | | | |
| Acetyl CoA Carboxylase (ACCase) | Overexpression | *C. cryptica* | ~ 2-3 X ACCase activity No changes in lipid content | Dunahay et al., 1996 |
| | Overexpression | *D. salina* | 1.14 X Total lipid content | Talebi et al., 2014 |
| Acetyl CoA Synthase (ACS) | Overexpression | *Schizochytrium* sp. | 1.3 X Biomass productivity and improved FA profile | Yan et al., 2013 |
| | Overexpression | *C. reinhardtii* | 2.4 X TAG content | Rengel et al., 2018 |
| Acyl-ACP reductase | Overexpression | *C. merolae* | 3 X TAG accumulation | Sumiya et al., 2015 |
| **Manipulation of Carbon partitioning:** | | | | |
| Malic enzyme (ME) | Overexpression | *P. tricornutum* | 2.5-2.7 X Lipid content | Xue et al., 2015 |
| ADP-glucose pyrophosphorylase (*sta6*) | Suppression | *C. reinhardtii* | 10 X TAG content | Li et al., 2010[a] |
| | Suppression | *Coccomyxa* sp. | Higher lipid content | Takahashi et al., 2018 |
| Isoamylase (*sta7-10*) | Suppression | *C. reinhardtii* | ~1.8 X total lipid | Work et al., 2010 |
| Pyruvate dehydrogenase Kinase (PDK) | Suppression | *P. tricornutum* | + 82% Neutral lipid | Ma et al., 2014 |
| Phosphofructo-2-kinase (PFK2) | Overexpression | *T. pseudonana* | Increased glycolytic activity | Abbriano et al., 2018 |
| Citrate Synthase (CIS) | Suppression | *C. reinhardtii* | ~ 3 X TAG productivity | Deng et al., 2013[a] |
| Phosphoenolpyruvate Carboxylase (PEPC 1) | Suppression | *C. reinhardtii* | + 20% TAG level | Deng et al., 2014 |
| Phosphoenolpyruvate Carboxylase 2 | Suppression | *C. reinhardtii* | + 14-28% Oil content | Deng et al., 2011 |
| UDP-Glucose | Suppression | *P. tricornutum* | 45 X TAG accumulation | Daboussi et al., 2014 |
| Pyrophosphorylase (UGPase) | Suppression | *P. tricornutum* | + 25% Total lipid content | Zhu et al., 2016 |
| Glycerol kinase | Overexpression | *F. solaris* | + 12% Lipid productivity | Muto et al., 2015 |
| | Overexpression | *S. quadricauda* | 1.6 X G3P content | Gomma et al., 2015 |
| | Overexpression | *S. quadricauda* | 1.9 X intracellular G3P | Gomma et al., 2015 |
| Glycerol-3-Phosphate Dehydrogenase (G3PDH) | Overexpression | *P. tricornutum* | 1.9 X Neutral lipid content | Yao et al., 2014 |
| | Overexpression | *C. minutissima* | + 30-40% TAG content | Hsieh et al., 2012 |
| **Increasing intracellular reducing equivalents:** | | | | |
| NAD(H) Kinase | Overexpression | *C. pyrenoidosa* | ~ 1.6 X lipid content | Fan et al., 2015 |
| Ferrodoxins (FDX) | Overexpression | *C. reinhardtii* | 2.5 X lipid level | Huang et al., 2015 |
| **Blocking TAG hydrolysis:** | | | | |
| TGL1 (Triglyceride Lipase 1) | Suppression | *P. tricornutum* | Increased TAG level in lipid extracts | Barka et al., 2016 |
| PNPLA3 | Overexpression | *P. tricornutum* | 1.7 X Neutral lipid content | Wang et al., 2015 |
| | Overexpression | *P. tricornutum* | 1.55 X TAG content | Wang et al., 2018[a] |
| Thaps3_264297 | Suppression | *T. pseudonana* | ~ 3.3-4.1 X lipid content | Trentacoste et al., 2013 |

(*Continued*)



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**Table 2.3** Continued

| Gene | Mode of action | Species | Observation | Ref. |
|---|---|---|---|---|
| LIP (Diacylglycerol lipase) | Suppression | *C. reinhartdii* | Delay in TAG hydrolysis | Li et al., 2012 |
| cht7 (TAG lipase) | Suppression | *C. reinhartdii* | 10 X TAG level | Tsai et al., 2014 |
| Lipid droplet protein (StLDP) | Overexpression | *P. tricornutum* | Increased lipid droplet accumulation | Yoneda et al., 2018 |
| Oleosin protein 3 (AtOLEO3) | Overexpression | *P. tricornutum* | 1.4 X TAG content | Zulu et al., 2018 |
| **Increasing TAG content (single gene):** | | | | |
| GPAT | Overexpression | *C. reinhardtii* | ~1.5 X TAG content | Boyle et al., 2012 |
| | Overexpression | *C. minutissima* | + 30-40% TAG content | Hsieh et al., 2012 |
| LPAAT | Overexpression | *C. reinhardtii* | + 20% TAG | Yamaoka et al., 2016 |
| | Overexpression | *C. minutissima* | + 30-40% TAG content | Hsieh et al., 2012 |
| PAP | Overexpression | *C. reinhardtii* | + 7.5% to 21.8% lipid content | Deng et al., 2013[b] |
| | Overexpression | *C. minutissima* | + 30-40% TAG content | Hsieh et al., 2012 |
| DGAT | Overexpression | *N. oceanica* | + 69% lipid content | Li et al., 2016 |
| | Overexpression | *C. reinhardtii* | 2X TAG content | Ahmad et al., 2015 |
| | Overexpression | *T. pseudonana* | 1.9X TAG content | Manandhar-Shrestha et al., 2015 |
| | Overexpression | *C. minutissima* | + 30-40% TAG content | Hsieh et al., 2012 |
| | Overexpression | *S. obliquus* | ~ 2X lipid content | Chen et al., 2016 |
| | Overexpression | *C. reinhardtii* | Unchanged lipid content | La Russa et al., 2012<br>Iwai et al., 2014 |
| | Overexpression | *C. reinhardtii* | 2.5X TAG content | Niu et al., 2013 |
| | Overexpression | *P. tricornutum* | Increased neutral lipid content | |
| | Overexpression | *T. chui* | + 40-115% TAG content | Úbeda-Mínguez et al., 2017 |
| PDAT | Overexpression | *C. minutissima* | + 30-40% TAG content | Hsieh et al., 2012 |
| | Overexpression | *C. reinhardtii* | 32% increase in TAG content | Zhu et al., 2018 |
| **Increasing TAG content (multiple gene):** | | | | |
| Quintuple construct containing G3PDH, GPAT, LPAAT, PAP, DGAT, and PADAT | Overexpression | *C. minutissima* | ~1.8 X TAG content | Hsieh et al., 2012 |
| Co-expression of GPAT and DGAT | Overexpression | *P. tricornutum* | 2.6 X Total lipids | Zou et al., 2018 |
| Co-expression of DGAT and Oleosin | Overexpression | *P. tricornutum* | 3.6 X TAG content | Zulu eta al., 2018 |
| **Manipulation of Transcription regulators:** | | | | |
| CONSTANS like | Suppression | *C. reinhartdii* | + 25% TAG level | Deng et al. 2015 |
| P-II Like | Suppression | *C. reinhartdii* | ~ 1.3 X TAG content | Zalutskaya et al., 2015 |
| bHLH2 | Overexpression | *N. salina* | + 33% FAME productivity | Kang et al., 2015 |
| Wrinkled1 (WRI1) | Overexpression | *N. salina* | + 64% FAME yield | Kang et al., 2017 |
| basic leucine zipper (bZIP) | Overexpression | *N. salina* | + 203% Neutral lipids | Kwon et al., 2017 |
| PSR1 | Overexpression | *C. reinhardtii* | 7 X lipid content | Ngan et al., 2015 |
| ZnCys | Suppression | *N. gaditana* | + 103% Lipid productivity | Ajjawi et al., 2017 |
| Dof | Overexpression | *C. ellipsoidea* | ~ 1.5 X lipid content | Zhang et al., 2014 |
| | Overexpression | *C. reinhardtii* | ~ 2 X Total lipid | Ibanez-Salazar et al., 2014 |

*(Continued)*



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**Table 2.3** Continued

| Gene | Mode of action | Species | Observation | Ref. |
|---|---|---|---|---|
| **Modifying Fatty acid profile:** | | | | |
| Acyl-ACP thioesterase | Overexpression | *Nannochloropsis* | ~1.5 X C 14:0 & C 16:1 fatty acids | Ozaki et al., 2016 |
| C 12 thioesterase | Overexpression | *P. tricornutum* | ~1.2 X C 12:0 FA accumulation | Radakovits et al., 2011 |
| C 14 thioesterase | Overexpression | *P. tricornutum* | 2 X C 14:0 Fatty acid | Radakovits et al., 2011 |
| | Overexpression | *D. tertiolecta* | 2 X C 14:0 & C 12:0 FA content | Lin et al., 2018 |
| Stearoyl-ACP desaturase (SAD) | Overexpression | *C. reinhardtii* | 2.7 X C 18:1 FA content | Hwangbo et al., 2013 |
| | Suppression | *C. reinhardtii* | 2 X C 18:0 FA in TAG | de Jaeger et al., 2017 |
| Delta-12 desaturase (Δ12D) | Overexpression | *N. oceanica* | 4 X C 18:2 FA level | Kaye et al., 2015 |
| Delta-5 desaturase (Δ5D) | Overexpression | *P. tricornutum* | + 64-75% PUFA and MUFA | Peng et al., 2014 |
| Delta-6 FA desaturase (Δ6D) | Overexpression | *P. tricornutum* | + 47.66% in EPA (C 20:5) content | Zhu et al., 2017 |
| Omega-3 fatty acid desaturase (ω-3 FAD) | Overexpression | *C. Vulgaris* | Enhanced C18:3, n3 FA accumulation | Norashikin et al., 2018 |
| Delta-5 elongase (Δ5E) | Overexpression | *P. tricornutum* | 8 X DHA (22:6, n-3) content | Hamilton et al., 2014 |

'+' increase in quantity percentile; 'X' fold change; FAME, fatty acid methyl ester; FA, fatty acid; PUFA, polyunsaturated fatty acid; MUFA, monounsaturated fatty acid; TAG, triacylglycerol; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

Many such promoters have been identified in microalgae, including one with light-responsive elements in *Dunaliella* (Park et al., 2013; Baek et al., 2016).

Besides the manipulation of carbohydrate metabolism- and lipid metabolism-related genes for increasing cellular neutral lipids (TAG), the overexpression of acyltransferases has also yielded interesting outcomes (**Table 2.3**). The Kennedy pathway for TAG assembly includes several steps catalyzed by different acyltransferases, including acyl-CoA: glycerol-3- phosphate acyltransferase (GPAT), acyl-CoA: lysophosphatidic acyltransferase (LPAAT), and acyl-CoA: DGAT. These TAG assembly genes were found to be worthy targets in lipid pathway engineering (Bhowmick et al., 2015; Maravi et al., 2016).



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In the green microalgae *Chlorella minutissima*, simultaneous expression of five acyltransferases (phosphatidic acid phosphatase, LPAAT, glycerol-3-phosphate dehydrogenase, GPAT, and DGAT) resulted in a two-fold increase in lipid content (Hsieh et al., 2012). These instances of co-expressing multiple enzymes exemplify the effectiveness of system level control of metabolic flux toward lipid overproduction. Transcriptional regulation can have a similar effect on the systemic metabolomic flux as transcription factors can target multiple regulatory points in a metabolic pathway. Overexpression of genes encoding transcription factors targeting the upregulation of downstream lipid biosynthesis genes can result in increased oil content. In this realm, higher plants have been in the spotlight with numerous literature highlighting the benefit of transcription factor engineering for enhanced lipogenesis (Cernac and Benning, 2004; Mendoza et al., 2005). However, in microalgae, the major focus of transcriptional regulation studies is limited to a select microalgal species (**Table 2.3**). In this context, the identification of endogenous transcription factors and their subsequent manipulation in their host would be more viable to trigger lipid accumulation (Tsai et al., 2014; Ngan et al., 2015; Kwon et al., 2017). Although the manipulation of transcription factors like PSR1 and Compromised Hydrolysis of Triglycerols 7 (CHT7) have led to enhanced lipid accumulation without compromising biomass production, weak carbon partitioning for lipid synthesis still remains a bottleneck which may be overcome by finding other potential lipid-triggers (Chen et al., 2018). In one such groundbreaking effort, the knockdown of a single transcription regulator ZnCys in *Nannochloropsis gaditana* resulted in a 103% increase in lipid content, indicating a lipid yield to the tune of ~5 g/m2/day (Ajjawi et al., 2017).

Even though the overexpression of endogenous transcription factors for increasing oil content in



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microalgae is very promising, the lengthy functional characterization process greatly limits its applications. A more direct approach is to consider the heterologous expression of a transcription factor of plant origin to regulate the microalgal lipid biosynthetic pathway. Several lipogenesis promoting transcription factors from higher plants were overexpressed in microalgae and showed to have a remarkable impact on the lipid accumulation pattern (**Table 2.3**). In addition to engineering for enhanced oil content, it is also important to improve the quality of oil for better biodiesel fuel properties. The carbon chain length and degree of unsaturation of the FAs present in oil affect the cold flow and oxidative stability properties of the fuel. Oils derived from microalgal feedstocks commonly contain FAs of chain length between 14 and 20, mostly C16:0, C16:1, and C18:1, while the ideal should be C12:0 and C14:0 (Radakovits et al., 2010). The key factor that determines FA chain length is the thioesterase enzyme, which catalyzes the release of the FA chain from the FA synthase complex. Several acyl-ACP thioesterases specific to short FA chain length have been identified, and engineering the expression of these enzymes can successfully modify the fuel properties. Transgenic microalgae containing exogenous short- chain length biased FA acyl-ACP thioesterases have directed an increase in percent composition of myristic (C14:0) and lauric (C12:0) acids in the overall FA profile (**Table 2.3**). A seamless biodiesel fuel also requires a balanced coalescence of MUFAs, SFAs, and PUFAs in oil (Durrett et al., 2008). The scant presence of MUFAs in the microalgal lipid profile (Patil et al., 2007) requires biotechnological interventions to modify the degree of unsaturation. The desaturase enzyme, which catalyzes the formation of unsaturated FAs, was targeted to manipulate the FA profile primarily in MUFA and PUFA contents (**Table 2.3**).



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Enhancements in PUFA entities like linoleic acid and eicosapentaenoic acid are particularly noteworthy considering their high nutritional value. Apart from biodiesel, microalgal oils can also be used to produce gasoline and jet fuel, which requires FAs with even shorter chain lengths. Even though it is possible to chemically synthesize suitable feedstocks for gasoline or jet fuel by breaking down the long chains into shorter hydrocarbon chains, the genetic engineering of microalgae to synthesize short-chain FAs will significantly reduce the production cost (Radakovits et al., 2010). For instance, the overexpression of 8:0- and 10:0-biased thioesterases from *Cuphea hookeriana* in Canola has reportedly enhanced the synthesis of short-chain FAs (Dehesh et al., 1996). Replication of this achievement of raised short-chain FA profile in different oleaginous microalgal species would have a high impact.

#### 2.4.2.2 Enhancing the biomass yield

Enhancing the biomass yield is very important as the total energy output relies on both energy density and the total biomass content (Barry et al., 2015). In photosynthetic organisms including microalgae, abiotic stress, $CO_2$ fixation rate, and light utilization efficiency are the primary factors that govern biomass productivity (Chu, 2017). Engineering microalgal strains for stress tolerance and higher photosynthetic efficiency can ensure the cost-effective production of biofuel. A number of studies report transgenic microalgal strains tolerant to abiotic stress through enhanced reactive oxygen species (ROS) scavenging, hypertolerance to DNA damage, and polyploidization (**Table 2.4**). Kotchoni et al. (2016) reported the manipulation of intracellular steady-state ATP levels for cold adaptation of microalgal cells. The identification of key transcription regulators and enzymes, as well as stress-responsive promoters through omics analysis, can serve as the toolbox for future genetic engineering designs.



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Manipulation of carbon fixation is vital to improving the photosynthesis rate. The Calvin cycle is the initial pathway for carbon fixation in all photosynthetic organisms, and strategies seeking improvement in the photosynthetic efficiency require a breakthrough in the regulation of this pathway. Carboxylation of ribulose 1, 5-bisphosphate (RuBP) and its subsequent regeneration are the checkpoints in the Calvin cycle. The enzymes that catalyze these regulatory steps are ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), fructose 1,6-bisphosphate aldolase (aldolase), and sedoheptulose 1,7-bisphosphatase (SBPase), and these three enzymes are prime targets for manipulating the Calvin cycle owing to their high flux control coefficient values (Raines, 2003; Yang et al., 2017). Among the three enzymes, Rubisco is the primary target as the carboxylation capacity of Rubisco majorly influences the rate of carbon assimilation. However, efforts to directly manipulate this enzyme have been met with limited success, owing to the complex enzyme kinetics of Rubisco, which challenges the operational understanding and makes it difficult to spot a change in the phenotype upon manipulation (Tcherkez et al., 2006). Therefore, efforts are being made to target factors that regulate Rubisco activity instead of the direct manipulation of the enzyme itself. One such target is the Rubisco activase (RCA) enzyme, which regulates the activity of Rubisco by regenerating the catalytic sites (Hazra et al., 2015). Ultimately, RCA determines the rate of carbon fixation by maintaining a high proportion of catalytically competent active sites of Rubisco. An attempt to overexpress RCA in an oleaginous microalga, *Nannochloropsis oceanica*, resulted in elevated photosynthetic activity accompanied by enhanced biomass and lipid accumulation (Wei et al., 2017). Apart from Rubisco, aldolase and SBPase are also crucial to improve carbon fixation as these enzymes are involved in the regeneration of precursor substrates for Rubisco.



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As a proof of concept, the overexpression of genes encoding aldolase and SBPase in different microalgal strains resulted in enhanced photosynthetic efficiency and biomass production (**Table 2.4**).

Abiotic factors such as availability of photon energy also affect the efficiency of carbon fixation. Photosynthetic microalgae have developed large photosynthetic antenna systems to maximize the photon absorption and conversion efficiency as an adaptation to its habitat where low light intensity is a growth limiting factor. However, the sustainability of microalgal biomass production requires large-scale cultures with high cell density. In such dense cultures, the high pigment density due to the large antenna systems limits the penetration of light into the deeper layers of the culture. Under such conditions, cells at the surface receive an excess amount of photon energy, which quickly saturates the photosynthesis process, and dissipate the excess energy through non-photochemical quenching (NPQ). At the same time, cells in the deeper layers are exposed to a low-light intensity, which compels the cells to perform respiration instead of photosynthesis (Formighieri et al., 2012). This uneven distribution of light energy leads to suboptimal photosynthetic efficiency, which in turn reduces the overall biomass productivity of the culture. One approach to enhance the photosynthetic yield is by reducing the size of light capturing antenna systems in microalgae to minimize the energy loss due to NPQ. In biological terms, antennas, or light-harvesting complex (LHC), are pigment-binding proteins, which capture the light energy and relay it to photosynthetic reaction centers. In green microalgae, they bind the majority of pigments and, therefore, are mainly responsible for the optical density of the culture. Wild type photosynthetic microalgae harbor a vast number of chlorophyll molecules associated with both photosystems I and II; however, only a few of these are essential to carry out the vital functions of photosynthesis (Simionato et al.,



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2013). Therefore, it is possible to improve light transmission and light absorption capacities by reducing the number of chlorophyll molecules from the LHC in microalgal cells. Mutants have been developed with truncated antenna systems in different microalgal strains through downregulating the genes encoding LHC pigment binding-proteins, which showed a marked reduction in energy losses by NPQ and increased biomass production under laboratory scale culture conditions (**Table 2.4**). Mass culture of these truncated antenna mutants is expected to fine-tune light absorption characteristics influencing higher biomass productivity and the eventual reduction in the production cost. However, susceptibility of such strains with the shrunk antenna system to photodamage by intense solar radiation is a great limitation. de Mooij et al. (2015) found a smaller antenna size that made the mutants vulnerable to high light intensity while tailoring the antenna size in *Chlamydomonas reinhardtii*. The reduced fitness due to impaired photoprotection mechanisms triggered by altered antenna size lead to insignificant changes in the biomass productivity of mutants. Therefore, future strategies for antenna size reduction in microalgae should address the unintended side effects of antenna size on mutants. Interestingly, transgenic *C. reinhardtii* strains generated by Perrine et al. (2012), having variation in antenna sizes and reduced chlorophyll (Chl) b content, showed a higher growth rate in mutants with intermediate antenna size. The results conform to the hypothesis that reduction but not the elimination of Chl b content would result in the optimal photosynthesis process.

Among the abiotic factors that influence rate of carbon fixation, the most critical is the availability of inorganic carbon, as the concentration of $CO_2$ in the vicinity of Rubisco affects its carboxylase property. Microalgae have developed a $CO_2$ concentrating mechanism (CCM) to alleviate the stress caused by limited $CO_2$ in aquatic ecosystems (Wang et al., 2011).



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Microalgae elevate the $CO_2$ concentration at the site of Rubisco through the operation of CCM. Since Rubisco also has the inherent capacity to divert the carbon pool toward unimportant photorespiratory pathways, an elevated $CO_2$ concentration favors carboxylation, thereby increasing the rate of carbon fixation (Singh P. et al., 2016). Inorganic carbon (Ci) import, enzymatic catalysis of the imported carbon to form $CO_2$, and compartmentalized Rubisco systems are the functional components of microalgal CCM. Environmental conditions such as the Ci content, ratio of $[CO_2]/[O_2]$, and dissolved $CO_2$ concentration are ascribed for the regulation of microalgal CCM (Morales et al., 2018). Several studies in *C. reinhardtii* have identified various factors such as CIA5, which acts at the cellular level and regulates the transcription of multiple genes having a role in CCM (Fukuzawa et al., 2001; Yoshioka et al., 2004; Fang et al., 2012b).

Tapping these regulatory factors in photosynthetic microalgae has assumed significance in enhancing fitness toward naturally occurring low $CO_2$ conditions (Price et al., 2013). Besides these, modulations in other functional components of CCM such as Ci transporters and carbonic anhydrases can also facilitate enhancements in the carboxylation reaction, which in turn increases the photosynthetic performance and biomass yield. However, except a few filed patents (**Table 2.4**), to date, there are no reports of successful CCM engineering in microalgae. Thus, suppression of the oxygenase activity of Rubisco through tailoring $CO_2$ capture mechanism in microalgae remains a challenge to be addressed for improving the carbon fixation process.



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**Table 2.4** Summary of genes manipulated for increasing the biomass yield.

| Gene | Mode of action | Species | Observation | Ref. |
|---|---|---|---|---|
| **Abiotic Stress tolerance:** | | | | |
| Superoxide dismutase (SOD1) | Overexpression | *Schizochytrium* sp. | Enhanced PUFA production without compromising growth | Zhang et al., 2018 |
| Ubiquitin (UBC2) | Overexpression | *C. reinhardtii* | Increased cell lipid and growth rate | Fei et al., 2017 |
| Diploid *C. reinhardtii* (CMD ex1 & ex4) | Polyploidization | *C. reinhardtii* | Accumulated two times more biomass and FAME yield. | Kwak et al., 2017 |
| AMP deaminase (AMPD) | Suppression | *C. reinhardtii* | Displayed higher growth rate and lipid productivity | Kotchoni et al., 2016 |
| **Manipulation of Calvin cycle:** | | | | |
| Rubisco (rbcL & rbcS) | Overexpression | *S. elongatus* | 1.4 fold increase in Rubisco activity | Atsumi et al., 2009 |
| | Overexpression | *Synechocystis* | Increased growth rate and photosynthesis | Liang and Lindblad, 2017 |
| Rubisco type-I | Overexpression | *Synechococcus* sp. | Rubisco activity was improved by 4 fold | Iwaki et al. 2006 |
| RuBisCO activase (RCA) | Overexpression | *N. oceanica* | Biomass and lipid productivity increased by 46% and 41% respectively | Wei et al., 2017 |
| Sedoheptulose 1,7-bisphosphatase (SBPase) | Overexpression | *D. bardawil* | Improved photosynthetic performance | Fang et al., 2012[b] |
| Fructose 1,6-bisphosphate aldolase (aldolase) | Overexpression | *C. vulgaris* | Increased photosynthetic capacity by 1.2-fold and enhanced cell growth | Yang et al., 2017 |
| **Optimizing light use efficiency:** | | | | |
| tla3 (CpSRP43) | Suppression | *C. reinhartdii* | Improved solar energy conversion efficiency and photosynthetic productivity | Kirst et al., 2012[a] |
| tla1 | Suppression | *C. reinhartdii* | Higher photosynthetic productivity | Polle et al., 2003 |
| tla2 (CpFTSY) | Suppression | *C. reinhartdii* | Improved solar energy conversion efficiency | Kirst et al., 2012[b] |
| tla4 (CpSRP54) | Suppression | *C. reinhartdii* | Higher photosynthetic productivity | Jeong et al., 2017 |
| Stm3LR3 (NAB 1) | Suppression | *C. reinhartdii* | Higher photosynthetic quantum yield | Mussgnug et al., 2007 |
| Chlorophyllide a oxygenase (CAO) | Suppression | *C. reinhartdii* | Two-fold increase in photosynthetic rate | Perrine et al., 2012 |
| LHCP translocation defect (LTD) | Suppression | *C. reinhartdii* | Culture accumulated higher cell density | Jeong et al., 2018 |
| Knockout of Seven LHC genes | Suppression | *N. gladitana* | Up to ~50% reduction in photosynthetic antennae size | Verruto et al., 2018 |
| **Enhancing carbon concentrating mechanism:** | | | | |
| Pyr-decarboxylase | Overexpression | Not mentioned | Aims to improve carbon fixation | Allen and Dupont, 2014 |
| Bicarbonate transporter (ictB) | Overexpression | Not mentioned | Enhancing photosynthetic rate is the target | Wang et al., 2014 |
| ATP-dependent bicarbonate anion transporter (HLA3) | Overexpression | Not mentioned | Target is to enhance $CO_2$ fixation | Sayre et al., 2017 |



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### 2.4.2.3 Genome editing in microalgae for strain improvement

Metabolic pathway engineering is crucial for enhancing the productivity of a microalgal strain, and for this purpose, gene editing offers a powerful and easy mechanism to overcome the genetic inadequacies (Ng et al., 2017). Until recently, the RNAi technology was frequently used as a tool for gene silencing and proved efficient in pathway engineering and gene function alteration. However, RNAi has its limitations, which include incomplete suppression, silencing of the RNAi transgene, and inconsistent suppression in different transformants (Banerjee et al., 2018). In contrast, the emergence of genome editing bypasses the limitations of RNAi, offering new avenues to modify and edit the genome of cells. The genome editing techniques based on engineered nucleases like clustered regularly interspaced palindromic sequences/CRISPR-associated protein 9 (CRISPR/Cas9), transcription activator-like effector nucleases (TALENs), and zinc-finger nucleases (ZFNs) provide the means for dissecting the operational organization of genes, gene families, and protein networks. These genome editing tools induce double- strand breaks at a specific locus in the genome, which get repaired through the non-homologous end joining machinery of the DNA repair process and introduce insertions or deletions at sites creating frameshift mutations (Gan and Maggs, 2017). Among the genome editing tools, CRISPR/Cas9 has gained much focus because of its simple, accurate, and efficient nature of operation (Jeon et al., 2017). In the CRISPR/Cas9 system, the Cas9 nuclease is directed by a single guide RNA (sgRNA) molecule, which binds to the target site in the genome following simple base-pairing rules. Steady progress in research on the CRISPR/Cas9 system has resulted in the development of many different variants of this technology. A mutated form of the Cas9 protein (dCas9) lacking the nuclease activity can be used with the CRISPR system to modulate the expression of specific target genes.



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Depending on the type of the effector molecule fused with dCas9/sgRNA, it is possible to precisely both stimulate and repress the activity of a target gene (Gilbert et al., 2013; Piatek et al., 2015). In addition to the expression modulation of a single gene, multiple genes can be simultaneously activated or silenced by the simple addition of guide RNAs for each of the targets into the dCas9/sgRNA variant of the CRISPR/Cas9 system (Kim and Kim, 2014). The versatility in the application of the CRISPR/Cas9 system makes the technique a remarkable and powerful tool in metabolic pathway engineering.

In microalgae, the utility of CRISPR is on the rise as it has considerable scope in microalgal trait improvement for biofuel and nutraceutical applications. Various advances in CRISPR/Cas and other genome editing tools have led to several successful attempts in many microalgae species (**Table 2.5**), which endorse this technology for its effectiveness in generating targeted mutants. One prominent advantage of applying the CRISPR technology in microalgae is the ease of multiplexing, which, unlike the conventional mutagenesis and RNAi mediated knockout and knockdown approaches, facilitates a less complicated and more programmable approach for manipulating metabolic pathways. In case of lipid engineering in oleaginous microalgal strains, this technique can improve the lipid profile of the microalgal strain by simultaneously blocking the metabolic routes competitive to lipid production such as starch generation, lipid degradation, and β-oxidation. Other than gene silencing, a dCas9 variant can be recruited to activate stress responsive elements of the lipid synthesis pathway under non-stress conditions, thus bypassing the inhibitory effects on the biomass yield. An activator molecule fused with dCas9 can be used to stimulate supportive pathways such as FA synthesis, which facilitates the production of precursors for lipogenesis. Functional characterization of a novel gene is another aspect of utilizing the



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CRISPR technology in addition to gene editing. Annotating novel genes encoding proteins significant for lipid production can broaden the spectrum of target selection for superior biofuel production.

Indigenous microalgal strains promoted for biofuel production have some limitations for commercial scale production, which include suboptimal lipid profile and light harvesting efficiency, among others. However, adjusting these limiting attributes is not recommended as it interferes with the normal physiology of microalgae. For example, generating truncated LHC in microalgae is associated with susceptibility to photodamage (de Mooij et al., 2015). Therefore, it is advantageous to have a system that can detect a trigger such as the presence of a chemical or a variation in light intensity; therefore, once the culture is grown for some time, the trigger can be activated resulting in improved productivity. Development of a dCas9 variant that can be activated by light or chemicals can facilitate a tool for the conditional modulation of molecular intricacies, bypassing the physiological interference of the change in cell metabolism (Polstein and Gersbach, 2015; Zetsche et al., 2015). Despite several advantages, this system has its share of challenges in the form of cytotoxic effects of the Cas9 nuclease in some of the microalgae species, which have limited the full-scale utilization of this system. Off-target effects of the Cas9 protein have been linked with cytotoxicity in cells transformed with the Cas9 gene construct.

However, modifications in the Cas9 protein delivery through the ribonucleoprotein (RNP) complex has been reported to reduce the off-target problems associated with the Cas9 protein. Replacing the Cas9 protein with a Cas12a variant is also an alternative to consider as it has been reported to solve the cytotoxicity in cyanobacteria (Naduthodi et al., 2018).



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**Table 2.5** Overview of the application of different genome editing tools in photosynthetic microalgae.

| Species | Genome editing tool | Mode of action (Efficiency) | Target gene | Ref. |
|---|---|---|---|---|
| *Chlamydomonas reinhardtii* | CRISPR/Cas9 | Knockout, low efficiency | *Hygro*, *mGFP*,*FKB12*, and *Gluc* | Jiang et al., 2014 |
| | CRISPR/Cas9 | Knockdown and knock-in | *MAA7*, *CpSRP43*, and *chlM* | Shin et al., 2016 |
| | CRISPR/Cas9 | Double Knockout | *ZEP* and *CpFTSY* | Baek et al., 2016 |
| | CRISPR/Cas9 | Knockout | Zeaxanthin epoxidase gene | Baek et al., 2018 |
| | CRISPRi | Knockdown | *PEPC1* and *RFP* | Kao and Ng, 2017 |
| | CRISPR/Cpf1 | Knockout (0.1-10%) | *CpFTSY*, *CpSRP43*, and *PHT7* | Ferenczi et al., 2017 |
| | Zinc-finger nuclease (ZNF) and CRISPR/Cas9 | Knockout (5 to 15%) | *COP1/2*, *COP3* (encoding channelrhodopsin 1 [ChR1]), *COP4* (encoding ChR2), *COP5*, *PHOT*, *UVR8*, *VGCC*, *MAT3*, and *aCRY* | Greiner et al., 2017 |
| | ZNF-mediated | Gene repair and gene knockout. | *aphVIII*, *COP3* | Sizova et al., 2013 |
| *Phaeodactylum tricornutum* | Meganucleases and TALEN-mediated | Knockout | *UGPase*/*NAT* gene | Daboussi et al., 2014 |
| | TALEN-mediated | Knockout (50%) | Blue-light dependent transcription factor Aureochrome1a (*PtAureo1a*) | Serif et al., 2017 |
| | TALEN-mediated | Knockout (24%) | Urease gene | Weyman et al., 2015 |
| | CRISPR/Cas9 | Knockout (31%) | Chloroplast signal recognition particle 54 (*CPSRP54*) | Nymark et al., 2016 |
| | CRISPR/Cas9 | Knockout (60%) | Urease gene, and eight genes involved in vanillin biosynthesis | Slattery et al., 2018 |
| | CRISPR/Cas9 | Knockout | A vacuolar protein,*Vtc2*, and a putative phosphate transporter, *Pho4* | Stukenberg et al., 2018 |
| *Thalassiosira pseudonana* | CRISPR/Cas9 | Knockout | Urease gene | Hopes et al., 2016 |
| | CRISPR/Cas9 | Knockout, highly efficient | Silacidin gene | Belshaw et al., 2017 |
| *Nannochloropsis oceanica* | CRISPR/Cas9 | Knockout (1%) | Nitrate reductase | Wang et al., 2016 |
| | CRISPR/Cas9 | Knockout | Nitrate reductase | Poliner et al., 2018 |
| | CRISPR/Cas9 | Knockout | Homolog of fungal Zn(ii)2Cys6 encoding gene (*ZnCys*) | Ajjawi et al., 2017 |
| *Nannochloropsis gaditana* | CRISPR/Cas9 | Knockout (~80%) | *Ble*, *GFP*, *Aco1*, *ZnCys*, and Seven LHC genes. | Verruto et al., 2018 |
| *Pseudochoricystis ellipsoidea* | TALEN-mediated | knockout | Uridine monophosphate synthetase (*UMPS*) | Kasai et al., 2015 |

Apart from these, the recent characterization of several other variants of the CRISPR system has extended the prospect of a genetic toolbox for microalgal genome engineering. Utilization of

these precise genome editing tools along with microalgal system biology can create an optimized platform customized for biofuel application and high-value product generation.

### 2.5 Genetic tools and techniques for transformation of microalgae

Genetic engineering of an organism requires availability of basic molecular tools and techniques such as gene transfer methods, efficient selection markers, and along with that availability of molecular information is also crucial (Hlavova et al., 2015). Further, for more complex applications such as knockdown of gene expression, multigene expression and endogenous gene expression requires availability of annotated genome sequences and suitable regulatory elements like promoters. Currently, the basic molecular tools are only available for few model microalga. However, genome sequencing and annotation projects of non-model microalgae species have enabled the reconstruction of metabolic pathways, and widespread use of RNA-Seq methods has allowed a more accurate prediction of microalgal metabolisms (Merchant et al. 2012). The recent development in functional analysis of genes involved in TAG biosynthesis and photosynthesis (Courchesne et al. 2009), expressed sequenced tag (EST) databases, prediction of de novo chloroplast TAG biosynthesis (Zienkiewicz et al. 2016), and information on transcription factors involved in these cellular metabolisms has brought new opportunities to harness the benefits of algal biotechnology (Bajhaiya et al. 2017). Thus, it is necessary to develop a reliable DNA transfer method in microalgae to improve the wild strains, understand basic metabolic processes underpinning growth, photosynthesis, and oil biosynthesis, and the production of high-value bioactive metabolites and recombinant proteins (Ng et al. 2017). However, except for a few model algae (Mini et al. 2018) and diatoms (Han et al. 2020), the method of introducing DNA into eukaryotic microalgae is still far from routine (Sharma et al. 2018).



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### 2.5.1 Transformation techniques

A variety of transformation methods have been employed to transfer foreign DNA into microalgal cells (Radakovits et al. 2010), involving electroporation (Lee et al. 2020), biolistic (George et al. 2020), and *Agrobacterium*-mediated gene transfer (Dehghani et al. 2020), however, the progress is mostly confined to the model alga, *Chlamydomonas reinhardtii* (Doron et al. 2016). The electroporation and biolistic gene transfer methods have their drawbacks, including potential cell damage and often transferring multiple copies of the transgene. Among the DNA transfer methods, *Agrobacterium*-mediated gene transfer is the most preferred method due to its ability to transfer low copies of the transgene and integrate foreign DNA preferentially into transcriptionally active genomic regions and feasibility to transfer large DNA segment with minimal rearrangement. Most importantly, the procedure is inexpensive and straight forward (Bakshi et al. 2011). However, only a few microalgal species have been successfully transformed by the *Agrobacterium*-mediated gene transfer method, which includes the model alga, *C. reinhardtii* (Kumar et al. 2004; Pratheesh et al. 2014; Mini et al. 2018), and the non-model algal species *H. pluvialis* (Kathiresan et al. 2009), *Isochrysis* sp. (Prasad et al. 2014), *C. vulgaris* (Cha et al. 2012), *Dunaliella* (Srinivasan and Gothandam 2016), *Scenedesmus acutus* (Suttangkakul et al. 2019) and *C. sorokiniana* (Gomez-Espinoza et al. 2018). However, in *C. sorokiniana*, the published methods (Gomez-Espinoza et al. 2018) lacked the confirmation of stable transgene integration by Southern hybridization and recombinant protein.

### 2.5.2 Nuclear genetic engineering

In comparison to the transgene expression in other organelle, nucleus has few distinct advantages such as it facilitates post-translational modification and stringent regulation of transgene



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expression through triggering regulatory elements (Rasala and Mayfield, 2015). Nuclear expressed recombinant proteins can be targeted to a specific location through signaling elements. With the advancement of nuclear genomic engineering, new tools such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) or associated programmable guide RNAs (CRISPR/Cas) have been developed (Gaj et al., 2013). These new development in nuclear genetic engineering can facilitates manipulations of precise metabolic pathways for industrial applications.

## 2.6 Challenges and future prospects

Microalgae as an alternative energy source hold immense potential to revolutionize the biofuel production system without putting much pressure on agriculture and the forest ecosystem. Despite the promises, commercialization of the microalgal biofuel technology is far from real, owing to its high production cost. Development of economically feasible technologies, such as microalgal strain improvement for improved oil production, holds the future for commercial scale production of algal biofuel. As summarized by Chung et al. (2017), biotechnological interventions could reduce the microalgal biofuel production cost by 15–20% in comparison with traditional approaches. Accordingly, implementation of key molecular schemes targeting pivotal cost-contributing attributes comprising superior feedstocks, oil extraction procedures, and quality of biodiesel can ease the financial burden imparted by these factors. The successful realization of these approaches can make microalgal biofuel production competitive with fossil fuel. To materialize the goal of gaining economic parity with fossil fuel, recent progress in microalgal biotechnology particularly in the field of biocatalyst engineering, synthetic biology, and genome editing has facilitated the necessary tools to design novel microalgal strains as per the culture condition. Furthermore, merging the primary goal of biofuel production with the intended



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coproduction of value-added products, such as antioxidants, nutraceuticals, and pharmaceuticals, could help in generating returns for financial investments (Jagadevan et al., 2018). Additionally, the adaptation of a consolidated biorefinery and phycoremediation approaches are also projected to diversify the utility of microalgal biomass (Rizwan et al., 2018). However, the sustainability of these approaches largely depends on the cost incurred during the culturing process of microalgal strains. As large-scale open pond culture is the most economic method for microalgae biomass production, the majority of the commercial microalgae are cultured by this system (Kumar et al., 2018). Thus, open pond culturing of genetically modified (GM) microalgae appears more promising in cutting down the cost; however, the impact on human health and environmental risks form the major concerns with transgenic microalgae if exposed to natural ecosystems (Rastogi et al., 2018). Being one of the primary producers in aquatic ecosystems, any involuntary introduction of GM microalgae could result in an ecological calamity (Singh S.K. et al., 2016). Strict monitoring and risk assessment analysis are, therefore, necessary to design the biosafety regulations for GM microalgae. Apart from these, techniques for the bio-containment of transgenes with codon reassignment and mutagenesis might be helpful in mitigating environmental risks through the deletion of genes crucial for survival in the wild but lack importance for culture (Henley et al., 2013; Gressel et al., 2014; Young and Purton, 2016). Additionally, a long-term comprehensive evaluation of the impact of non-indigenous and engineered microalgal strains on the native ecosystem could be helpful in eliminating the ambiguities around regulations on the cultivation of GM algae. In one notable case, Szyjka et al. (2017) reported that when a microalgal species, *Acutodesmus dimorphus*, was cultured in an open pond, neither the transgenic nor the wild type counterpart of the microalgae species were successful in outcompeting the native strains.



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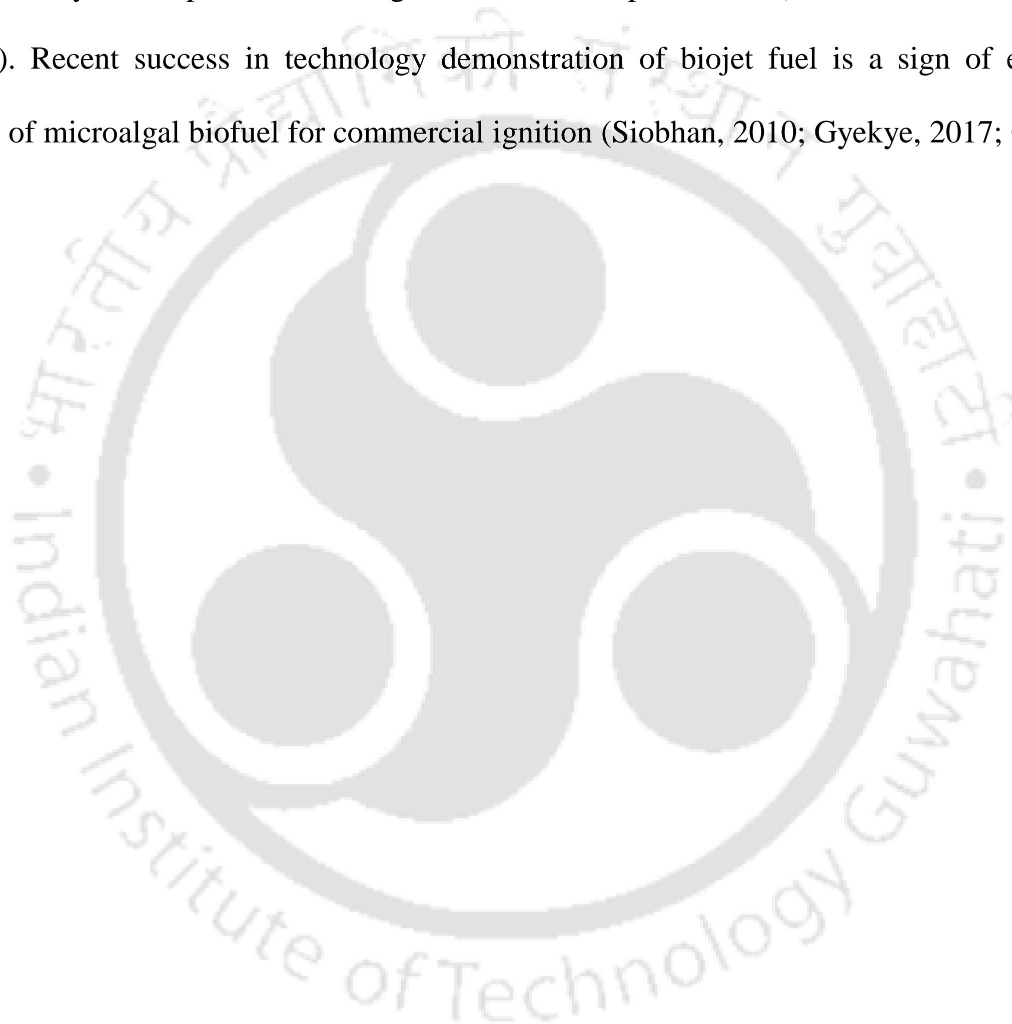
The study concluded that the outdoor culturing of GM microalgae fails to affect the microalgal diversity in the native ecosystem. However, before drawing any conclusion, extensive studies should be conducted as it is evident that regulatory certainty would be critical in the development of economically viable processes for algae based biofuel production (Glass, 2015; Randhawa et al., 2017). Recent success in technology demonstration of biojet fuel is a sign of emerging prospects of microalgal biofuel for commercial ignition (Siobhan, 2010; Gyekye, 2017; Chandra, 2018).


Indian Institute of Technology Guwahati



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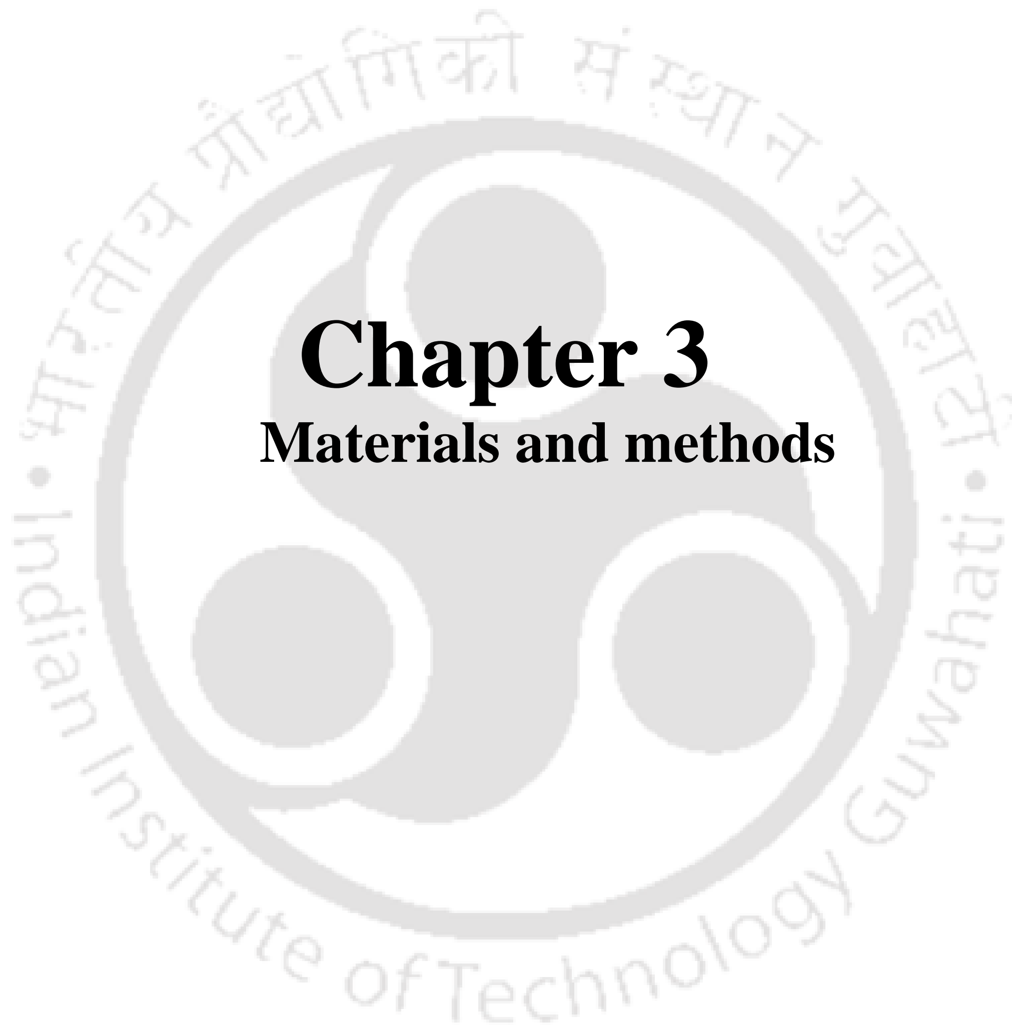

# Chapter 3

## Materials and methods

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## 3.1 Bacterial and Algal strains used in this work

### 3.1.1 *Chlorella sorokiniana*

*C. sorokiniana* (strain CG12) used in this study was collected from Prof. Vaibhav V. Goud (Center for Energy, IIT Guwahati, India). The strain was maintained as axenic cultures under the culturing conditions described later.

### 3.1.2 *Agrobacterium tumefaciens*

*Agrobacterium tumefaciens vir* helper strain EHA105 was used in this study.

### 3.1.3 *Escherichia coli*

*E. coli* DH5α strain was used in the cloning experiments in this study.

## 3.2 Growth and handling of microalgae cultures

The microalgae cultures were maintained as axenic cultures in liquid and solid BG11 medium (Stanier et al. 1971) and TAP medium (Sueoka 1960). The cultures were maintained at 25 °C under a 16-h light/8-h dark photo-period regime (50 ± 5 µmol photons m $^{-2}$ s$^{-1}$) in an orbital shaker (120 rpm).

### 3.2.1 Growth media recipes

The routine culture medium was Tris-acetate–phosphate (TAP) liquid or agar-solidified medium (Sueoka 1960) containing $NH_4Cl$ (375 mg/L), $MgSO_4.7H_20$ (100 mg/L), $CaCl_2.2H_2O$ (50 mg/L), $K_2HPO_4$ (108 mg/L), $KH_2PO_4$ (54 mg/L), Tris (hydroxymethyl) aminomethane (2.42 g/L) (pH 7.0), glacial acetic acid (1 mL/L) and trace metal solution (1 mL/L). The stock solution of trace metals was prepared using the following ingredients: EDTA disodium salt (50 g/L), $ZnSO_4.7H_2O$ (22 g/L), $H_3BO_3$ (11.4 g/L), $MnCl_2.4H_2O$ (5.06 g/L), $CoCl_2.6H_2O$ (1.61 g/L), $Cu.SO_4.5H_2O$ (1.57 g/L), $(NH_4)_6MoO_3$ (1.10 g/L), and KOH (17 g/L). For microalgae transformation and antibiotic



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selection experiments, a liquid or agar-solidified BG11 culture medium (Stanier et al. 1971) was used. The media composition of BG11 medium (pH 7.1) consists of $NaNO_3$ (1.5 g/L), $K_2HPO_4$ (40 mg/L), $MgSO_4.7H_20$ (75 mg/L), $CaCl_2.2H_2O$ (36 mg/L), Citric acid (6 mg/L), Ammonium ferric citrate green (6 mg/L), EDTA disodium salt (1 mg/L), $Na_2CO_3$ (20 mg/L) and trace metal solution (1 mL/L). The composition of trace metal stock solution had $ZnSO_4.7H_2O$ (0.22 g/L), $H_3BO_3$ (2.86 g/L), $MnCl_2.4H_2O$ (1.81 g/L), $Co(NO_3)_2.6H_2O$ (0.05 g/L), $Cu.SO_4.5H_2O$ (0.08 g/L), $Na_2MoO_4.2H_2O$ (0.39 g/L).

### 3.2.2 Algal growth measurements

Growth kinetics parameters such as specific growth rate (μ) and cell doubling time (dt) were analyzed as described by Godoy-Hernandez and Vázquez-Flota (2006). Calculation of the growth parameters was made with the Eqs. (1) and (2):

$$\mu = (\ln X - \ln X_0)/t, \quad (1)$$

$$dt = \ln 2/\mu, \quad (2)$$

Where $X_0$ is the initial cell density, and $X$ is the cell density at time $t$.

### 3.2.3 Analysis of photosynthetic parameters

The chlorophyll fluorescence measurements were taken through a DUAL-PAM100 Chlorophyll Fluorometer (Heinz Walz Gmbh, Effeltrich, Germany), as described by Li et al. (2010[b]) with some modifications. In brief, the microalgal cell density was normalized to 10 µg of chlorophyll a per mL and cultured under standard culture conditions for several hours. After dark adaptation for 30 minutes, minimal fluorescence yield ($F_0$) was determined. Maximum fluorescence yield ($F_m$) was measured by applying a saturating light pulse. The maximal quantum efficiency of photosystem II (PSII) was measured as $F_v/F_m$, where $F_v = F_m - F_0$. Actinic light was applied to measure the



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parameters related to light-adapted conditions. Firstly, the steady-state fluorescence yield ($F_s$) was recorded, and subsequently, the stationary level of light-adapted maximum fluorescence ($F_{m'}$) was measured by applying a saturation light pulse. Then the quantum yield of PSII ($Y_{II}$) was calculated as $(F_{m'} - F_s)/F_{m'}$, and the non-photochemical quenching coefficient (NPQ) was determined as $(F_m - F_{m'})/F_{m'}$. Photochemical quenching coefficient (qP) was then calculated as $(F_{m'} - F_s)/ (F_{m'} - F_{0'})$, where $F_{0'} = F_0/ (F_v/F_m + F_0/F_{m'})$.

### 3.2.4 Preparation of cells for long-term storage

A large loop of microalgal cells from a two-week-old agar plate was inoculated into a 10 mL TAP medium and incubated for 30 min shaking at 25 °C until the mixture becomes homogenous. 1 mL of the algal culture was added to a cryovial containing 5% methanol and placed at -20 °C for 2 hours. The tubes are then briefly chilled in liquid nitrogen and finally transferred to a -80 °C freezer for long-term storage.

## 3.3 Nuclear transformation of *C. sorokiniana* using *A. tumefaciens*

For transformation experiments, *Agrobacterium* cells at early log-phase were used. A single bacterial colony was inoculated into 10 mL of liquid YEP medium supplemented with appropriate antibiotics and grown overnight at 28 °C in a shaker incubator at 180 rpm until $OD_{600}$ reached 1.0. The bacterial cells were collected by centrifugation at 4500 × g for 5 min in 50 mL falcon tubes. The supernatants were carefully decanted, and the pellets were resuspended in 20 mL of liquid BG11 or TAP medium to maintain the final $OD_{600}$ of *Agrobacterium* culture at 0.6. For virulence gene induction, acetosyringone (50–200 µM) was added to the *Agrobacterium* culture medium (BG11 or TAP) media and incubated at 28 °C for 1 hour with gentle shaking. Prior to cocultivation, $1 \times 10^6$ cells/mL of *C. sorokiniana* culture from log-phase culture were plated onto BG11/ TAP-



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agar media and precultured for a week at 25 °C. The cells were harvested and subsequently washed twice with the induction medium (BG11/TAP medium + 50 to 200 µM acetosyringone) on the day of cocultivation. After washing, the algal cells were finally resuspended in 1 mL of induction medium containing *Agrobacterium* cells and incubated at 28 °C for 30 min with mild agitation. The cell suspension was then spread onto the induction medium solidified with 1.2% (w/v) agar. Cocultivation was performed for 24–72 hours at 25 °C in the dark. Following cocultivation, cells were harvested with BG11 liquid medium supplemented with cefotaxime (500 mg/L) and incubated in the dark at 25 °C for two days to eliminate *Agrobacteria*. The algal cells were then collected and washed twice with ddH2O by centrifugation at 4500 × g for 5 min. The transformed algal cell pellet was resuspended into 5 mL of liquid selection media (BG11 containing 30 mg/L hygromycin) containing 500 mg/L cefotaxime and incubated at 25 °C in the dark for two days before transferring to light conditions for five more days. After this step, 100 µL of the homogenous cell suspension was inoculated into 5 mL of fresh BG11 liquid selection medium and incubated at 25 °C in light conditions for seven days. Subsequently, this step was repeated five times before plating the positively selected cells onto an agar-selection medium (75 mg/L hygromycin) to isolate individual resistant colonies. Resistant colonies were randomly selected and propagated on a non-selective TAP medium for 12 days.

## 3.4 Molecular biology techniques used in this work

### 3.4.1 DNA sequence manipulation software

Primer design and manipulation of DNA sequences in silico was done using the OligoAnalyzer online software which can be found at https://sg.idtdna.com.



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### 3.4.2 Genomic DNA isolation from *C. Sorokiniana*

Genomic DNA from transformed and wild-type (WT) *C. sorokiniana* was isolated using the CTAB method (Murray and Thompson 1980). 5 mL of mid-exponential phase culture of $OD_{680} = 1.0$ was harvested by centrifugation at 4500×g for 5 min. The pellet was washed twice with autoclaved distilled water and finally resuspended in 0.5 mL CTAB buffer (2% hexadecyltrimethylammonium bromide [Sigma], 0.1 M Tris-HCl pH 8 [Himedia], 1.4 M NaCl

[Himedia], 10 mM EDTA [Himedia], 2% β-mercaptoethanol [Himedia], RNase 100 µg/ml [Sigma]) and incubated for 1 hour at 65 °C with occasional shaking. The mixture was then purified with 0.5 mL of phenol:chloroform:isoamyl alcohol 25:24:1 [Himedia] by centrifugation at 9,000 x g for 5 min. The aqueous phase was then carefully transferred to a new 1.5 mL Eppendorf tube and precipitated with two volumes of chilled absolute ethanol and 0.3 M sodium acetate. The genomic DNA was spinned down by centrifugation for 20min at maximum speed. The pellet was then washed twice with 70% ethanol and finally dissolved in $ddH_2O$.

### 3.4.3 Construction of expression vectors

Full-length *Jatropha curcus diacylglycerol acyltransferase 1* (DGAT1) (Gene Bank: EU477378.1) cDNA was PCR-amplified with *XhoI* and *KpnI* sites on the 5′- and 3′- ends, respectively, using the forward primer (CTC GAG ATG ACG ATT TTG GAG ACC A) and reverse primer (GGT ACC CTA GTG ATT TCA TCT TAA TTC), and the PCR product was cloned as *XhoI– KpnI* fragment and maintained in the intermediate vector, pRT101. The entire gene cassette comprising the CaMV35S promoter, *JcDGAT1* gene, and the CaMV PolyA terminator was finally cloned into the *PstI* sites of the binary expression vector pCAMBIA-1301.



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The *JcDGAT1* construct was mobilized into the disarmed *Agrobacterium tumefaciens* strain EHA105 and used for transformation experiments.

Similarly, Full-length *Arabidopsis thaliana wrinkled 1* (WRI1) (Gene Bank: NM_001035780.3) cDNA was PCR-amplified with *BamHI* and *XbaI* sites on the 5′- and 3′- ends, respectively, using the forward primer (AGC GAC GGA TCC AAA CCA CTC TGC TTC CTC) and reverse primer (TGC ACC TCT AGA TCG CCC TTC AGG TAC CCA), and the PCR product was cloned as *BamHI – XbaI* fragment and maintained in the intermediate vector, pRT101. The entire gene cassette comprising the CaMV35S promoter, *AtWRI1* gene, and the CaMV PolyA terminator was finally cloned into the *HindIII* sites of the binary expression vector pCAMBIA 1301. The *AtWRI1* construct was mobilized into the disarmed *Agrobacterium tumefaciens* strain EHA105 and used for transformation experiments.

### 3.4.4 Preparation of competent cells and transformation of *E. coli*

The DH5α strain of *E. coli* was plated onto an LB agar plate and incubated overnight at 37 °C. A single colony was then inoculated in 10mL of fresh LB broth and incubated overnight at 37 °C. One mL of the culture was then inoculated into 100 mL of fresh LB broth and incubated for 2.5 hours at 37 °C shaken at 180 rpm. Then, the culture was kept on ice for 30 min and harvested in chilled 50 mL falcon tubes at 4500 x g for 10 min. Then, the cells were resuspended in 30 mL of chilled 50 mM $CaCl_2$ and kept on ice for 30 min. Afterward, the cell suspension was again pelleted at 4500 x g for 10 min and resuspended in 8 mL of freshly prepared chilled 50 mM $CaCl_2$. Finally, 3.5 mL of chilled 50% (v/v) glycerol was added to the suspension, and 500 µL aliquots were made in 1.5 Eppendorf tubes and frozen in liquid nitrogen before storing at -80 °C.



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### 3.4.5 Plasmid DNA isolation

Plasmid DNA was isolated from overnight grown *E. coli* cultures using a QIAprep Spin Miniprep Kit [Qiagen] according to the manufacturer's instructions.

### 3.4.6 Polymerase chain reaction (PCR) analysis

PCR analysis was conducted to detect the presence of transgenes using gene-specific primers (**Table 4.1, 5.1** and **6.1**) under the following conditions: 95° C for 5 min, 35 cycles of 95° C for 1 min, 58° C for 1 min, 72° C for 1 min, and then 72° C for 10 min. The recombinant plasmid pCAMBIA-1301 containing the transgenes were used as the positive control. The PCR-amplified products were analyzed on 1% agarose gel and visualized by ethidium bromide staining.

### 3.4.7 Southern hybridization

Randomly selected transgene PCR-positive *C. sorokiniana* transformants were further analyzed for stable T-DNA integration by Southern hybridization. The transformants were previously propagated in standard TAP medium up to nine times to avoid any false-positive cases, and then genomic DNA (gDNA) was extracted from 2 g (wet cell weight) of transgenic and control wild-type cells using the NucleoSpin 8 Plant II Maxi kit (Macherey-Nagel, Duren, Germany). Approximately 100 µg of gDNA was digested with specific restriction enzymes. The restriction enzyme digested samples were separated on a 0.6% agarose gel and transferred onto a positively charged Zeta-Probe nylon membrane (Bio-Rad, USA) using a vacuum-assisted blotting procedure (Gross et al. 1988). The blot was then hybridized with a 0.5-Kb DIG-labeled probe corresponding to the transgene. The high hybridization buffer used for the pre-hybridization and hybridization washes consisted of 5XSSC, 1% blocking solution, 0.1% (w/v) N-lauroyl sarcosine, 0.02% (w/v) sodium dodecyl sulfate. The washing and detection were carried out according to the DIG-



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labelling and detection system (Roche Applied Science, Mannheim, Germany).

### 3.4.8 RNA isolation and quantitative RT-PCR analysis

The quantitative real-time PCR (qRT-PCR) was performed to examine the expression of the transgene. The total RNA was isolated from the stable T-DNA integrated transgenic lines and wild-type untransformed cells using Trizol (Invitrogen, USA) and quantified with nanodrop spectrometer (Nanodrop, USA). The cDNA was prepared with one μg of total RNA using a reverse transcription kit (ThermoScientific, USA). The quantitative RT-PCR was then performed with SYBR Green PCR Master Mix (Invitrogen) using transgene-specific primers (**Table 4.1, 5.1,** and **6.1**). The mRNA expression level was normalized using the *actin* gene as the internal control. The relative fold change in the transgene transcript was estimated by the comparative $\Delta\Delta CT$ method (Pfaffl 2006; Schmittgen and Livak 2008) with corresponding standard errors (Hoebeeck et al. 2007).

### 3.4.9 Western blotting

Western blotting was carried out to detect the expression of the transgene. Total protein was extracted from freshly harvested algae following the procedure described by Weis et al. (2002). Protein concentrations were assessed using the Bradford protein assay (He 2011). Equal amounts of protein were loaded onto a 12% SDS-PAGE gel and electrophoresed on a Hoefer SE 260 gel unit (GE Healthcare, USA) at room temperature. The total proteins separated by SDS-PAGE were electrotransferred to polyvinylidene difluoride membrane (GE Healthcare, Freiburg, Germany) and blocked with 3% BSA in TBST buffer (20 mM Tris–HCl, 120 mM NaCl, 0.1% Tween 20) for two hours. The membrane was probed with polyclonal primary antibodies (AB clonal Technology, Woburn, MA, USA) against the GUS protein at 4 °C for 12 hours.



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Following the washing, the blot was incubated with HRP-labeled secondary antibodies (Sigma-Aldrich, Darmstadt, Germany) for two hours. The blots' signals were detected using the Super Signal West Dura Trial Kit (Thermo Scientific, IL, USA).

## 3.5 Biochemical assay

### 3.5.1 Estimation of soluble protein

Extraction of soluble protein from the microalgae cultures was carried out by grinding approximately 100 mg (wet cell weight) of biomass in 1.5 mL of chilled 0.1 M phosphate buffer (pH 7.0) and then centrifuged at 9000 x g at 4 °C for 15 min. The aqueous fraction was then pooled, and the amount of protein was estimated through the Bradford method using three technical replicates. Graded concentrations of bovine serum albumin (BSA) standards were used to construct the standard curve.

### 3.5.2 Estimation of carbohydrate

The total carbohydrate content was measured by the procedure described by Van Wychen and Laurens (2017). Graded concentrations of D-glucose standards were used to construct the standard curve. All the measurements were performed in three technical replicates and represented as the percentage of fresh biomass.

### 3.5.3 Lipid extraction

Total lipids were extracted with the method described by Bligh and Dyer (1959). For the extraction of total lipids, 100 mg (DCW) of lyophilized microalgal biomass was used. The lyophilized biomass was homogenized in mortar-pestle, and lipids were extracted from the organic phase, which is then dried in a vacuum dryer. The total lipid content (% DCW, w/w) was measured as the gravimetric percentage of dry cell weight (DCW).



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## 3.6 Lipid analysis of *C. sorokiniana*

### 3.6.1 Analysis of TAG by thin layer chromatography

The extracted lipids were fractionated using thin-layer chromatography, and which were resolved on a silica gel plate (TLC Silica Gel 60 F254, Merck) using the hexane: diethyl ether: acetic acid (70:30:1, v/v/v) solvent system. The TAG spots were visualized by staining with iodine vapor.

### 3.6.2 Fatty acid methyl ester (FAME) preparation

FAME was prepared from 10 mg of lipids using the KOH/methanol method. Initially, total lipids were dissolved in methanol and 0.5 M Potassium hydroxide. Then, the mixture was shaken and incubated at 60 °C for 30 min. The FAME produced during the reaction was extracted with hexane and washed twice with dH2O. The pooled organic phase was then dried in a vacuum dryer and stored at 4 °C for further analysis.

### 3.6.3 Nile red staining

Nile red (NR) staining was used to identify intracellular lipids in microalgae. The NR stock (1 mg/mL) was made in DMSO and then diluted to 1 µg/mL for the final working concentration. The staining of the microalgal cells was done according to Storms et al. (2014). During the whole staining process, a 1:100 dye-to-algal cell ratio was maintained.

### 3.6.4 FACS

The NR stained cells were washed twice in phosphate-buffered saline (PBS; 0.1M pH 7.4) and finally resuspended in PBS, then analyzed using a flow cytometer (FACS AriaI, Becton Dickinson, France) to determine the NR fluorescence signal for cytosolic neutral lipids (NL). A 488 nm solid-state laser was used in the cytometer. NL's emission signal was collected using a 586/42 nm



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(PE-A) FL2 optical filter. The autofluorescence of chlorophyll also overlaps with the polar lipids; hence, unstained cells were used as an auto-fluorescence monitor.

### 3.6.5 Fluorescence microscopy

Nile red stained microalgae cells were examined for the presence of intracellular lipid droplets using fluorescence microscopy (Carl Zeiss AXIO SCOPE A1, Germany). Oil droplets were visualized at 570-630 nm emission with 559 nm excitation.

### 3.6.6 $^{1}$H NMR spectroscopy

NMR analysis of the lipids and FAMEs were carried out on a Bruker Avance III spectrometer at 600.13 MHz NMR proton frequencies. The extracted lipids and the FAME samples were dissolved in $CDCl_3$ (deuterated chloroform) and measured for relaxation time (T1) and $^{1}$H spectra with a 5 mm inverse triple resonance probe. In a time interval of 24 seconds, a total of 16–32 scans were performed, and the echo time (TE) measurements were taken at a temperature of 25 °C. The TMS (tetramethylsilane) peak's chemical shift was set to 0 ppm, and for baseline correction, the $CDCl_3$ peak was set to 7.20 ppm. All NMR data were processed and analyzed through the version of Mnova 11.0.2 (Mestrelab Research).

### 3.6.7 CD spectroscopy

The far-UV circular dichroism (CD) spectra of microalgae lipids dissolved in hexane were determined (JASCO; Tokyo, Japan) according to Li et al. (2018). Samples (0.1 mg/mL) were loaded onto a quartz cell with a 0.1 cm path length. The scan was performed at a 200 nm/min rate with a bandwidth of 1 nm from 170 to 600 nm. Hexane was used as a blank for baseline correction. The data in this analysis was expressed in terms of ellipticity (millidegree), and the spectra presented were the average of three scans.



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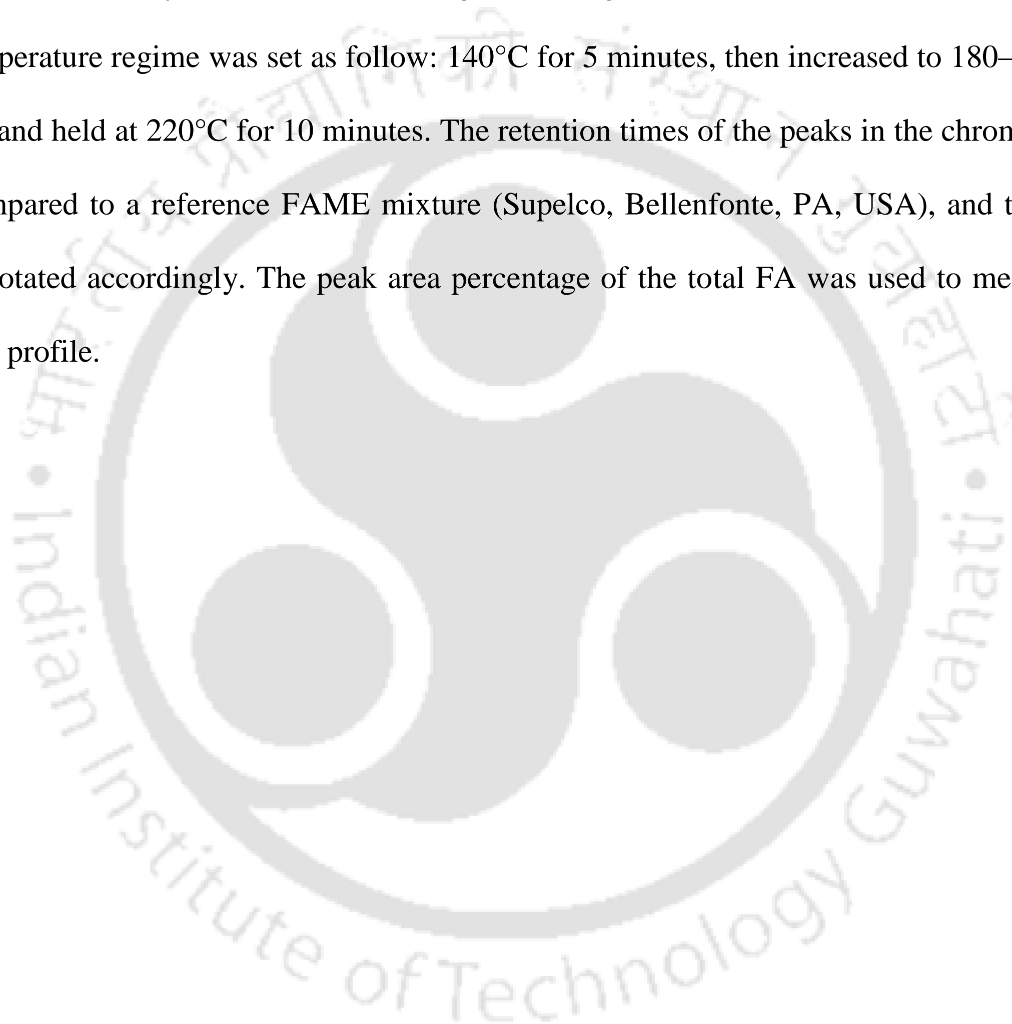
### 3.6.8 FAME analysis by Gas Chromatography

For FAME analysis, Varian 450-GC (Varian Capillary Column CP-SiL8 CB, 30 m 0.25-mm i.d., 0.25-µm film thickness) equipped with flame ionization detectors (FID) was used to separate and track FAMEs. A steady flow rate of the Nitrogen carrier gas was maintained at 0.4 mL $s^{-1}$, and the oven temperature regime was set as follow: 140°C for 5 minutes, then increased to 180–240°C at 3°C/min and held at 220°C for 10 minutes. The retention times of the peaks in the chromatogram were compared to a reference FAME mixture (Supelco, Bellenfonte, PA, USA), and the peaks were annotated accordingly. The peak area percentage of the total FA was used to measure the fatty acid profile.





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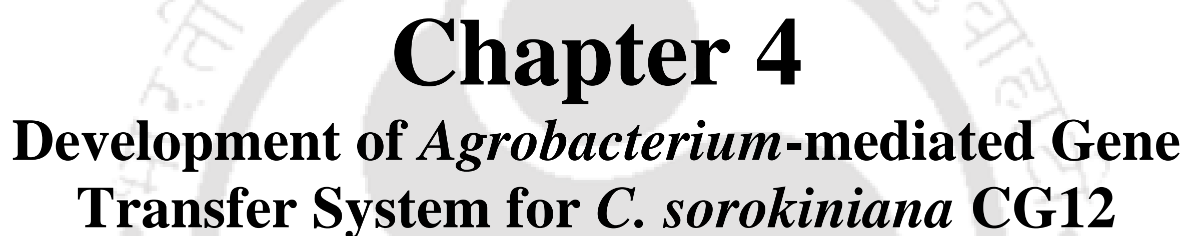

# Chapter 4

## Development of *Agrobacterium*-mediated Gene Transfer System for *C. sorokiniana* CG12

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The green oleaginous microalgae, *Chlorella sorokiniana*, is a highly productive *Chlorella* species and a potential host for the production of biofuel, nutraceuticals, and recombinant therapeutic proteins. The lack of a stable and efficient genetic transformation system is the major bottleneck in improving this species. We report an efficient and stable *Agrobacterium tumefaciens*-mediated transformation system for the first time in *C. sorokiniana*. Cocultivation of *C. sorokiniana* cells (optical density at $\lambda_{680}$=1.0) with *Agrobacterium* at a cell density of $OD_{600}$=0.6, on BG11 agar medium (pH 5.6) supplemented with 100 µM of acetosyringone, for three days at 25 ± 2 °C in the dark, resulted in significantly higher transformation efficiency (220 ± 5 hygromycin-resistant colonies per $10^6$ cells). Primarily, the transformed cells were selected on BG11 liquid medium with 30 mg/L hygromycin followed by homogenous colony selection of transformants on BG11 agar medium with hygromycin (75 mg/L). PCR analysis confirmed the presence of *hptII*, and the absence of *virG* amplification which ruled out the possibility of *Agrobacterium* contamination in transformed microalgal cells.

Further, the southern hybridization confirmed *hptII* gene integration into the genome of transformed *C. sorokiniana*. The qRT-PCR and Western blot analyses confirmed *hptII* and *GUS* gene expression in the transgenic cell lines. The specific growth rate, biomass doubling time, PSII activity, and fatty-acid profile of transformed cells were unaffected by the transgene expression. This protocol can facilitate opportunities for future production of biofuel, carotenoids, nutraceuticals, and therapeutic proteins.



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### 4.1 Optimization of the antibiotic selection for *C. sorokiniana* transformation

Determining the sensitivity of a microalgal species to a particular antibiotic is crucial for selecting microalgal cells transformed with an antibiotic selection marker gene. The growth of wild-type *C. sorokiniana* cells at the exponential phase was tested for their sensitivity to hygromycin on BG11 agar medium supplemented with varying concentrations of hygromycin (0–75 mg/L) (**Figure 4.1a**) and in BG11 liquid medium with hygromycin (0–30 mg/L) (**Figure 4.1b**). After 15 days of culture on the selection medium, the growth of *C. sorokiniana* cells was determined. The lowest hygromycin concentration that completely inhibited *C. sorokiniana* cells' growth in BG11 agar medium was 75 mg/L and 30 mg/L in liquid BG11 medium (**Figure 4.1 a, b**). Therefore, BG11 liquid medium with 30 mg/L hygromycin was used for primary selection, and BG11 agar medium with 75 mg/L hygromycin was used for subsequent selection of homogenous colonies.

### 4.2 *Agrobacterium*-mediated transformation of *C. sorokiniana*

The binary vector pCAMBIA-1301 harboring the *hptII* gene as a selection marker and *gus* reporter was mobilized to *A. tumefaciens* vir helper strain EHA105. A single clone of recombinant *A. tumefaciens* was used for the transformation of *C. sorokiniana*. Preliminary experiments were carried out before serial transformation of *C. sorokiniana* for optimizing different parameters of *the Agrobacterium*-mediated transformation process. In earlier studies, *A. tumefaciens* showed the ability to transform different microalgae (Naing et al. 2016; Karami 2008; Rathod et al. 2013; Cha et al. 2012). However, the transformation efficiency of different recipient cell types does not show the same consistency. The choice of cocultivation conditions mainly, cocultivation duration, type of cocultivation media, and acetosyringone concentration, greatly determine the transformation efficiency. Hence, it is crucial to standardize the cocultivation conditions to



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design a reliable and efficient gene delivery system. In this study, three crucial cocultivation parameters were evaluated, which included the type of cocultivation media (BG11 and TAP), cocultivation duration (1, 2, and 3 days), and acetosyringone concentration (50, 100, 150, and 200 µM). Based on previous literature, cocultivation was performed on solid media as T-DNA transfer was unsuccessful in liquid media (Cha et al., 2012). When cocultivation was carried out on BG11 agar media, it produced the most transformed colonies than TAP-agar media (**Figure 4.2**). On the contrary, TAP media in both cocultivation and the selection stage have resulted in higher transformation efficiency in model microalgae (Kumar et al. 2004; Mini et al. 2018; Anila et al. 2011). Based on our results, BG11 media was selected as the cocultivation medium for *C. sorokiniana* transformation.

The cocultivation period is one of the crucial parameters that influence the transformation efficiency (Naing et al. 2016; Cha et al. 2012), and the optimal duration of cocultivation is dependent on the *Agrobacterium* strain, host cell type, and the cocultivation medium (Barik et al. 2005; Kumar et al. 2012; Hu et al. 2006). The highest number of hygromycin-resistant colonies was observed after three days of cocultivation (**Figure 4.2**). This observation was similar to previous findings wherein three days of cocultivation was optimal for the transformation of *Chlorella* (Cha et al. 2012), *Isochrysis* (Prasad et al. 2014), and *C. reinhardtii* (Pratheesh et al. 2014). Our results indicated that *Agrobacterium*-mediated transformation of *C. sorokiniana* was optimal when cocultivation medium was supplemented with 100 µM of acetosyringone as the number of transformed colonies dramatically increased with an increase in acetosyringone concentration from 50 to 100 µM (**Figure 4.2**).



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Further increase in acetosyringone concentration above 100 µM did not appear to increase the transformation efficiency. Phenolic compounds such as acetosyringone are known to induce *Agrobacterium* virulence and stimulate bacterial surface attachment to the host cells, thereby enhancing the transformation efficiency (Naing et al. 2016; Karami 2008). Interestingly, the transformation of certain microalgal species, such as *Chlamydomonas* (Kumar et al. 2004), *Haematococcus* (Kathiresan et al. 2009), and *Parachlorella kessleri* (Rathod et al. 2013), was possible without the inclusion of acetosyringone. However, a study in *Chlorella* reported that T-DNA transfer was not feasible in the absence of acetosyringone (Cha et al. 2012), while a high concentration of acetosyringone negatively affected transformation efficiency (Chakrabarty et al. 2002; Zhu et al. 2009). The optimal acetosyringone thus depends on the microalgal species, cocultivation media, and cocultivation duration. Our results revealed that the cocultivation of *C. sorokiniana* cells ($OD_{680}$ = 1.0) on BG11 agar medium with pH 5.6, supplemented with 100 µM of acetosyringone, for three days at 25 ± 2 °C in the dark, with *Agrobacterium* at a cell density of $OD_{600}$ = 0.6 yielded the best result. Our method's transformation efficiency was determined to be 220 ± 5 colonies per $10^6$ cells, significantly more efficient than the previous report described for other *Chlorella* species (Sanitha et al. 2014). Here, the observation of high number of antibiotic resistant colonies in respect of other green microalgae lineage is indicative of an inherent antibiotic resistance property (as discussed in 4.1).

### 4.3 Detection of putative transformants and *Agrobacterium*-contaminants

Two putative hygromycin-resistant *C. sorokiniana* colonies were randomly selected and cultured on liquid BG11 medium containing 500 mg/L cefotaxime to eliminate *Agrobacterium* contamination. Genomic DNA was extracted from the putative transformants and subsequently



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subjected to PCR analysis (**Figure 4.3a**). Expected amplification of a 500 bp DNA fragment with *hptII* specific primers (**Table 4.1**) in each of the transformants analyzed confirmed the presence of *hptII* in the genome of *C. sorokiniana* transformants (**Figure 4.4c**). No amplification was observed in the wild-type cell lines.

Further, the possibility of *Agrobacterium* contamination in the transgenic lines was checked by observing the growth of *Agrobacterium* in YEP medium inoculated with transformed *C. sorokiniana* culture (**Figure 4.7**). As the growth rate of *Agrobacteria* tends to be faster than that of microalgae, this preliminary test helped to check the bacterial contamination in microalgal cultures (Srinivasan and Gothandam 2016; Prasad et al. 2019). No bacterial growth was observed in the cultures inoculated with the two transformed microalgal cultures viz, CsTr-9 and CsTr-16, even after 48 h of incubation at 28 °C. Both of the transformants were also assessed by PCR analysis using *virG*-specific primers to rule out bacterial contamination. No amplification of *virG* was detected in the *C. sorokiniana* transformed cultures (**Figure 4.3b**), revealing the absence of *Agrobacterium* contamination within the transformants (CsTr-9 and CsTr-16). This result also supported the fact that the *hptII* PCR analysis of the transgenic cell lines showed no *Agrobacterium* cells containing pCAMBIA 1301 plasmid in the transgenic *C. sorokiniana* cultures. PCR-based detection of marker genes located in the non-T-DNA region of the Ti-plasmid or *virC*/*virG* (virulence) genes in the *Agrobacterium* resident vir helper plasmid is, therefore, a good indicator for the presence of *Agrobacterium* contamination. Previous studies on *Agrobacterium*-mediated transformation of various microalgae such as *P. purpureum* (Prasad et al. 2019), *C. reinhardtii* (Mini et al. 2018), *Dunaliella salina* (Srinivasan and Gothandam 2016) have shown PCR-based detection of non-T-DNA localized *npt* or *virC*/*virG* to check the presence of *Agrobacterium* DNA



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in the transformed cultures.

### 4.4 Southern hybridization analysis for T-DNA integration

Southern hybridization analysis determined the stable integration of the transgene into the nuclear genome of *hptII* PCR-positive *C. sorokiniana* transformants. The two *C. sorokiniana* transformants showed signals corresponding to hybridization with *hptII*, indicating stable T-DNA integration with single transgene copy insertion. The different hybridization patterns observed for two different restriction enzymes indicated random integration of the T-DNA into the genome (**Figure 4.4a**). No hybridization signal was detected in wild-type *C. sorokiniana* cells.

### 4.5 Expression analysis of *hptII* gene in *C. sorokiniana* transgenic lines

The relative expression of *hptII* in the two *C. sorokiniana* transformants was analyzed by qRT-PCR (**Figure 4.4d**). *C. sorokiniana actin* gene was used to normalize the expression patterns (**Figure 4.4e**). The transformed lines with *hptII* transcript levels < 5-fold were categorized as low, while those with more than a fivefold increase in expression were considered high expression lines. Among the transformed lines checked, the CsTr-16 line showed high expression (**Figure 4.4f**). The wild-type line showed no expression of the *hptII*. However, no correlation was found between the *hptII* expression level and the transgene copy number in the transformants, which could be attributed to the position effects of the T-DNA integration sites (Gelvin 2003; Steinbrenner and Sandmann 2006; Liu et al. 2013). The integration of the *hptII* in the transcriptionally active locus of the CsTr-16 genome could have led to a higher expression of the *hptII* in the transformed line (Prasad et al. 2019; Gelvin 2003).



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### 4.6 Expression analysis of *gus* gene in *C. sorokiniana* transgenic lines

The transformed lines were further subjected to Western blot analysis to detect GUS expression using anti-GUS antibodies. The whole-cell protein from wild-type cell lines was used as a negative control. An approximately 70-kDa band was observed in the protein samples of the transformants. In contrast, no such bands were observed in wild-type samples (**Figure 4.4g**), confirming the active functionality of *gus* transgene in the two transformed lines of *C. sorokiniana*.

### 4.7 Analysis of growth related parameters in *C. sorokiniana* transgenic lines

To evaluate the possible interference of transgene expression on growth and cellular metabolism of the genetically transformed *C. sorokiniana* cells, the specific growth rate, biomass doubling time, photosynthetic efficiency of PSII, and fatty-acid profile of transformed cells were compared with the wild-type untransformed cells. The results of this study (**Figure 4.5, 4.6**) suggested that the transformants had a similar growth pattern and metabolic processes as that of wild-type cell lines, and the random integration of transgene had no adverse effect on growth and cellular metabolism. This finding correlated with previous reports (Prasad et al. 2019; Liu et al. 2013), which observed a similar growth pattern in the wild type and the transformants.

### 4.8 Conclusion

To harness the benefit of microalgal biotechnology for biofuel application, genetic manipulation of metabolic pathways is essential, requiring an efficient genetic transformation method. Besides, an efficient gene transfer system in microalgae would allow a way to understand cellular metabolism regulation by characterizing the genes involved through a reverse genetics approach. *A. tumefaciens*-mediated genetic transformation is a method of choice for ease in transformation and its ability to precisely integrate low copy number transgene into transcriptionally active



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genomic regions. However, in *C. sorokiniana*, the lack of a reliable and efficient *Agrobacterium*-mediated gene transfer method limits its potential uses in commercial-scale utilization. We described an efficient *A. tumefaciens*-mediated genetic transformation in *C. sorokiniana*. For the first time in *C. sorokiniana*, it highlighted the reliable detection of stable transgene integration and expression in *C. sorokiniana*, which opens up limitless possibilities in biofuel production and other commercially valuable commodities. Furthermore, considering the crucial role of *Agrobacterium* vir gene induction on effective T-DNA transfer, we optimized crucial cocultivation conditions. We found cocultivation of *C. sorokiniana* cells ($OD_{680}$ = 1.0), on BG11 agar medium with pH 5.6, supplemented with 100 µM of acetosyringone, for three days at 25 ± 2 °C in the dark, with *Agrobacterium* at a cell density of $OD_{600}$ = 0.6 resulted in the efficient transformation of *C. sorokiniana*.

Our finding opens up new possibilities for microalga with commercial potentials such as *C. sorokiniana* through genetic manipulation for specific target genes associated with diverse cellular pathways.



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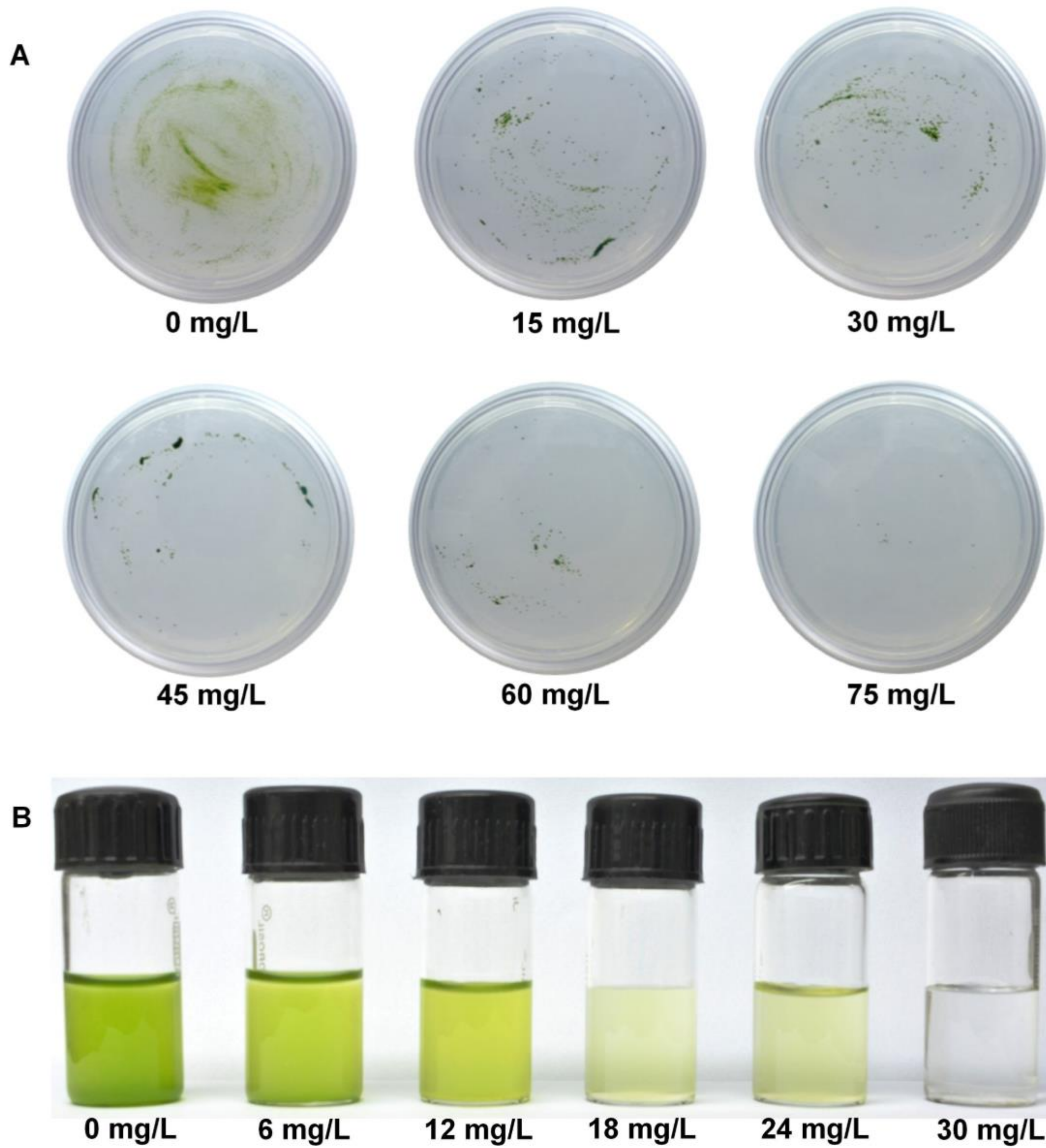


**Figure 4.1 Optimization of the hygromycin selection system for *C. sorokiniana* in** (a) BG11 agar medium and (b) BG11 liquid medium with different concentrations of hygromycin.



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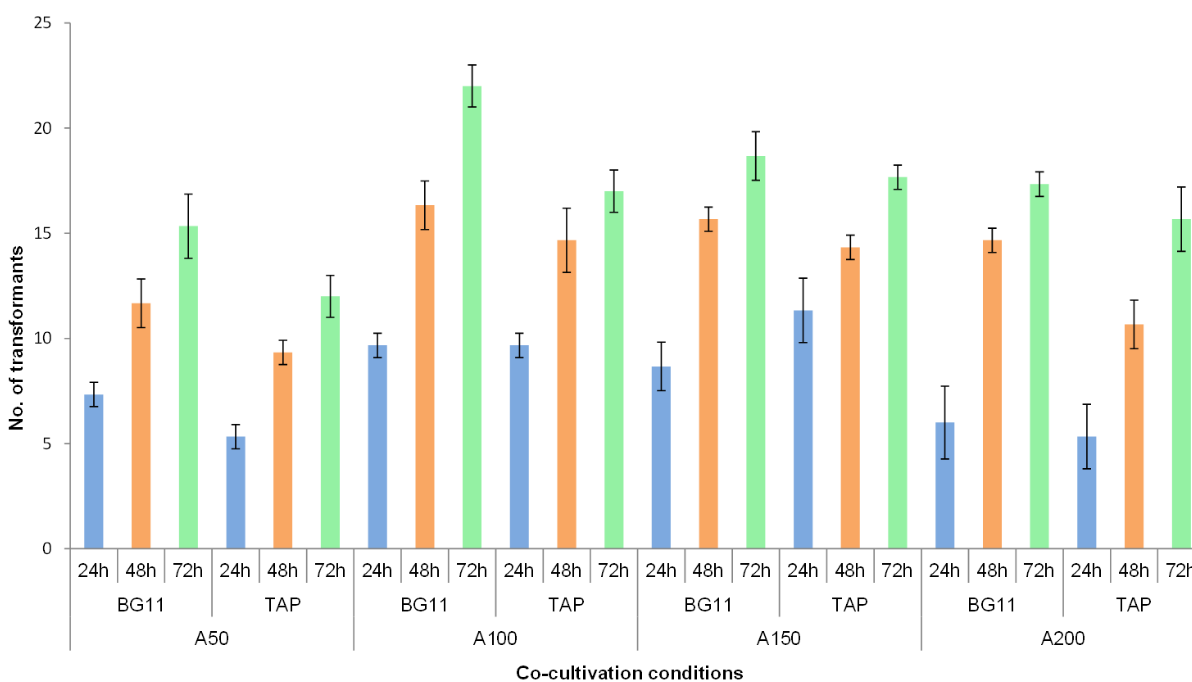


**Figure 4.2 Effect of different cocultivation parameters on the transformation efficiency in *C. sorokiniana*.** The tested cocultivation conditions include type of cocultivation media (BG11 and TAP), acetosyringone concentrations (A:50 µM; A:100 µM; A:150 µM; and A:200 µM), and cocultivation periods (24 h, 48 h, and 72 h). The difference between each of the instances tested for the different cocultivation conditions was significant at $P < 0.05$ by Tukey test, where values represented as mean ± SD (n = 3).

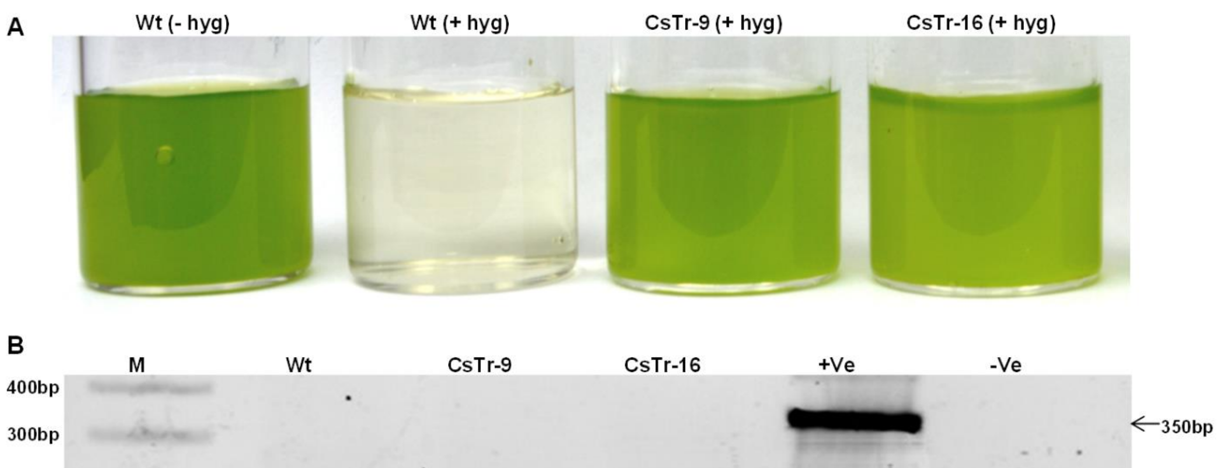


**Figure 4.3 Tolerance of wild-type and transgenic lines of *C. sorokiniana* against hygromycin in** (A) BG11 liquid media with hygromycin (30 mg $L^{-1}$): wild-type cells under hygromycin selection [Wt(+ hyg)] and without hygromycin selection [Wt(−hyg)], Transgenic cell lines under hygromycin selection [CsTr-9(+ hyg) and CsTr-16(+ hyg)]. (B) PCR amplification of the 350 bp-fragment of *VirG* gene for the detection of *Agrobacterium* contamination in the transgenic cell lines of *C. sorokiniana*: lane M, molecular marker; lane Wt, wild type; lane CsTr-9/16; transgenic lines; lane + Ve, *Agrobacterium* strain EHA105 (positive control); lane − Ve, negative control.

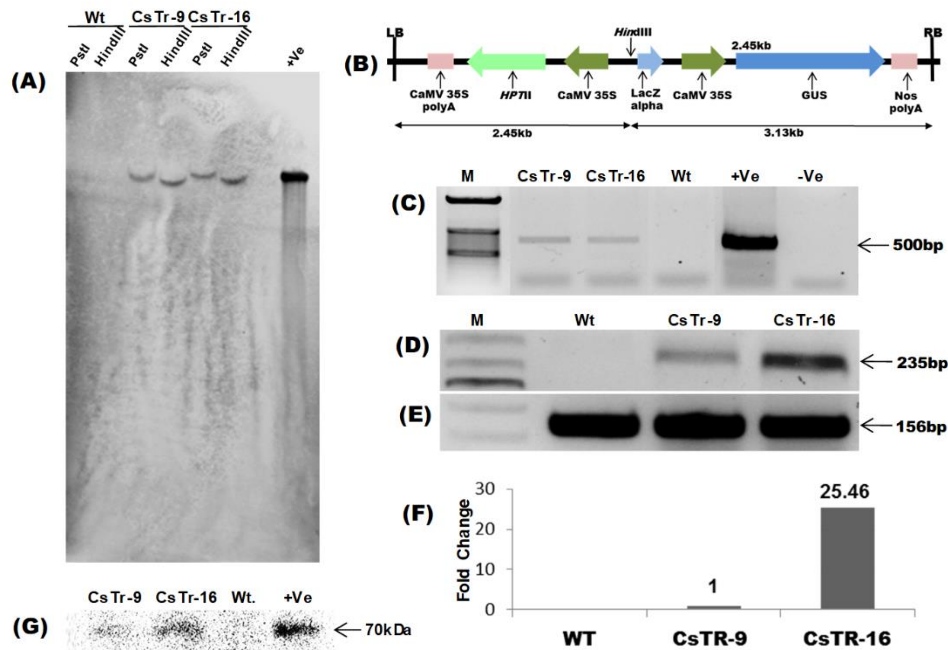


**Figure 4.4 Molecular analysis to detect transgenic lines of *C. sorokiniana*.** (a) Southern blot hybridization of *PstI* and *HindIII* digested genomic DNA of wild-type (Wt) and transgenic lines (CsTr-9/16) of *C. sorokiniana* hybridized to the hptII probe; lane + Ve, *HindIII* digested pCAMBIA1301 (11 Kb); (b) T-DNA region of pCAMBIA1301 binary vector used for transformation experiment; (c) PCR amplification of 634 bp-fragment of *hptII* gene; lane M, molecular marker; lane CsTr- 9/16, transgenic lines; lane Wt, wild type; lane + Ve, positive control; lane −Ve, negative control; (d) Transcript abundance of *hptII* gene in *C. sorokiniana* transformants. Expression analysis was carried out by semi-quantitative PCR using *actin* as an internal control (e); (f) Quantitative RT-PCR for analysis of relative abundance of hptII transcripts; (g) Western blot of *C. sorokiniana* transformants (CsTr-9 and CsTr-16) and wild-type (Wt.) cell lines; lane + Ve, positive control (GUS protein).



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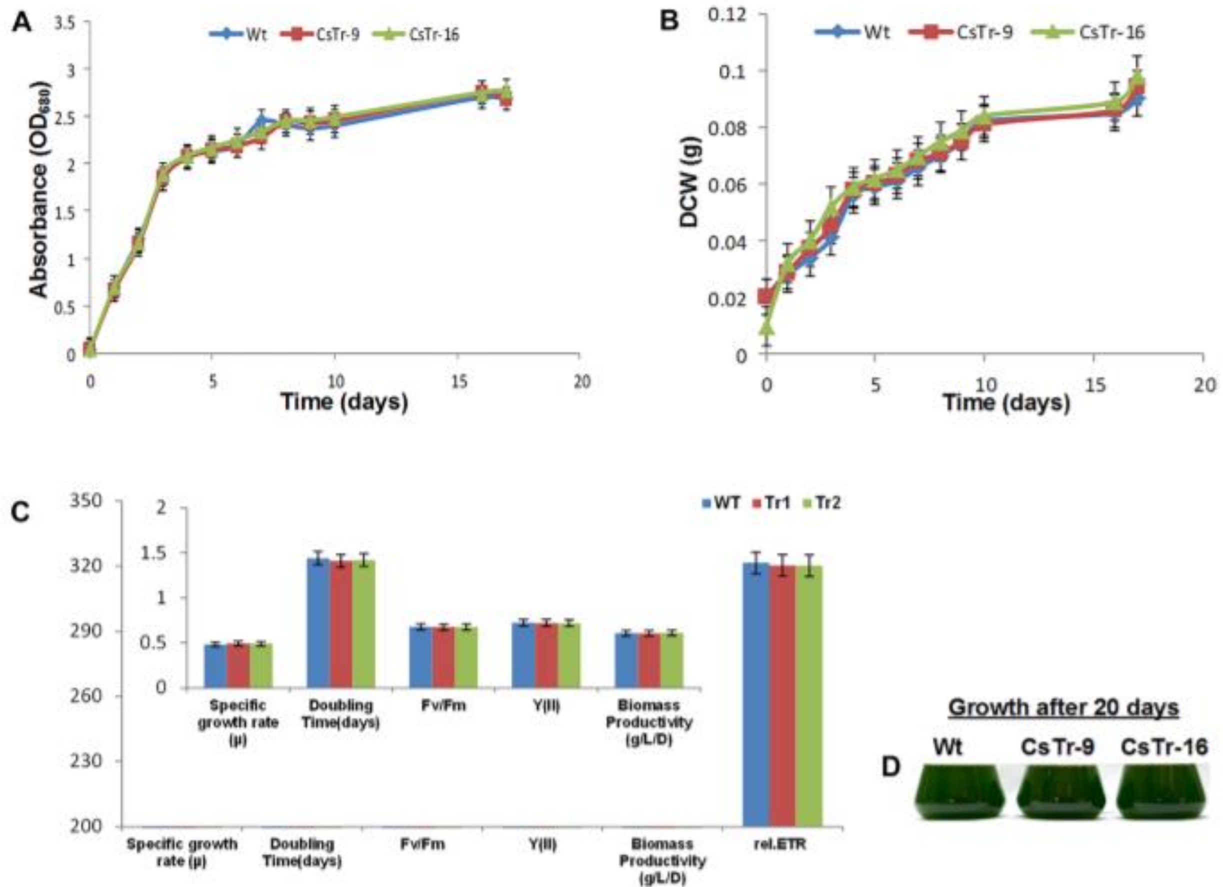


**Figure 4.5 Growth rate and photosynthetic productivity-related parameters of transgenic (CsTr- 9/16) and wild-type (Wt) cell lines of *C. sorokiniana* under optimal growth conditions.** (a) Growth curve of *C. sorokiniana* cell lines; (b) Biomass productivity of the *C. sorokiniana* cell lines measured through dry cell weight (DCW); (c) Comparison of different photosynthetic parameters among transgenic (CsTr-9/16) and wild-type (Wt) cell lines of *C. sorokiniana*. (d) Transgenic (CsTr-9/16) and wild-type (Wt) cell lines of *C. sorokiniana* cultured in TAP liquid media for a period of 20 days.



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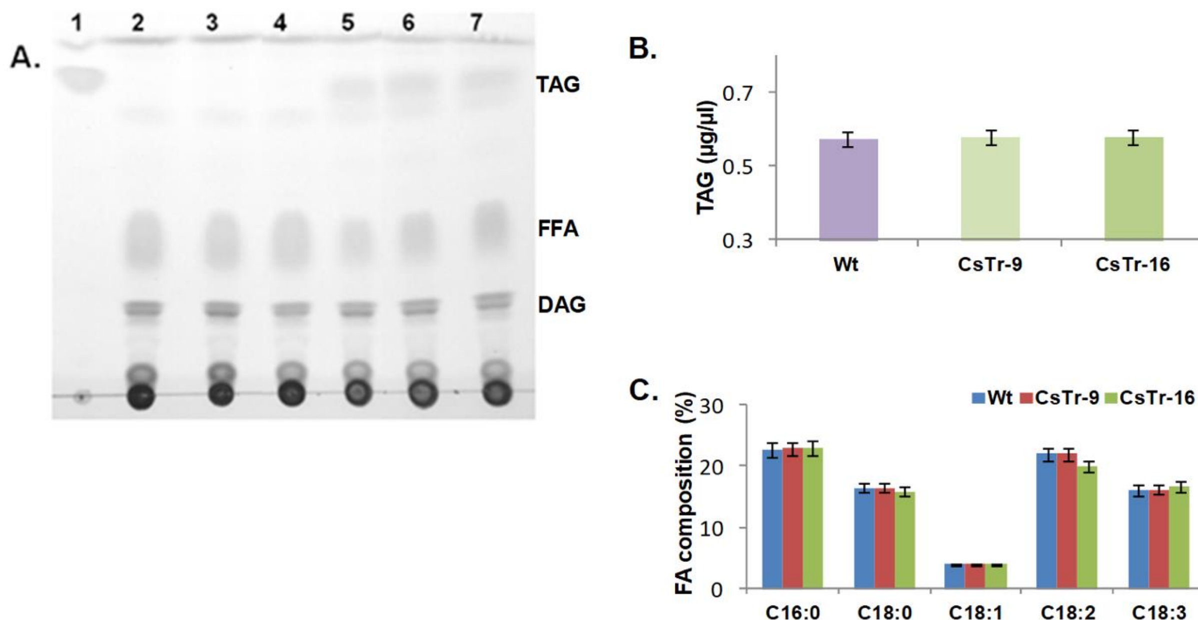


**Figure 4.6 Triacylglycerol accumulation and Fatty acid profile of wild-type and transgenic *C. sorokiniana* cell lines.** (a) TLC separation of neutral lipids: lane 1, trioleate standard; lanes 2–4, wild type, CsTr-9 and CsTr-16, respectively (cell lines cultured under nitrogen- sufficient conditions); lanes 5–7, wild type, CsTr-9 and CsTr-16, respectively (cell lines cultured under nitrogen-deficient conditions). Neutral lipid was fractioned by Silica Gel plates into triacylglycerol (TAG), free fatty acids (FFA) and diacylglycerol (DAG) fractions; (b) TAG content in wild-type (Wt), transgenic cell lines (CsTr-9 and CsTr-16) cultured under nitrogen-deficient conditions; (c) fatty acid (FA) profile of transgenic (CsTr-9 and CsTr-16) and wild-type (Wt) *C. sorokiniana*.

**Figure 4.7 Test for *Agrobacterium*-contamination by culturing in YEP medium.** Lane EHA105, *A. tumefaciens* strain EHA105; lane Wt, wild-type; lane CsTr-9 and CsTr-16, transgenic *C. sorokiniana* cell lines; lane Blank, negative control.



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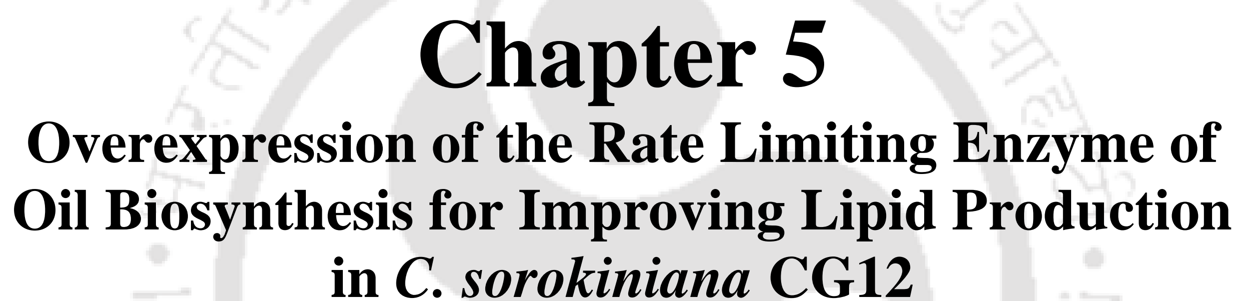
**Table 4.1 List of primers used in the study.**

| Sl. No | Target Gene | Forward Primer (5’-3’) | Reverse Primer (5’-3’) | Product size |
|---|---|---|---|---|
| 1. | *hptII* | ATCCTTCGCAAGACCCTTCCT | GGTGTCGTCCATCACAGTTTG | 634 bp |
| 2. | *RT_hptII* | GGTAAATAGCTGCGCCGATGGTTTCTAC | ATCACAGTTTGCCAGTGATACACATGGG | 235 bp |
| 3. | *CsAct1* | CTCGGTCAGGATCTTCATCAT | ATGACGCAGATCATGTTTGA | 156 bp |
| 4. | *virG* | GAACTGCTTGCTGTCGGC | GCGGTGCGACAATAGGCG | 350 bp |



# Chapter 5

## Overexpression of the Rate Limiting Enzyme of Oil Biosynthesis for Improving Lipid Production in *C. sorokiniana* CG12

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Microalgae have emerged as a potential feedstock for biodiesel production due to its high cellular lipid content, essential for achieving sustainability in biofuel production. The TAG biosynthetic pathway in microalgae is considered to be similar to that of higher plants. In the de novo TAG biosynthetic pathway, diacylglycerol acyltransferase (DGAT; EC 2.3.1.20) catalyzes the process's final and rate-limiting step. The DGAT enzyme is thought to be universal among all organisms in de novo TAG biosynthesis. Type 1 and type 2 DGAT enzymes are the two most studied classes of these enzymes. Although both forms of DGATs perform similar roles, their sequences and preferences have developed separately. Both forms of DGATs occur in plants, with DGAT1 being a significant contributor to TAG accumulation in seeds. In previous studies, ectopic expression of plant DGAT1 resulted in an increase in seed oil content and a shift in fatty acid composition, demonstrating the pivotal role of DGAT in TAG synthesis (Maravi et al., 2016). Further, *Jatropha curcus* being one of the oleaginous crop traditionally used as a biodiesel feedstock. Therefore, manipulating Jatropha *DGAT1* gene expression could be beneficial for microalgae's metabolic engineering for increased oil content and customized fatty acid (FA) composition. The green microalgae *Chlorella sorokiniana* was genetically engineered with a rate-limiting enzyme of TAG biosynthesis pathway, diacylglycerol acyltransferase 1 from *Jatropha curcas* (*JcDGAT1*), to enhance the lipid content. The *JcDGAT1* gene integration, expression, and functionality in the transgenic cells were analyzed by PCR, Southern blot, and spectro-fluorometric analysis of Nile red-stained cells. The transformed microalgal cells showed a 1.22 fold increase in total lipid content than that of untransformed control. Further, an increase in mono and poly-unsaturated fatty acids, primarily linolenic acid content by 1.16 fold in the transformed cells than WT, makes the transgenic microalgae-derived biofuels ideal for combustion. The enhanced lipid content in



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transformed lines did not significantly impair growth and photosynthetic rate. The study suggests a strategy for enhancing oil production by genetic means and might facilitate a platform strain with industrial potential.

### 5.1 Heterologous expression of *JcDGAT1* in *C. sorokiniana*

Several putative transformed colonies were obtained after our optimized protocol for *C. sorokiniana* (Sharma et al., 2021); however, many of the putative transformants were lost during the successive antibiotic-aided selection process. Among the phenotypically consistent colonies, four putative transformants (**Figure 5.1**), namely D5(i)2 [D1], BDT(i)B3 [D2], CDT(ii)C6 [D3], and DGAT13 [D4], were randomly selected to screen for the transformation event through PCR and southern blotting.

### 5.2 Molecular characterization of *JcDGAT1* overexpressing *C. sorokiniana* transgenic lines

The mechanism of lipid biosynthesis in microalgae could be subjected to rate-limiting steps with unknown feedback regulations, affirmed by the overexpression of three type-2 *DGAT* homolog genes in *C. reinhardtii*, which neither enhanced any intracellular TAG aggregation nor altered the fatty acid profile (La Russa et al., 2012). Numerous reports of homologous gene overexpression in microalgae enhance TAG accumulation (Zhang et al., 2021; Chungjatupornchai and Fa-aroonsawat, 2021). However, scarce *DGAT* gene characterization studies in *C. sorokiniana* encourage the use of heterologous origin of *DGAT* gene. Moreover, using a heterogeneous gene may bypass the feedback inhibition when inserted into an organism due to differences in the enzyme's active site (Ahmed et al., 2015). In the present study, transgenic *C. sorokiniana JcDGAT1* lines were made and subsequently confirmed by PCR of genomic DNA



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using *JcDGAT1* (**Figure 5.2B**) and *hptII* (**Figure 5.2C**) specific primers (**Table 5.1**), which do not produce unspecific bands in the wild-type *C. sorokiniana* samples. The integration of the T-DNA region comprising the *JcDGAT1* expression cassettes in *C. sorokiniana* was confirmed by southern hybridization. Among the four transgenic colonies tested for T-DNA integration, only two *C. sorokiniana* transformants showed stable integration with single transgene copy insertion. Moreover, different banding patterns were observed in the two transformants, which indicated independent transgenic events resulting in random integration of the T-DNA into the genome (**Figure 5.2D**). No hybridization signal was observed in the wild-type cells. The relative transcript abundance of *JcDGAT1* was determined using qPCR (**Figure 5.2F** and **5.2G**) using *CsActin* as a reference gene (**Figure 5.2E**). As shown in **Figure 5.2G**, the relative transcript abundance of *JcDGAT1* was significantly enhanced up to 1.2-fold in the transformants than the wild-type cells, indicating functionally active *JcDGAT1* gene integration into the genome of *C. sorokiniana* transformants. Moreover, the two transformants displayed different transcript abundance levels, which might be due to the epigenetic silencing effects on a transgene (Cerutti et al., 1997).

### 5.3 Analysis of lipid content in *JcDGAT1* transgenic lines

In vivo, Nile Red fluorescence assay was used to assess the lipid accumulation in the *JcDGAT1* transgenic lines to evaluate the impact of transgene expression. Previous literature revealed a progressive increase in lipid accumulation from day 2 to day 6 under nitrogen deplete conditions (Li et al., 2015). Hence, in the present study, the transgenic and the wild-type cells were cultivated in TAP-nitrogen sufficient media to early stationary phase and then shifted to TAP-nitrogen limited conditions and maintained for six more days before analyzing lipid content. Fluorescence microscopy revealed a higher number of lipid droplets (LDs) in the transgenic cells



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than the wild-type cells (**Figure 5.3A**). Intense yellow-color lipid bodies were observed in transgenic lines D1 and D2, whereas lipid bodies of WT were light yellow-color. The red-color background represents autofluorescence due to chlorophyll. Furthermore, the intracellular lipid accumulation was relatively compared through flow cytometry, which exhibited a higher FL2 mean fluorescence in the transgenic lines than the WT cells (**Figure 5.3B**). The results suggest an enhanced neutral lipid accumulation in the transgenic cells when concomitantly compared to WT. Similarly, several studies also summarized that higher intracellular Nile-red-based fluorescence strongly indicates higher neutral lipid turnover (Katayama et al., 2019; Tale et al., 2018).

Total cellular lipids were extracted to assess the neutral lipid reserve in the genetically engineered microalgae cells. The results showed that total lipid content was increased 1.22 fold in the transgenic lines than WT cells (**Figure 5.3C**). However, as TAG is a vital component for biodiesel production, it is crucial to quantify the TAG content in total lipids. The fraction of TAG content in total lipids was assessed with TLC (**Figure 5.3D**), and the band intensities associated with TAGs were subsequently quantified (**Figure 5.6**). The results showed a significant enhancement in TAG content in both transgenic lines than that of the WT. It was also evident that TAG is the most significant fraction of the lipids extracted from *C. sorokiniana*. Previously, heterologous overexpression of a yeast diacylglycerol acyltransferase (*ScDGA1*) in the microalgae *Phaeodactylum tricornutum* observed a dramatic increase in both total lipids and TAG content in comparison to the wild type (Zulu et al., 2017), which justified the findings of the present study. In brief, lipid analysis data suggests *JcDGAT1* overexpression markedly improved TAG content in the microalga.



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### 5.4 Spectropolarimeteric analysis of oils derived from transgenic *C. sorokiniana*

In the TAG molecule, the fatty acyl groups are covalently bound to the glycerol backbone by ester bonds in one of the three stereospecifically different sites, namely sn-1, sn-2, and sn-3 (IUPAC-IUB Commission on Biochemical Nomenclature, 1977). The TAG biosynthesis includes a metabolic network that overlaps directly with essential membrane lipid biosynthesis, involving several DAGs and acyl donor substrate pools and various TAG biosynthetic acyltransferases (Li-Beisson et al., 2013; Bates, 2016). The DGAT enzyme is known to catalyze the final acylation step in the TAG biosynthetic pathway by assigning a fatty acyl group from acyl-CoA to the sn-3 position of diacylglycerol (DAG) to form TAG (Shockey et al., 2016). Thus, the TAGs formed by esterifying the terminal sn-3 hydroxyl group of the glycerol backbone with different fatty acids have chiral asymmetries (Kalpio et al., 2020).

Moreover, the stereospecific distribution of fatty acids in TAGs of natural fats and oils are genetically regulated, implying that fatty acid positional distribution is not random (Vichi et al., 2007). Hence, overexpression of a transgenic DGAT1 enzyme may change the chirality of the oils derived from the transgenic lines of *C. sorokiniana*. The changes in the chirality of the oils derived from the wild-type and transgenic lines were evaluated through a CD spectropolarimeter. The results showed significant changes in the optical properties of the oils derived from transgenic lines compared to that of wild-type (**Figure 5.3H**). The observation implies that the transgenic DGAT1 enzyme has different fatty-acyl specificities than that of the endogenous DGAT enzymes, resulting in a difference in the oils' chirality from transgenic lines and wild-type *C. sorokiniana*.

### 5.5 FAME analysis of *JcDGAT1* transgenic lines

Generally, the oil content of most algal species is not suitable to be used as a direct substrate for



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premium quality biodiesel production, as in most cases, the saturated and unsaturated fatty acids are not present in a sufficient proportion. For instance, biodiesel with a high percentage of saturated short or medium-chain FAs may become more resistant to oxidation, but it will likely have a lower cloud point and produce wax at lower temperatures (Ahmed et al., 2015). The FA composition of the transgenic lines was tested by transesterifying the TAGs to fatty acid methyl esters (FAME) to address this concern. Evaluation of the transesterification process was done through $^{1}$H NMR spectroscopy to determine the composition of FAME samples (**Figure 5.3E**) by comparing them with the 1H NMR spectra of oils (**Figure 5.3F**) derived from *C. sorokiniana*. The doublet of doublets peaks at 4.27-4.33 ppm and 4.11-4.17 ppm are the characteristic peaks of glyceryl methylenes of the TAG molecule.

Simultaneously, a single peak at 3.66-3.68 ppm is the characteristic peak of methyl esters of the FAME molecule (Anderson and Franz, 2012). In **Figure 5.3E**, the presence of a prominent singlet peak at 3.5 ppm and the absence of doublet of doublets peaks in the vicinity of 4.27-4.33 ppm and 4.11-4.17 ppm region shows the efficient conversion of TAGs into FAMEs. The FAME samples were then analyzed using GC–MS. The palmitic acid (C16:0), stearic acid (C18:0), linolenic acid (C18:3), and linoleic acid (C18:2) are the most abundant FAs in the TAG (**Figure 5.3G**). The linolenic acid content in the transformants (D1 and D2) compared to wild-type was increased by 1.16 fold; wherein the saturated FAs like palmitic acid was reduced by 1%, and a slight but evident increase in the proportion of monounsaturated FAs such as oleic acid (C18:1) was observed in the transformants. In the present study, oils derived from the transgenic lines showed a reduction in saturated fats and an increment in unsaturated fats, a few of the desired qualities to source premium quality biodiesel. Increased unsaturated FAs content in oils is good for producing biodiesel that



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suits cold weather, but it may need an oxidative stabilizer to circumvent oxidation and rancidification (Ahmed et al., 2015).

Similar reports of FA profile modification were previously observed in *Tetraselmis chui* through overexpression of a Boraginaceae *EpDGAT1*, which increased the PUFA (18:3n3) content (Úbeda-Mínguez et al., 2017). In addition to utilization in biofuels, PUFA is emerging as an effective dietary supplement. Hence, oils derived from the transgenic lines (D1 & D2) may have more commercially valuable utilization as dietary supplements other than biofuel. Thus, the introduction of *JcDGAT1* improved the neutral lipid content in transgenic *C. sorokiniana* than in control. This strategy can be applied to commercially important microalgal strains to accumulate higher neutral lipid content and improve the oil quality by introducing the jatropha *JcDGAT1*.

### 5.6 Physiochemical characterization of *JcDGAT1* transgenic lines

To assess the possible interference of *JcDGAT1* overexpression on the growth and primary metabolism of the transformed cell lines, we analyzed the growth curve of the transformants under nitrogen-sufficient (+N) conditions. Both transformants (D1 & D2) showed an overall similar growth pattern compared to the wild-type, while slightly lower growth during the initial exponential phase (**Figure 5.4**). However, the transformants' biomass productivity and specific growth rate were not significantly different from wild-type (**Figure 5.5**). Furthermore, besides growth parameters, it is of interest to determine whether enhanced neutral lipid accumulation in the transformants had any metabolic compensations. Previous studies dealing with lipogenic transgene overexpression showed a marked decrease in the transformants' total carbohydrate and protein content than the wild-type (Muñoz et al., 2019; Wang et al., 2020). Hence, we compared the total cellular carbohydrate and protein content in the transformants to the wild-type. The results



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showed a significant reduction in carbohydrate and protein content in the transformants than in the wild-type (**Figure 5.5**). As hypothesized by Wang et al. (2018b), carbon precursors and metabolic machinery might have been redirected to lipogenesis in transgenic lines; the present study results also apparently follow a similar mechanism.

Besides this, to elucidate the potential ill-effects of enhanced neutral lipid accumulation on photosynthetic electron transport in photosystem II (PSII), the chlorophyll fluorescence parameters of the transformants and WT cells were compared (**Figure 5.5**). The results suggest a minute difference between the transgenic lines and WT in Fv/Fm and Fv′/Fm′ ($Y_{II}$) values. On the other hand, both transformants had significantly higher qP values and similar NPQ values than WT cells. The observation is indicative of a slightly reduced electron transport in the transformants and a similar thermal dissipation of PSII compared to the WT. This finding was per Li et al. (2016), which observed similar photosynthetic behavior of the transgenic cell lines.

## 5.7 Conclusion

The present study demonstrated that heterologous expression of the *JcDGAT1* gene could enhance neutral lipid accumulation and at the same time balance the fatty acid composition in *C. sorokiniana* for better quality biodiesel production without compromising the cell growth rate. The *JcDGAT1* gene could be further investigated using a stress-inducible promoter to enhance TAG accumulation in other transformable microalgae species under different stress conditions. The study also illustrated a high-throughput method to detect any modifications in oil through CD spectropolarimetry. In conclusion, this research provides proof of concepts to make microalgae an economically viable source for biodiesel production. The technique may be helpful for the biosynthesis of high-value nutraceuticals in microalgae.



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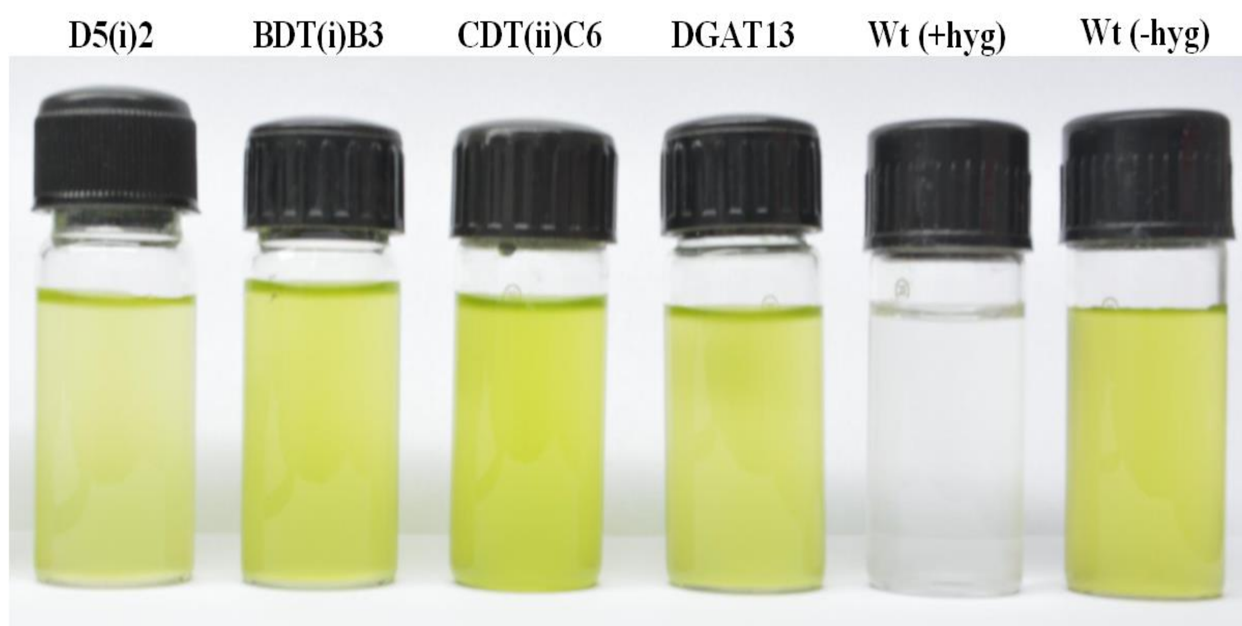


**Figure 5.1 Screening of hygromycin resistant colonies of *C. sorokiniana* in BG11 selection medium.**

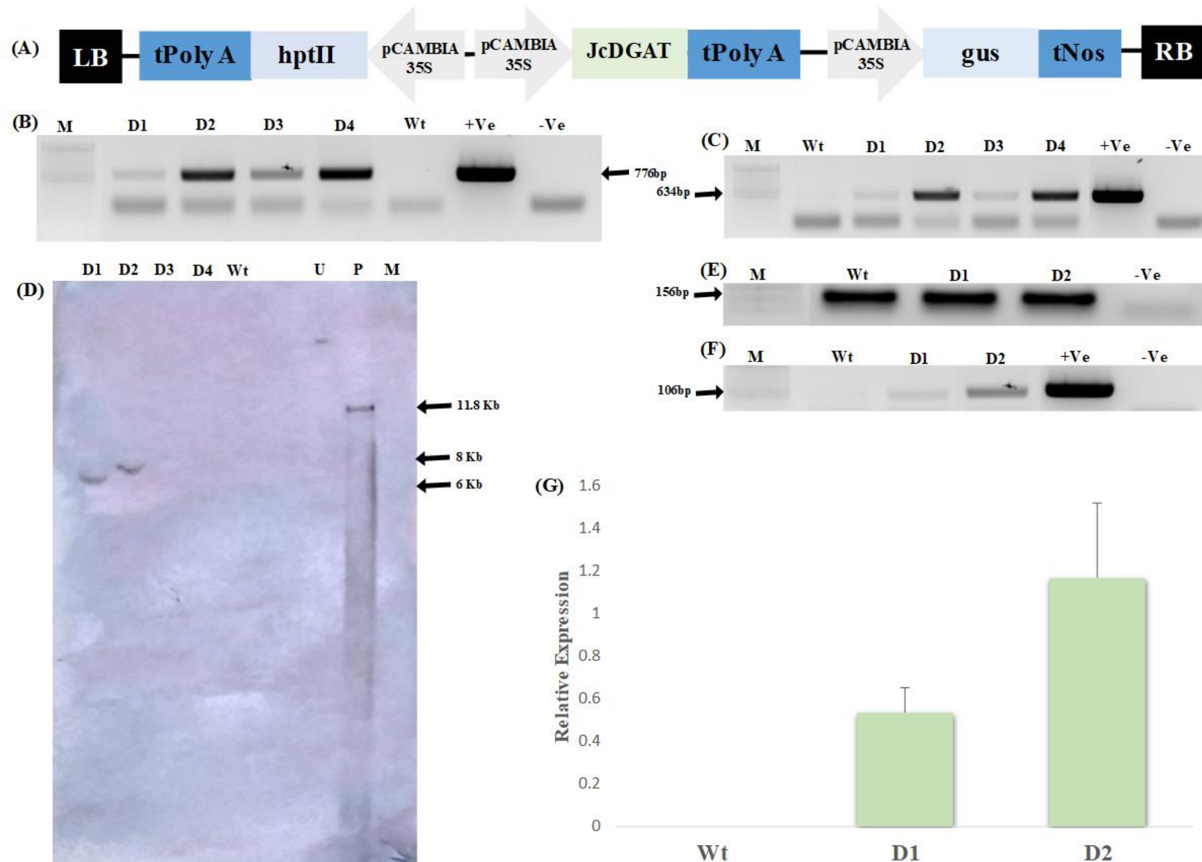


**Figure 5.2 Molecular analysis to detect transgenic lines of *C. sorokiniana*.** (A) T-DNA region of pCAMBIA1301 binary vector with *JcDGAT1* transgene used for the transformation experiment; (B) PCR amplification of 776 bp-fragment of *JcDGAT1* gene and 634 bp-fragment of *hptII* gene (C); lane M, molecular Marker; lane D1-D4, transgenic lines; lane Wt, wild type; lane +Ve, positive control; lane −Ve, negative control; (D) Southern blot hybridization of *HindIII* digested genomic DNA of wild-type (Wt) and transgenic lines (D1-D4) of *C. sorokiniana* hybridized to the hptII probe; lane P, *Hind*III digested pCAMBIA1301 (11.8 Kb); lane U, undigested gDNA of D1 transgenic line; lane M, molecular marker; (F) Transcript abundance of *JcDGAT1* gene in *C. sorokiniana* transformants. Gene expression analysis was carried out by semi-quantitative PCR using *actin* as an internal control (E); (G) Quantitative RT-PCR for analysis of the relative abundance of *JcDGAT1* transcripts.

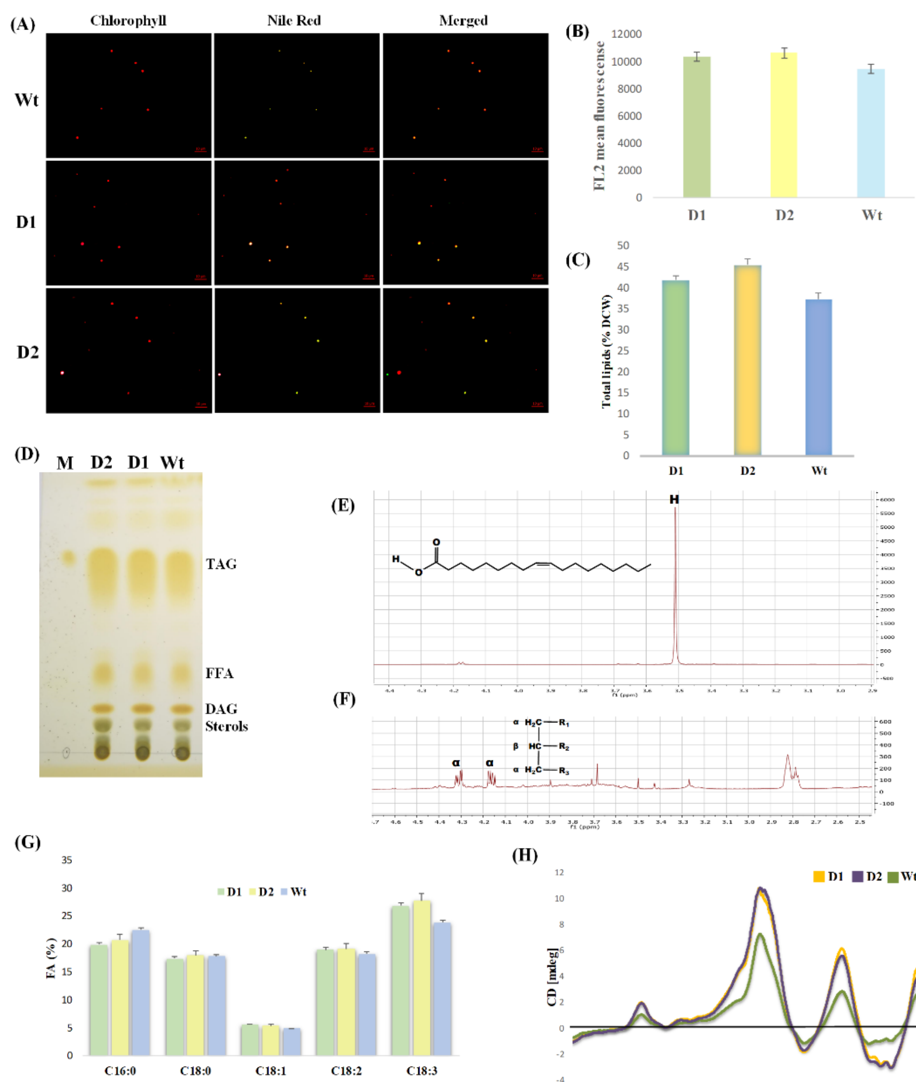


**Figure 5.3 Lipid analysis of *C. sorokiniana* transgenic lines.** (A) Detection of neutral lipid in the transformants (D1 and D2) and wild-type (Wt) using Nile red fluorescence microscopy during N-limited growth conditions; (B) Comparative *in vivo* lipid accumulation in *C. sorokiniana* using flow cytometry; (C) Total lipid content (% DCW) in transgenic lines and wild-type; (D) TLC separation of neutral lipids: lane M, trioleate standard; lanes D2 & D1, transgenic lines; lane Wt, wild-type; Neutral lipid was fractioned by Silica Gel plates into triacylglycerol (TAG), free fatty acids (FFA) and diacylglycerol (DAG) fractions; (E) $^1$H NMR spectra of FAME samples derived from oils (F) extracted from *C. sorokiniana*; (G) Fatty acid profile of transgenic (D1 and D2) and wild-type (Wt) *C. sorokiniana* were analyzed using GC-FID. Each value represents mean ± SD (n = 3), and the difference between the transformants and wild type were significant at $p < 0.05$ by Tukey test (*t*-test); (H) Spectropolarimeteric analysis of oils derived from transgenic (D1 and D2) and wild-type (Wt) *C. sorokiniana*.



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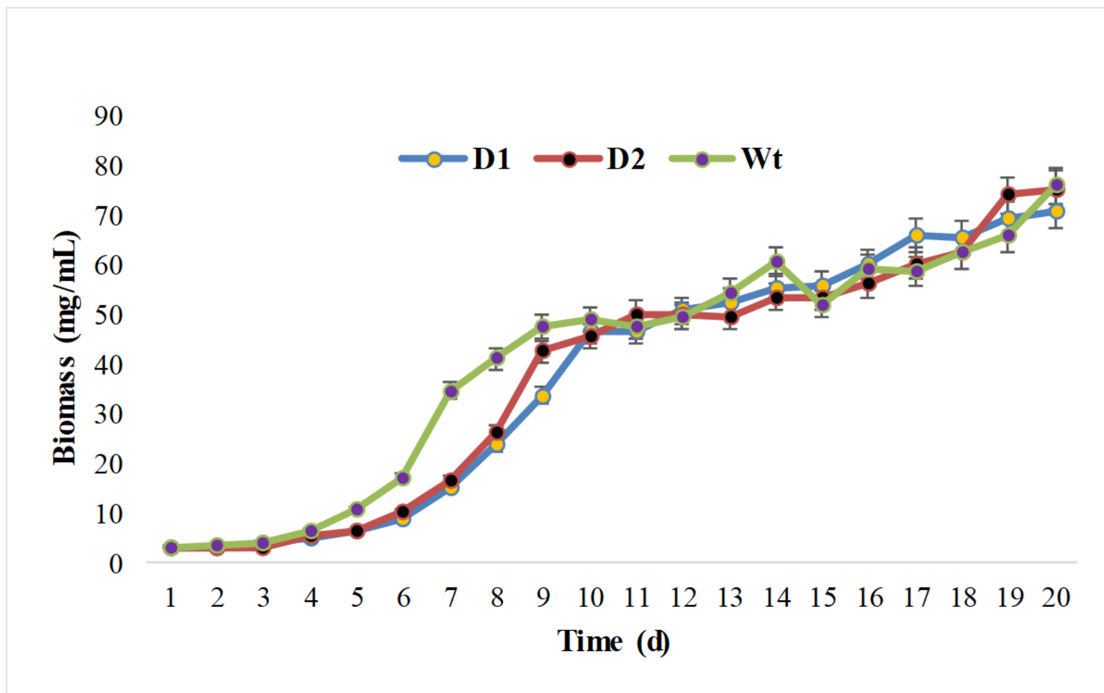


**Figure 5.4 Growth curve of *C. sorokiniana* transformants (D1 and D2) and wild-type (Wt) during N-sufficient growth condition.** Each value represents mean ± SD (n = 3).



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| | D1 | D2 | Wt |
|---|---|---|---|
| Biomass Productivity (mg/mL/d) | 3.56 | 3.79 | 3.822 |
| Specific growth Rate ($d^{-1}$) | 0.17 | 0.17 | 0.17 |
| Total Protein (%) | 39.12 | 37.23 | 53.64 |
| Total Carbohydrate (%) | 22.64 | 18.66 | 24.52 |
| Fv/Fm | 0.55 | 0.56 | 0.58 |
| Y(II) | 0.3 | 0.37 | 0.39 |
| NPQ | 0.07 | 0.05 | 0.07 |
| qP | 0.7 | 0.75 | 0.81 |

**Figure 5.5 Physiochemical characterization of *C. sorokiniana* transgenic lines (D1 and D2) and wild-type (Wt).** Each value represents mean±SD (n=3).



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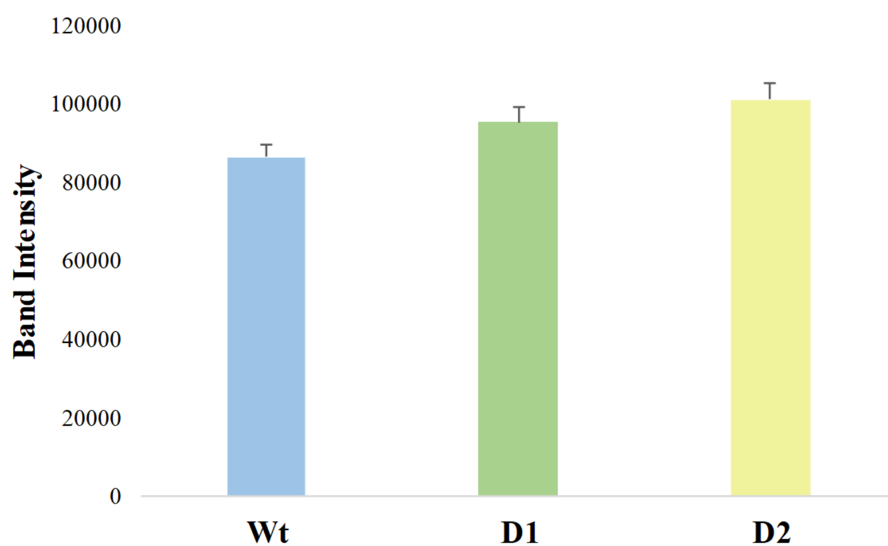


**Figure 5.6 Relative TLC-plate band intensities of neutral lipid (TAG) in the oil samples derived from transgenic lines of *C. sorokiniana* (D1 and D2) and wild-type (Wt).**
Each value represents mean±SD (n=3).



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**Table 5.1 List of primers used in the study.**

| Sl. No | Target Gene | Forward Primer (5'-3') | Reverse Primer (5'-3') | Product size (bp) |
|---|---|---|---|---|
| 1. | *hptII* | ATCCTTCGCAAGACCCTTCCT | GGTGTCGTCCATCACAGTTTG | 634 |
| 2. | *JcDGAT1* | ATGACGATTTTGGAGACCACTAC | GAGTTGGCAATGGGTCTCAT | 776 |
| 3. | *CsAct1* | CTCGGTCAGGATCTTCATCAT | ATGACGCAGATCATGTTTGA | 156 |
| 4. | RT_*DGAT1* | GGGTTGACCAAGAATGCCGA | CCATTGGTCGGGATCATCAA | 100 |



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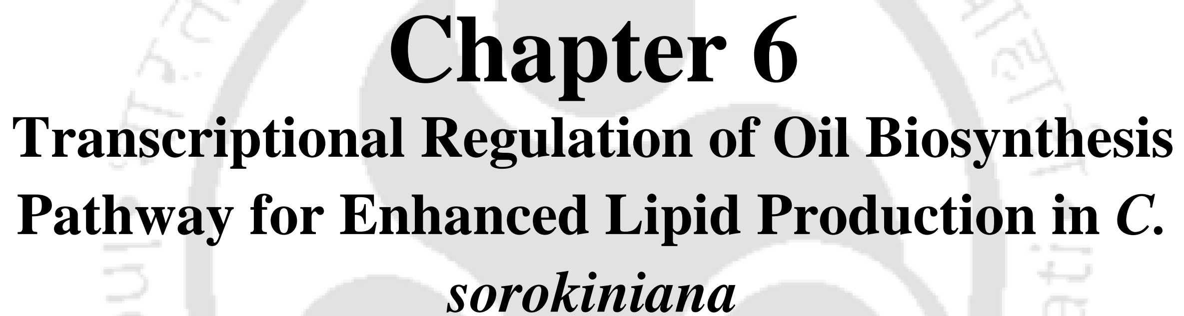

# Chapter 6

## Transcriptional Regulation of Oil Biosynthesis Pathway for Enhanced Lipid Production in *C. sorokiniana*

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# CHAPTER 6

Microalgae has emerged as a potential feedstock for biofuel production that has showed potential to replace fossil fuels. A prerequisite to materialize this idea is to genetically engineer microalgae to reduce the production cost. Transcription factor engineering has shown promise as crucial regulators of multiple genes involved in lipogenic pathways, which can effectively enhance biofuel production from microalgae. The WRINKLED1 (*WRI1*) transcription factor is a positive regulator of oil biosynthesis in plants. However, there is scarce information on the role of WRI1 in *C. sorokiniana*, important industrial microalgae. Hence, in the present study, we tested the efficacy of heterologous expression of WRI1 from *Arabidopsis thaliana* (*AtWRI1*) in *C. sorokiniana* CG12 and obtained five transgenic *C. sorokiniana* cell lines with two lines having enhanced total lipid content than the wild-type. Further, it is also necessary to understand the acting mechanism of *AtWRI1* in *C. sorokiniana.* Hence, several genes involved in the lipid metabolism were selected (**Table 6.1**) based on previous literature (Kang et al. 2017). The selected target genes' transcript level was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). These results provided excellent proof of concept for heterologous expression of crucial regulatory TF from plants to improve lipid production in the industrial microalgae.

## 6.1 Heterologous expression of *AtWRI1* in *C. sorokiniana*

The *AtWRI1* gene was cloned into pCAMBIA-1301 binary vector and mobilized into *Agrobacterium* strain EHA105. Through *Agrobacterium*-mediated transformation five putative transformants were screened (W1-W5) through hygromycin selection media (**Figure 6.1A, 6.1B**) and molecular confirmation of the transgene integration was done by genomic PCR (**Figure 6.2A, 6.2B**). Amplification of a 634bp DNA fragment and 500bp with *hptII* specific primers (**Table 6.1**), respectively, in each of the transformants, confirmed the presence of *hptII* and *AtWRI1* in the



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genome of *C. sorokiniana* transformants. No amplification was observed in the wild-type cell lines.

The relative transcript abundance of *AtWRI1* was determined using qPCR (**Figure 6.2D, 6.2E**) using *CsActin* as a reference gene (**Figure 6.2C**). As shown in **Figure 6.2E**, the relative transcript abundance of *AtWRI1* in transgenic lines was significantly enhanced up to 0.5 to 1.8 ± 0.2-fold than the wild-type cells, indicating functionally active *AtWRI1* gene integration into the genome of *C. sorokiniana* transformants. The five transformants displayed different levels of *AtWRI1* transcript abundance, which might be due to the epigenetic silencing effects on a transgene (Cerutti et al., 1997).

## 6.2 Analysis of growth related parameters in *AtWRI1* transgenic lines

To assess the possible interference of *AtWRI1* overexpression on the growth and primary metabolism of the transformed cell lines, we analyzed the growth curve of the transformants under nitrogen-sufficient (+N) conditions. All of the transformants (W1-W5) showed an overall similar growth pattern compared to the wild-type (Wt), while slightly lower growth during the middle exponential phase (**Figure 6.3A**). However, the transformants' biomass productivity and specific growth rate were not significantly affected (**Figure 6.3B**). Similar observations were observed in *C. ellipsoidea* overexpressing *AtLEC1* (Liu et al., 2021). Besides growth parameters, it is of interest to determine whether enhanced neutral lipid accumulation in the transformants had any metabolic compensations. Hence, we compared the total cellular carbohydrate, protein, chlorophyll a (ChlA), chlorophyll b (ChlB), and carotenoid content in the transformants to the wild-type. The results showed a significant reduction in carbohydrate and protein content in two transformants (W1 and W3) than in the wild-type (**Figure 6.3B**).



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In the case of photosynthetic pigments, there were significant differences in the chlorophyll b content (reduction) and carotenoids (increased) in the W1 and W3 transgenic lines than the wild-type (**Figure 6.3B**).

Besides this, to elucidate the potential ill-effects of enhanced neutral lipid accumulation on photosynthetic electron transport in photosystem II (PSII), the chlorophyll fluorescence parameters of the transformants and WT cells were compared (**Figure 6.3B**). The results suggest a minute difference between the transgenic and WT in Fv/Fm and Fv′/Fm′ (YII) values. On the other hand, all transformants had similar qP values and NPQ values with WT cells. The observation indicates unaffected electron transport in the transformants and a similar thermal dissipation of PSII compared to the WT.

### 6.3 Analysis of lipid content in *AtWRI1* transgenic lines

Nile red fluorescence assay was used to investigate the impact of heterologous expression of the *AtWRI1* transcription factor on lipid biosynthesis. Previous literature has reported a significant enhancement in lipid production under the influence of WRINKLED1 transcription factor (Ji et al., 2018; Yang et al., 2015; Kang et al., 2017). Hence, in the present study, the Nile-red stained transgenic cells and the wild-type cells were visualized through fluorescence microscopy, which revealed a higher number of lipid droplets (LDs) in two of the transgenic cells (W1 & W3) than the wild-type cells (**Figure 6.4**). Bright yellow-color lipid droplets were observed in transgenic lines W1 and W3, whereas lipid bodies of WT and other transgenic lines (W2, W4, and W5) showed faint fluorescence indicating a reduced abundance of intracellular lipid droplets. Autofluorescence due to chlorophyll was represented as the red-color background.

Furthermore, the intracellular lipid accumulation was relatively compared through flow



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cytometry, which exhibited a higher FL2 mean fluorescence in the transgenic lines than the WT cells (**Figure 6.5A**). The results suggest an enhanced neutral lipid accumulation in the transgenic cells when concomitantly compared to WT. Previous studies also suggested that the presence of higher intracellular neutral lipid results in intense Nile-red-based fluorescence (Katayama et al., 2019; Tale et al., 2018).

In addition to examining the presence of intracellular lipid droplets, total cellular lipids were extracted to analyze the status of neutral lipid reserve in the transgenic lines. The results showed a significant rise in total lipids by 1.23-fold in the transgenic lines than WT cells (**Figure 6.5B**). However, as TAG is the primary source material for biodiesel synthesis, quantification of TAG content is crucial to analyze the quality of the extracted lipids. Hence, the TAG fraction present in total lipid was examined through thin-layer chromatography (**Figure 6.5C**), and then the band intensities corresponding to TAG were quantified (**Figure 6.5D**). The results showed a significant increase in TAG content in the W1 & W3 transgenic lines than that of the WT. It was also evident that TAG is the most significant fraction of the lipids extracted from *C. sorokiniana*.

### 6.4 FAME analysis of *AtWRI1* transgenic lines

In general, microalgal lipids are not regarded as a quality feedstock material for biofuel due to improper saturated and unsaturated fatty acid fractions. One such instance is, biodiesel made from oils with high saturated short and medium fatty acids may produce wax at lower temperatures (Ahmed et al., 2015). Hence, it is crucial to analyze the fatty acid profile of the extracted lipids. The FA composition of the lipids extracted from both transgenic lines and wild-type were tested by transesterifying to FAME and then analyzing the FAME samples using GC- MS. The palmitic acid (C16:0), stearic acid (C18:0), linolenic acid (C18:3), and linoleic acid (C18:2) are the most



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abundant FAs in the TAG (**Figure 6.5E**). The linolenic acid content in the transformants (W1-W5) compared to wild-type was increased up to 1.2-fold, wherein the saturated FAs like palmitic acid were reduced by 1% in the transformants. The results clearly showed a reduction in saturated FAs and an increase in unsaturated FAs, which is a good indicator of an excellent biodiesel source material (Ahmed et al., 2015). Thus, the study showed a technique applied to commercially important microalgal strains to accumulate higher neutral lipid content and improve the oil quality by introducing the *Arabidopsis WRI1* gene.

### 6.5 Molecular analysis of *AtWRI1*-regulated genes involved in lipid synthesis

Transcription factors act as a master switch for their downstream genes and effectively regulate an entire metabolic pathway. Similarly, to understand the mechanism by which overexpression of *AtWRI1* resulted in enhanced lipid synthesis in *C. sorokiniana*, based on previous literature, expression levels of genes involved in the lipid metabolism (**Table 6.1**) were analyzed (Kang et al. 2017). We identified homologs of the target candidates in *C. sorokiniana* and analyzed the expression of these genes with qRT-PCR (**Figure 6.6**). The downstream genes of *AtWRI1* that were studied include triacylglycerol lipase (*TAGL*) and lysophospholipase (*LPL*) that are involved in organic substance metabolic process; diacylglycerol kinase (*DAGK*) involved in phospholipid catabolic process; diacylglycerol acyltransferase family protein (*DGAT*) and lysophosphatidylglycerol acyltransferase 1 (*LPGAT1*) involved in transferring acyl groups; and pyruvate phosphate dikinase (*PPDK*) localized in chloroplasts. As expected with *AtWRI1* transgenic *C. sorokiniana* lines, all six genes showed an expression pattern consistent with the results of Kang et al. (2017). Interestingly, target genes involved in lipid biosynthesis showed the highest expression under the N limitation condition in most cases, consistent with the



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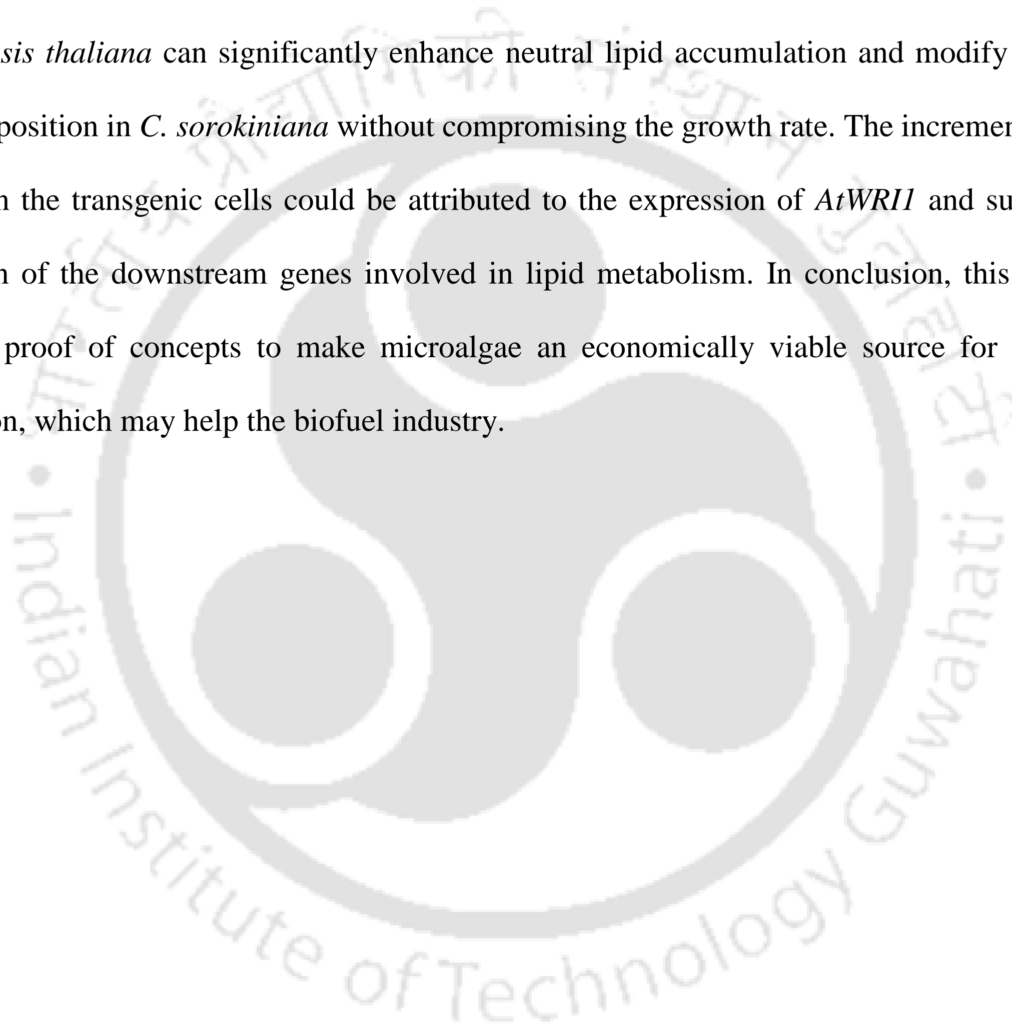
expression pattern of transgenic *AtWRI1*, which may have resulted in increased lipid accumulation under the same condition.

### 6.6 Conclusion

Heterologous expression of a gene encoding a transcription factor WRINKLED1 (*WRI1*) from *Arabidopsis thaliana* can significantly enhance neutral lipid accumulation and modify the fatty acid composition in *C. sorokiniana* without compromising the growth rate. The increment in lipid content in the transgenic cells could be attributed to the expression of *AtWRI1* and subsequent regulation of the downstream genes involved in lipid metabolism. In conclusion, this research provides proof of concepts to make microalgae an economically viable source for biodiesel production, which may help the biofuel industry.





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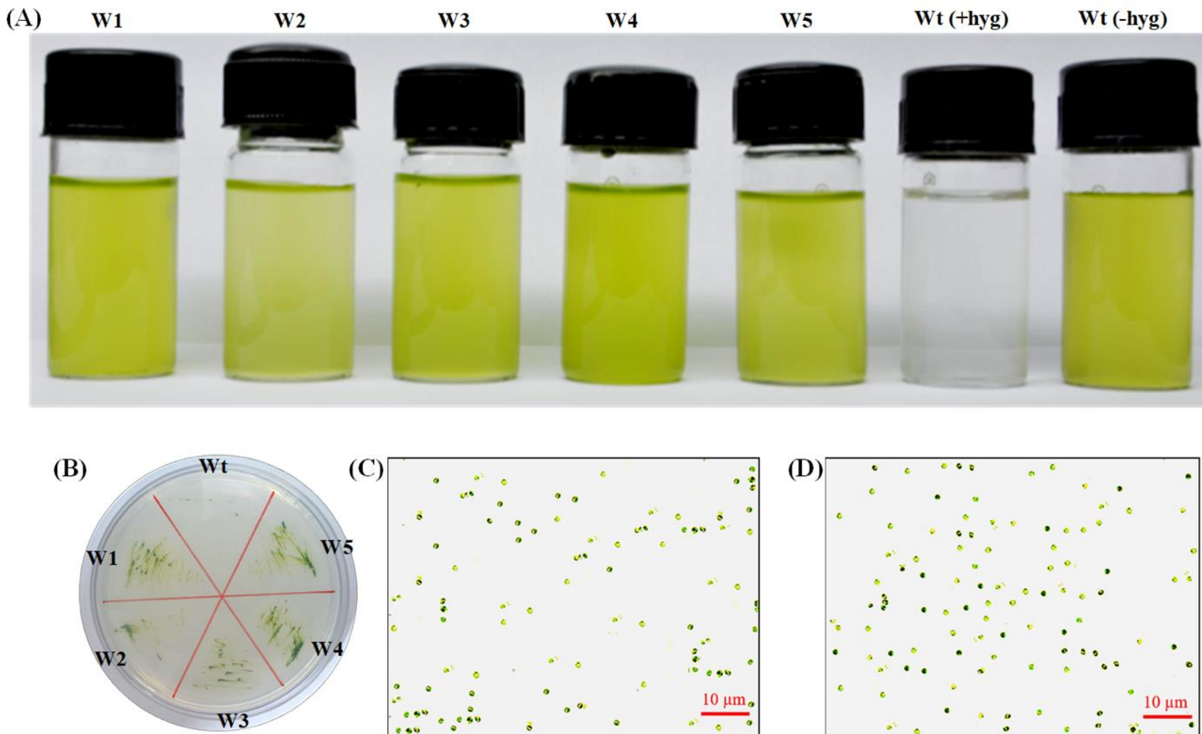


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**Figure 6.1 Screening of hygromycin resistant colonies of *C. sorokiniana*** in BG11 liquid (A) and solid (B) selection medium; (C) wild-type *C. sorokiniana* cells; (D) transgenic *C. sorokiniana* cells.



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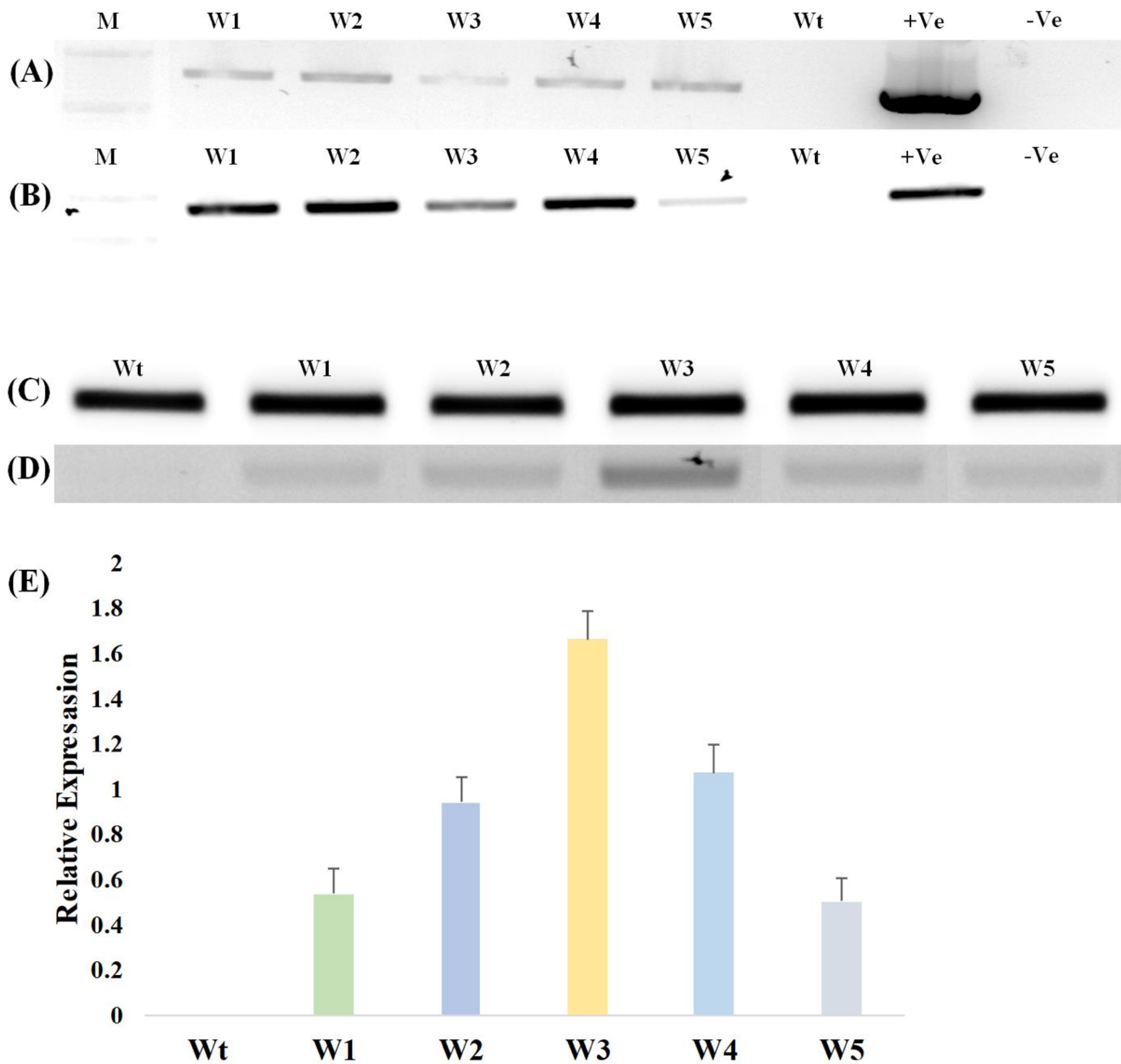


**Figure 6.2 Molecular analysis to detect transgenic lines of *C. sorokiniana*.** (A) PCR amplification of 500 bp-fragment of *AtWRI1* gene and 634 bp-fragment of *hptII* gene (B); lane M, molecular Marker; lane W1-W5, transgenic lines; lane Wt, wild type; lane +Ve, positive control; lane −Ve, negative control; (D) Transcript abundance of *AtWRI1* gene in *C. sorokiniana* transformants. Gene expression analysis was carried out by semi-quantitative PCR using *actin* as an internal control (C); (E) Quantitative RT-PCR for analysis of the relative abundance of *AtWRI1* transcripts.



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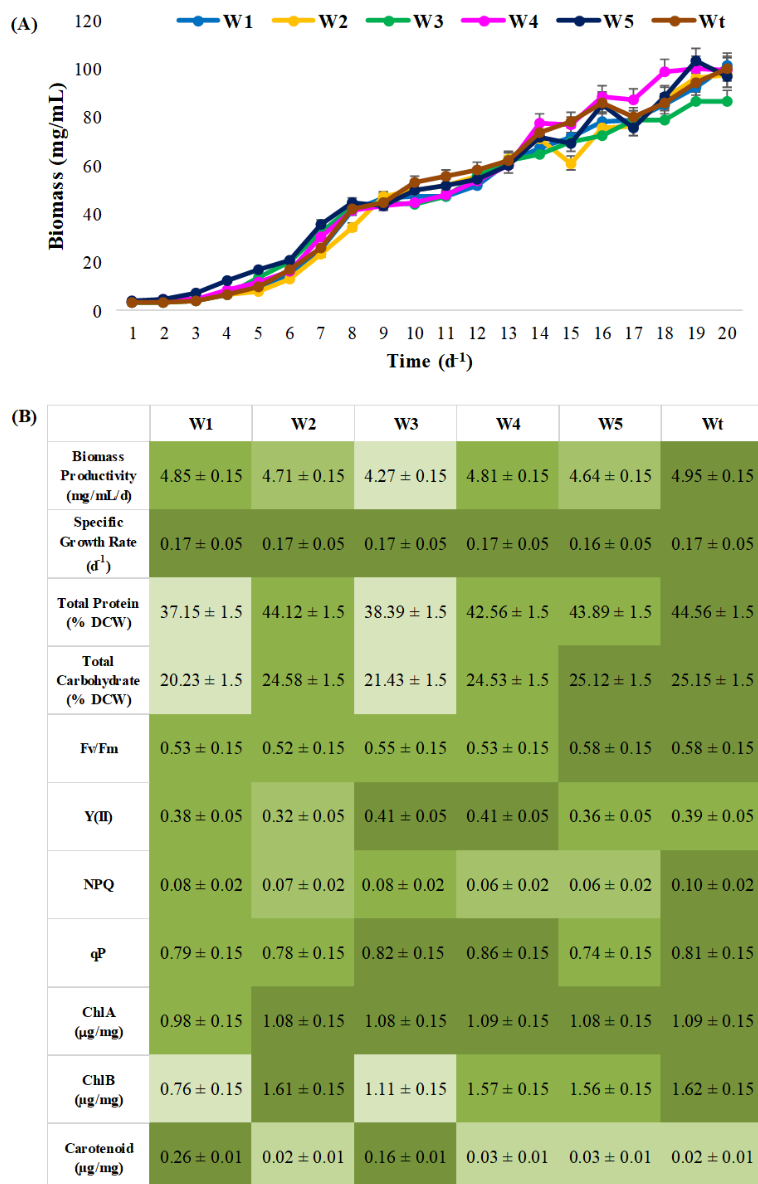


|  | W1 | W2 | W3 | W4 | W5 | Wt |
|---|---|---|---|---|---|---|
| Biomass Productivity (mg/mL/d) | 4.85 ± 0.15 | 4.71 ± 0.15 | 4.27 ± 0.15 | 4.81 ± 0.15 | 4.64 ± 0.15 | 4.95 ± 0.15 |
| Specific Growth Rate ($d^{-1}$) | 0.17 ± 0.05 | 0.17 ± 0.05 | 0.17 ± 0.05 | 0.17 ± 0.05 | 0.16 ± 0.05 | 0.17 ± 0.05 |
| Total Protein (% DCW) | 37.15 ± 1.5 | 44.12 ± 1.5 | 38.39 ± 1.5 | 42.56 ± 1.5 | 43.89 ± 1.5 | 44.56 ± 1.5 |
| Total Carbohydrate (% DCW) | 20.23 ± 1.5 | 24.58 ± 1.5 | 21.43 ± 1.5 | 24.53 ± 1.5 | 25.12 ± 1.5 | 25.15 ± 1.5 |
| Fv/Fm | 0.53 ± 0.15 | 0.52 ± 0.15 | 0.55 ± 0.15 | 0.53 ± 0.15 | 0.58 ± 0.15 | 0.58 ± 0.15 |
| Y(II) | 0.38 ± 0.05 | 0.32 ± 0.05 | 0.41 ± 0.05 | 0.41 ± 0.05 | 0.36 ± 0.05 | 0.39 ± 0.05 |
| NPQ | 0.08 ± 0.02 | 0.07 ± 0.02 | 0.08 ± 0.02 | 0.06 ± 0.02 | 0.06 ± 0.02 | 0.10 ± 0.02 |
| qP | 0.79 ± 0.15 | 0.78 ± 0.15 | 0.82 ± 0.15 | 0.86 ± 0.15 | 0.74 ± 0.15 | 0.81 ± 0.15 |
| ChlA (µg/mg) | 0.98 ± 0.15 | 1.08 ± 0.15 | 1.08 ± 0.15 | 1.09 ± 0.15 | 1.08 ± 0.15 | 1.09 ± 0.15 |
| ChlB (µg/mg) | 0.76 ± 0.15 | 1.61 ± 0.15 | 1.11 ± 0.15 | 1.57 ± 0.15 | 1.56 ± 0.15 | 1.62 ± 0.15 |
| Carotenoid (µg/mg) | 0.26 ± 0.01 | 0.02 ± 0.01 | 0.16 ± 0.01 | 0.03 ± 0.01 | 0.03 ± 0.01 | 0.02 ± 0.01 |

**Figure 6.3 Growth (A) and physiochemical characterization (B) of *C. sorokiniana* transgenic lines (W1-W5) and wild-type (Wt).** Each value represents mean±SD (n=3).

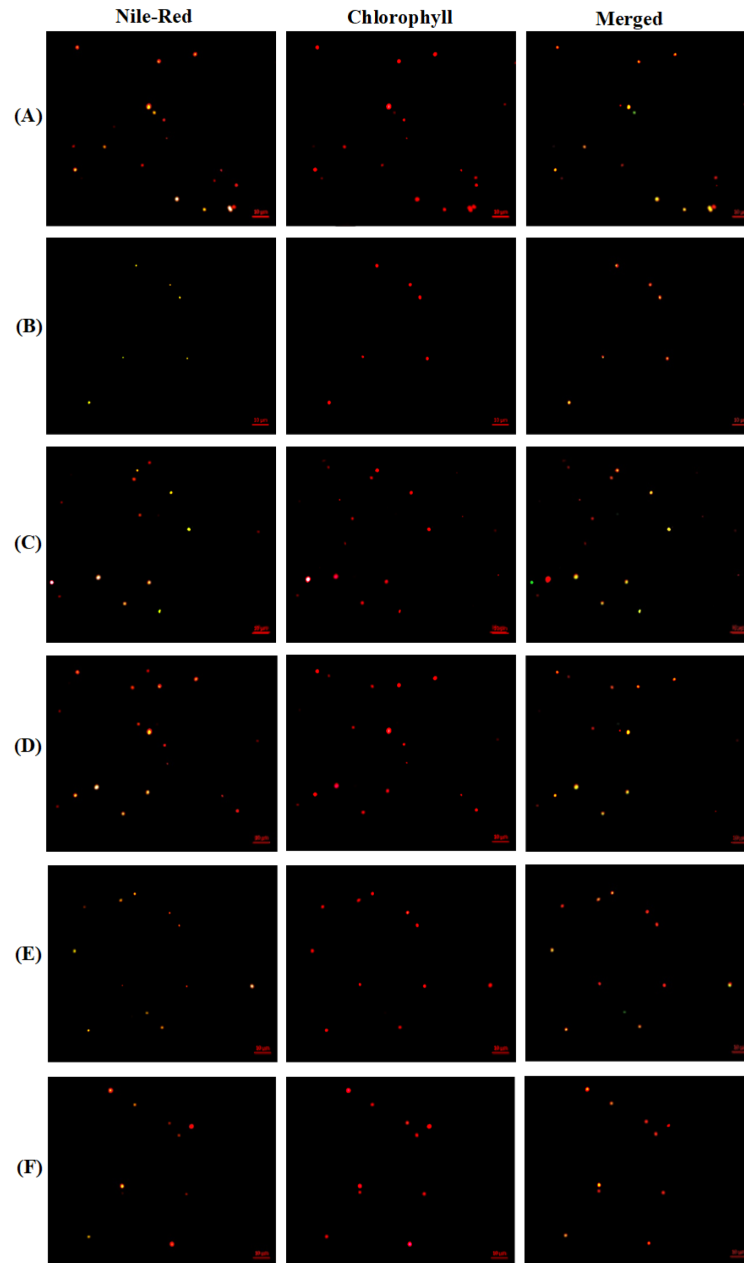


**Figure 6.4 Detection of neutral lipids in the transformants and wild-type using Nile red fluorescence microscopy during N-limited growth conditions.** (A) to (E) Nile-red fluorescence of transgenic lines, W1 to W5 respectively; (F) Nile-red fluorescence of wild-type.

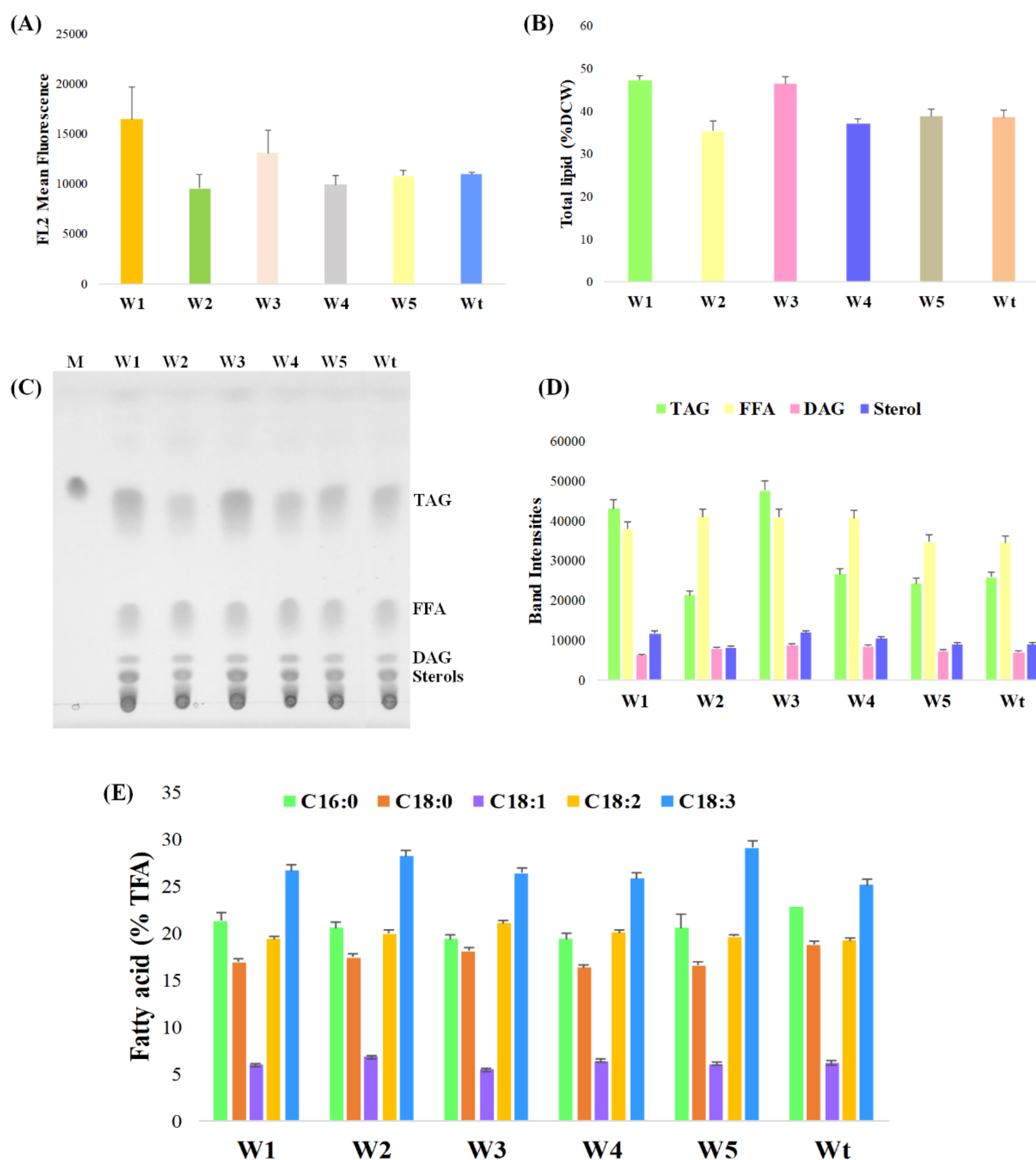


**Figure 6.5 Lipid analysis of *C. sorokiniana* transgenic lines.** (A) Comparative *in vivo* lipid accumulation in *C. sorokiniana* using flow cytometry; (B) Total lipid content (% DCW) in transgenic lines and wild-type; (C) TLC separation of neutral lipids: lane M, trioleate standard; lanes W1-W5, transgenic lines; lane Wt, wild-type; Neutral lipid was fractioned by Silica Gel plates into triacylglycerol (TAG), free fatty acids (FFA) and diacylglycerol (DAG) fractions and quantification of the respective band intensities (D); (E) Fatty acid profile of transgenic (W1-W5) and wild-type (Wt) *C. sorokiniana* were analyzed using GC-FID. Each value represents mean ± SD (n = 3), and the difference between the transformants and wild type were significant at $p < 0.05$ by Tukey test (*t*-test);



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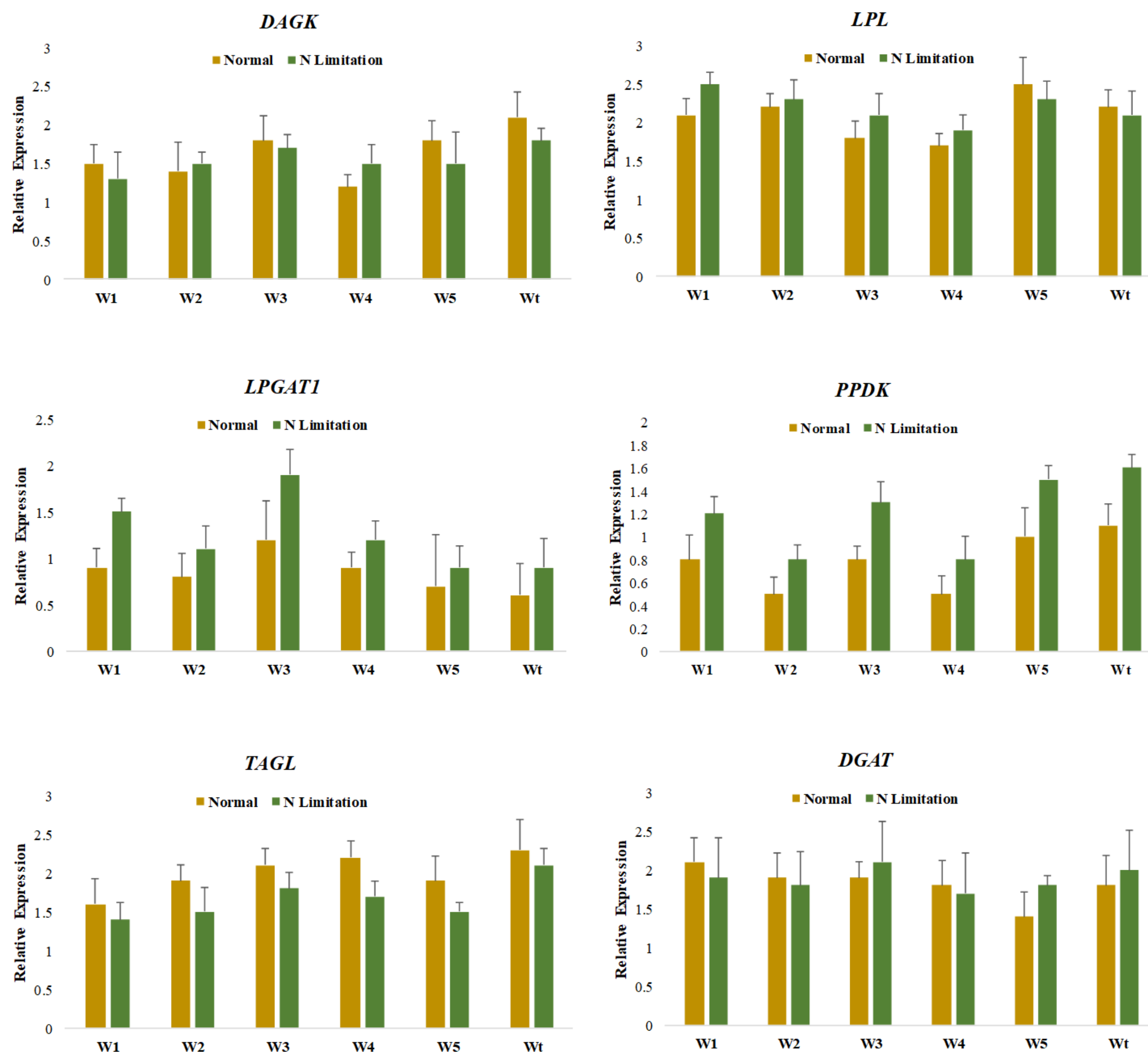


**Figure 6.6 Expression profiles of *AtWRI1*-regulated candidate genes involved in lipid synthesis in transgenic lines (W1–W5) under normal and N limitation conditions.** Each value represents mean ± SD (n = 3), and the difference between the transformants and wild type were significant at $p < 0.05$ by Tukey test (*t*-test).



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**Table 6.1 List of primers used in the study**

| Sl. No | Target Gene | Forward Primer (5'-3') | Reverse Primer (5'-3') | Product size (bp) |
|---|---|---|---|---|
| 1. | *hptII* | ATCCTTCGCAAGACCCTTCCT | GGTGTCGTCCATCACAGTTTG | 634 |
| 2. | *AtWRI1* | GCTTAACCACTTCCACTTGTTC | AGCTTCCTCCTGCGTATTATA | 500 |
| 3. | *CsAct1* | CTCGGTCAGGATCTTCATCAT | ATGACGCAGATCATGTTTGA | 156 |
| 4. | RT_*WRI1* | GCTTAACCACTTCCACTTGTTC | TGACTCCTCTGTAGATAGAGCT | 121 |
| 5. | *DAGK* | ATTCTTCCGCTACTGCTCCG | CCACGCACTGCCAGTACAAT | 140 |
| 6. | *LPL* | AGACCTCACCTTTGCCGAG | TCCAGATGAGCACGTTTGGC | 127 |
| 7. | *LPGAT1* | TGTGCATCATGGTTGAGCC | TGACAACCATCACCTCCACC | 110 |
| 8. | *PPDK* | CCACCCGCTTCTTCCTGTAT | TCCTGCTCCACCCAACCA | 99 |
| 9. | *TAGL* | CAACTACGCGGTGTCAGG | CCATGAAGTCGGTGTGGAAC | 121 |
| 10. | *DGAT* | GTGTGCATCCTCTGGCTCAA | CAGTAGCTCATCAACGCCTT | 137 |



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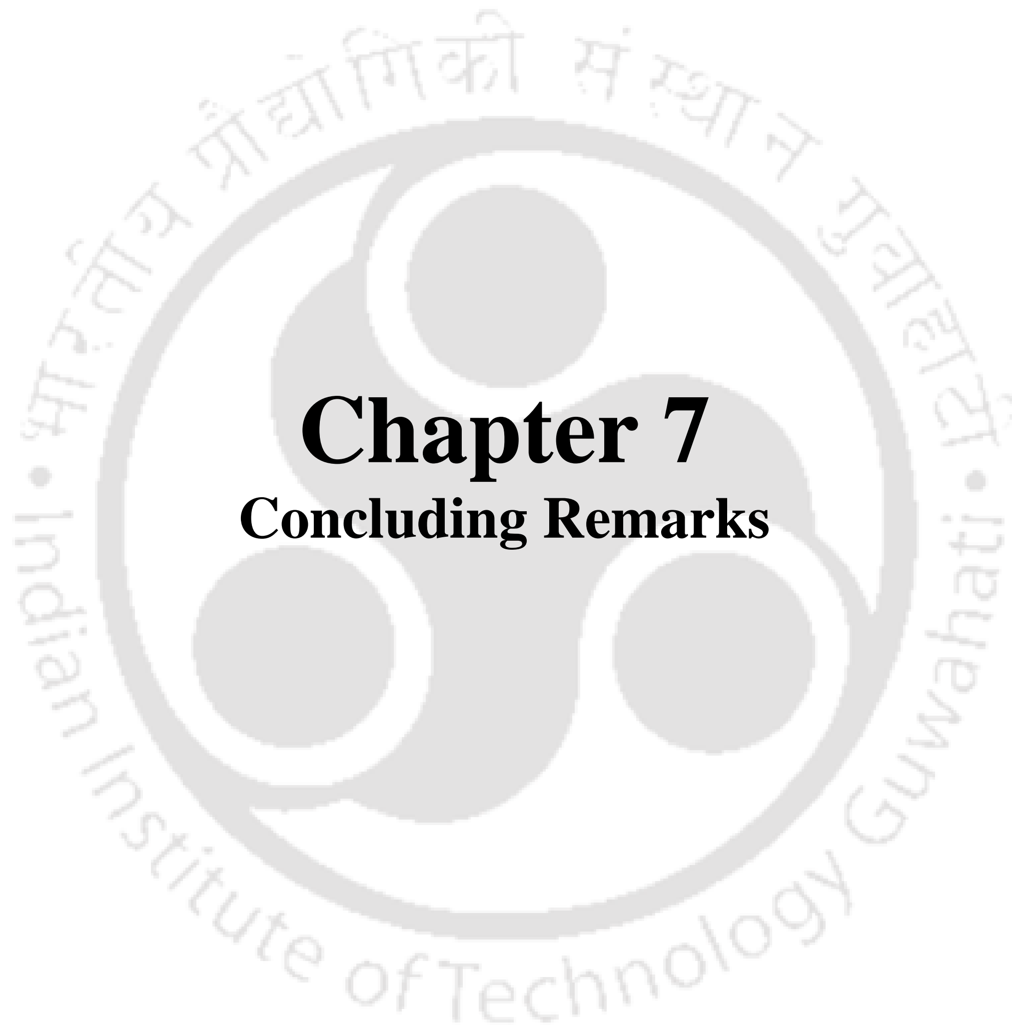

# Chapter 7

## Concluding Remarks

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### 7.1 Significance of the present study

To harness the benefit of microalgal biotechnology for biofuel application, genetic manipulation of metabolic pathways is essential, requiring an efficient genetic transformation method. Besides, an efficient gene transfer system in microalgae would allow a way to understand cellular metabolism regulation by characterizing the genes involved through a reverse genetics approach. *A. tumefaciens*-mediated genetic transformation is a method of choice for ease in transformation and its ability to precisely integrate low copy number transgene into transcriptionally active genomic regions. However, in *C. sorokiniana*, the lack of a reliable and efficient *Agrobacterium*-mediated gene transfer method limits its potential uses in commercial-scale utilization. We described an efficient *A. tumefaciens*-mediated genetic transformation in *C. sorokiniana*. For the first time in *C. sorokiniana*, it highlighted the reliable detection of stable transgene integration and expression in *C. sorokiniana*, which opens up limitless possibilities in biofuel production and other commercially valuable commodities.

Further, as higher lipid biosynthesis and accumulation are essential to achieve sustainable production of biofuel in microalgae. The green microalgae *Chlorella sorokiniana* was genetically engineered with a rate-limiting enzyme of neutral lipid biosynthesis, diacylglycerol acyltransferase 1 from *Jatropha curcas* (*JcDGAT1*) and a transcription factor WRINKLED 1 from *Arabidopsis thaliana* known to involve in lipid biosynthesis in higher plants, to enhance the lipid content. The results offer a valuable strategy for enhancing oil production and might facilitate a platform strain with industrial potential. Our results suggest genetic means to increase neutral lipids and unsaturated fatty acids in *C. sorokiniana* for biofuel production. In conclusion, this research provides proof of concepts to make microalgae an economically viable source for biodiesel



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production. A similar technique may be helpful for the biosynthesis of certain high-value compounds in microalgae.

### 7.2 The salient features of the present study

- For the first time, developed an efficient *A. tumefaciens*-mediated genetic transformation in *C. sorokiniana*.
- *C. sorokiniana* was genetically engineered with diacylglycerol acyltransferase 1 from gene from *Jatropha curcas* (*JcDGAT1*), which successfully enhanced the lipid content.
- Heterologous expression of a transcription factor, WRINKLED1 from *Arabidopsis* (*AtWRI1*) has shown to increase the lipid content and successfully modified the fatty acid composition in *C. sorokiniana*.

This study will provide guidelines for using genetic means to increase neutral lipids and unsaturated fatty acids in *C. sorokiniana* for sustainable biofuel production.



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### 7.3 Future prospects

To fully comprehend the potential of transgenic microalgae for lipid production, further enhancement in expression level of foreign genes is crucial alongwith ample of research on diversifying the uses of microalgae lipids such as sustainable aviation fuel (SAF). Besides these, thorough research on enhancing the transgene expression by experimenting with different strategies such as chloroplast transformation, using synthetic gene expression regulatory elements, and genome editing is also necessary. All of these strategies should be seriously considered in the future attempts to improve native microalgal strains for industrial use. Further, development of economically viable microalgae culturing techniques and downstream processing techniques are equally crucial to achieve sustainability in biofuel production from microalgae. Therefore, success of microalgae as a biofuel production platform depends on efficient collaboration between researchers dealing with both upstream and downstream sides of the process engineering.



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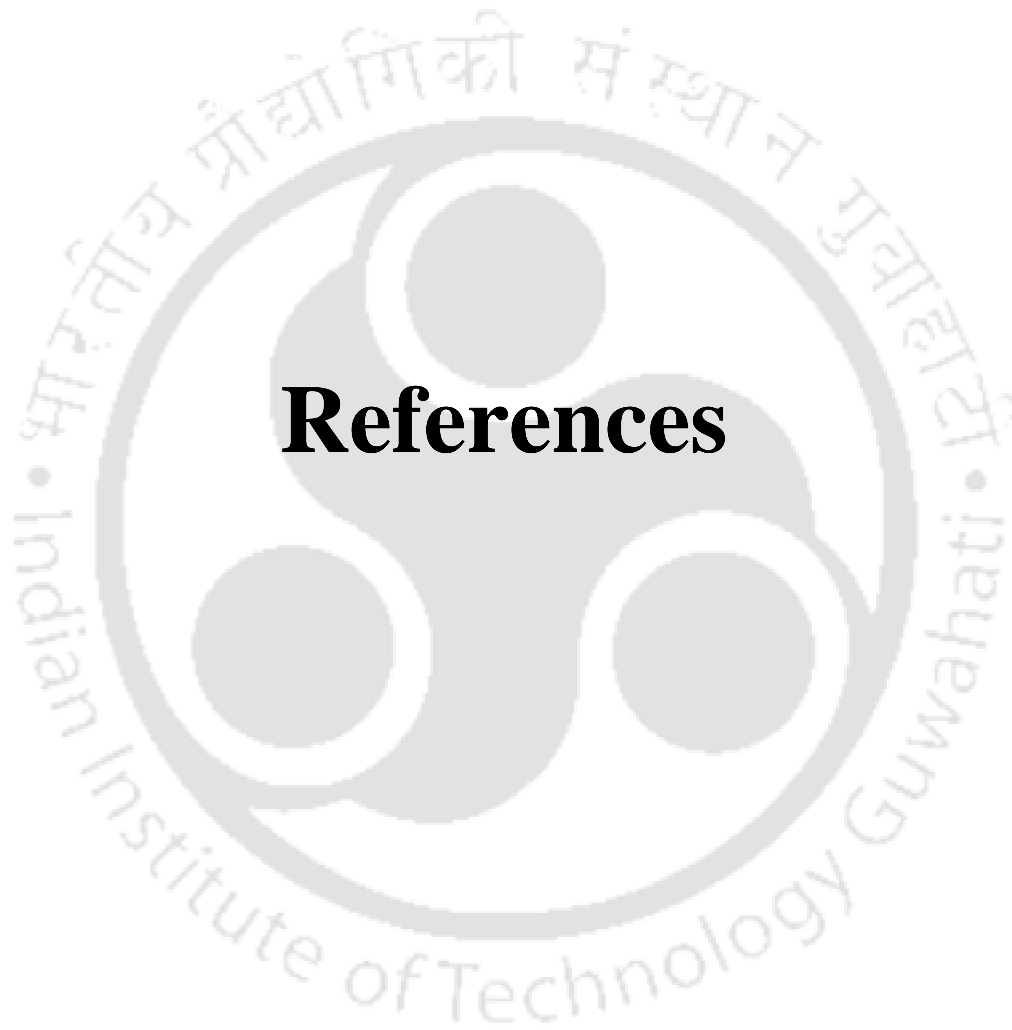
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# Appendix

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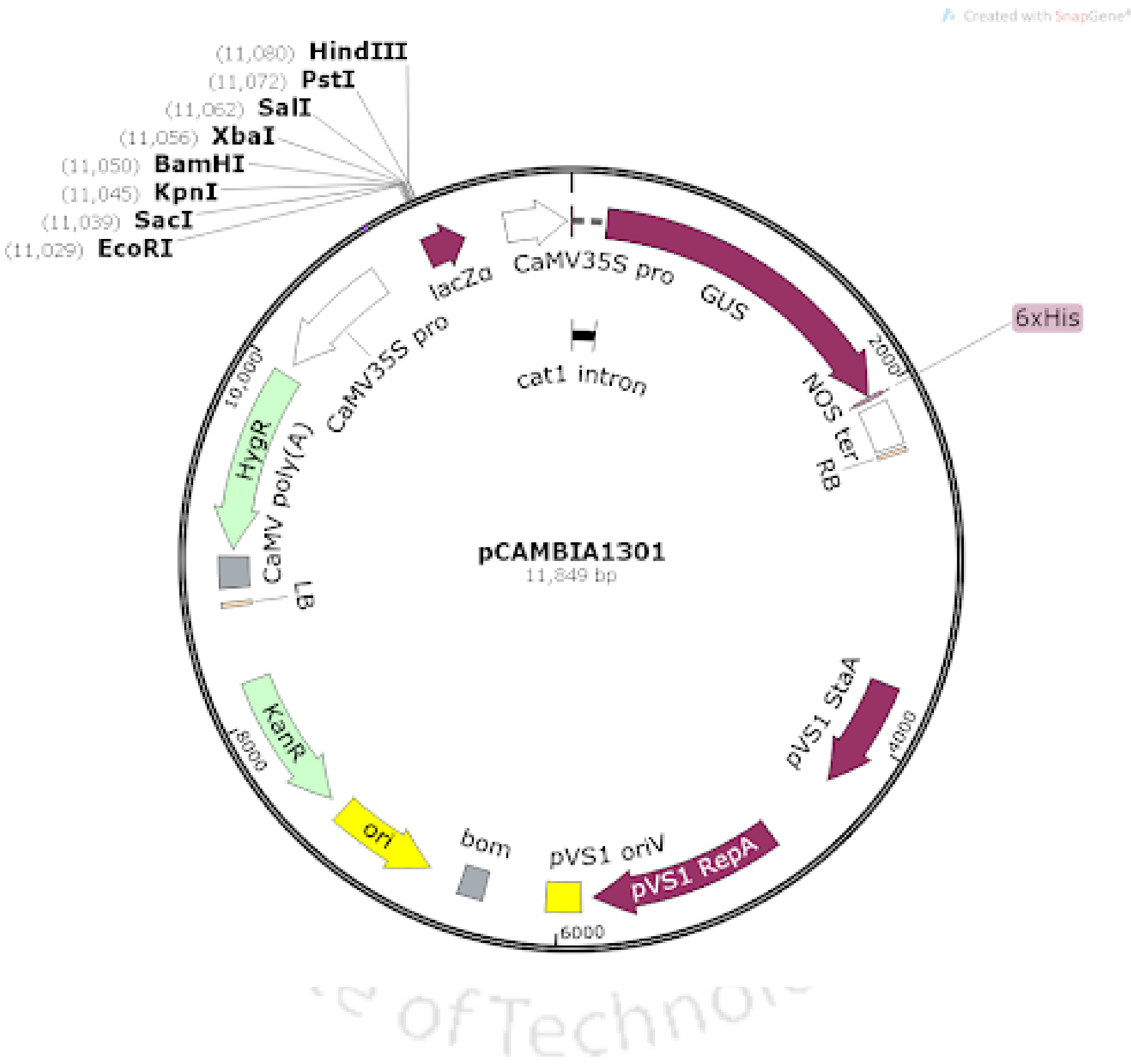
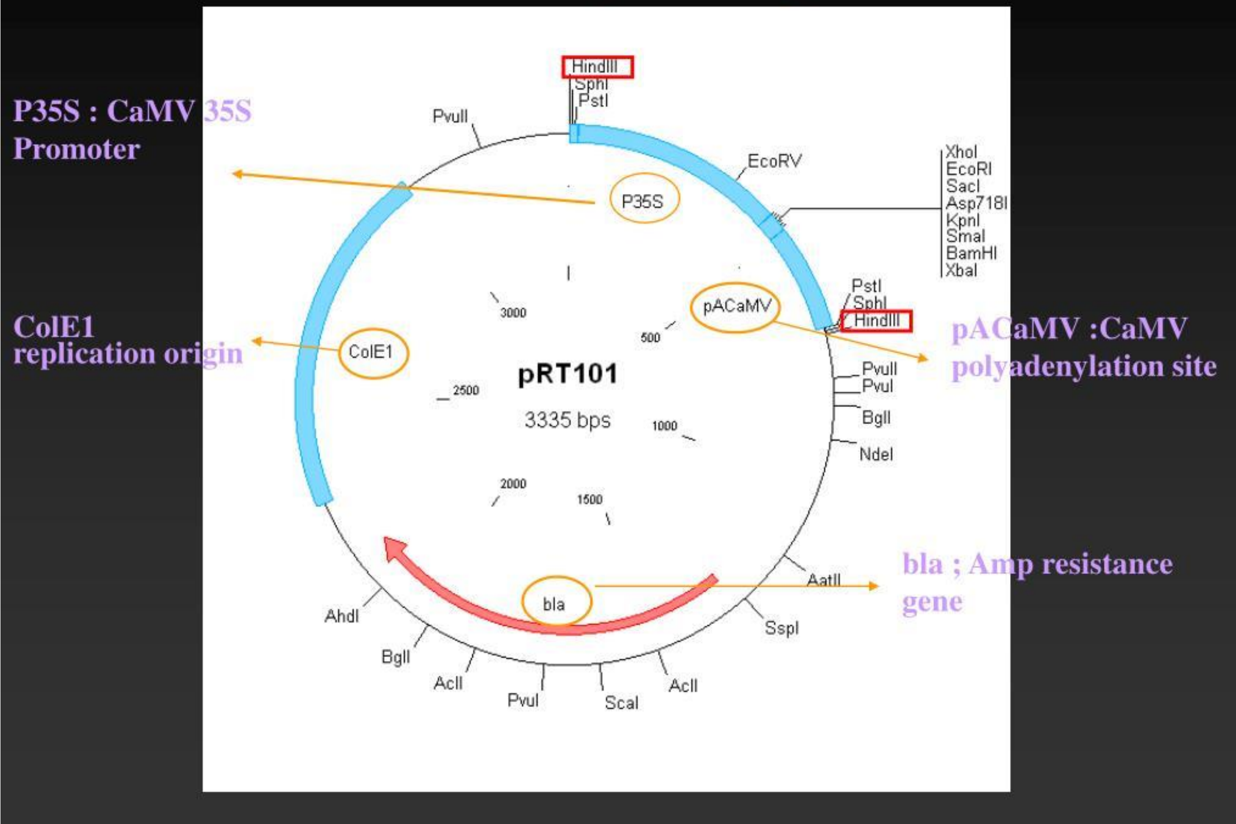
**Vector map: pCAMBIA1301**

(Source: https://www.snapgene.com/resources/plasmid-files/?set=plant_vectors&plasmid=pCAMBIA1301)



**Vector map: pRT101**

(Source: https://www.slideserve.com/gaetana/plant-expression-system)




stitute of Technology

**Table 1.** List of commercial kits

| Name | Use | Supplier |
|---|---|---|
| NucleoSpin Plasmid | Plasmid isolation | MN, Germany |
| NucleoSpin Gel and PCR clean up | Purification | MN, Germany |
| NucleoSpin RNA Plant | RNA isolation | MN, Germany |
| NucleoSpin Plant II Maxi | Genomic DNA isolation | MN, Germany |
| DIG Labellig and detection | Southern Hybridization | Roche Diagnostic, Mannheim, Germany |

**Table 2.** List of plasmid vectors

| Name | Use | Selection gene |
|---|---|---|
| pTZ57R/T | TA cloning | AmpR |
| pRT101 | Intermediate vector | AmpR |
| pCAMBIA1301 | Plant expression | KanR |

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# List of Publications

**Journals/Chapter/Published**

1. Maravi DK, Kumar S, **Sharma PK**, Kobayashi Y, Goud VV, Sakurai N, Koyama H, and Sahoo L (2016). Ectopic expression of AtDGAT1, encoding diacylglycerol O-acyltransferase exclusively committed to TAG biosynthesis, enhances oil accumulation in seeds and leaves of Jatropha. *Biotechnol Biofuels*., 9:226.
2. **Sharma PK**, Saharia M, Srivastava R, Kumar S and Sahoo L (2018). Tailoring microalgae for efficient biofuel production. *Front. Mar. Sci.* 5:382.
3. **Sharma PK**, Goud VV, Yamamoto Y, and Sahoo L (2021). Efcient *Agrobacterium tumefaciens*-mediated stable genetic transformation of green microalgae, *Chlorella sorokiniana*. *3 Biotech* 11:196.

**Submitted/Under review**

1. **Sharma PK**, and Sahoo L (2021). Enhanced lipid production and altered fatty acids in *Chlorella sorokiniana* by overexpression of a Jatropha diacylglycerol acyltransferase 1. **Journal of Applied Phycology** (Communicated).
2. **Sharma PK**, and Sahoo L (2021). Ectopic overexpression of WRINKLED 1 transcription factor for enhanced lipid biogenesis in *C. sorokiniana* CG12. **Journal of Phycology** (Communicated).

**Conferences and workshop**

1. **Sharma PK**, Kumar S, Saharia M, Srivastava R and Sahoo L "Enhanced lipid production and altered fatty acids in *Chlorella sorokiniana* by overexpression of a Jatropha *diacylglycerol acyltransferase* 1", International conference on Plant responses to light and

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stress: emerging issues in climate change, ICGEB New Delhi, Poster Presentation, **10th – 12th October, 2018**.

2. **Sharma PK** and Sahoo L "Enhanced lipid production and altered fatty acids in *Chlorella sorokiniana* by overexpression of a Jatropha *diacylglycerol acyltransferase* 1", International conference on Trends in Plants Sc. and Agrobiotechnology, IIT Guwahati, **14th -16th Feb. 2019**.
3. **Sharma PK**, Nishchal, Maravi DK, Goud VV, and Sahoo L "An integrated strategy for sustainable waste-water treatment and simultaneous biodiesel production using *C. sorokiniana*", International Symposium on Plant Biotechnology for Crop Improvement, IIT Guwahati, Poster Presentation, **20th – 21st January, 2017**.

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