



*Review*

## **Microalgae selection and improvement as oil crops: GM vs non-GM strain engineering**

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**Abstract:** Despite being established as a sustainable feedstock for biofuel production with tremendous potential, the microalgae biofuel industry still struggles to make large-scale production economically viable. An overriding aspect in microalgae oil production is strain selection, as it affects nearly all stages of production. This chapter presents the key traits that microalgae should possess for successful lipid production, as well as suitable isolation and selection strategies. It highlights the various metabolic engineering methods that are currently available for the biological improvement of microalgae strains, comparing GM vs non-GM approaches.

**Keywords:** biofuel; genetic engineering; metabolic engineering; microalgae; microalgal lipid

### **Highlights**

- Metabolic engineering and strain selection of microalgae have key implications on every aspect of production of algal oil.
- Key factors for selecting microalgae are a high productivity of extractable lipids and ease of harvest.
- Laboratory screening must always be followed by larger scale outdoor testing to ensure selection of a suitable species for commercial production.
- Non-GM methods for strain improvement such as mutation-selection programs are highly effective and quite rapid.

- GM methods are focused on improving photosystems, but transcriptomics studies have identified key metabolic and regulatory genes for genetic modification.
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## 1. Introduction

The use of microalgae as a sustainable feedstock for biofuel production has received much recent interest in an effort to confront depleting fuel reserves, global warming and climate change. Microalgae represent a renewable source of energy as they use photosynthesis to convert CO<sub>2</sub>, sunlight and water into energy that is stored as lipids and carbohydrates (e.g. starch). These can be converted into biofuels (biodiesel and bioethanol) with areal productivities that are significantly higher than traditional biofuel land crops, potentially without the use of precious arable land and freshwater [1,2]. While the potential of microalgae as a sustainable energy source, particularly biodiesel has been well established, many technical and biological barriers prevent large-scale economically viable production of microalgal biodiesel. So far, microalgae cultivation facilities such as those of Cyanotech Corporation in Hawaii, Earthrise Nutritionals in California and other large facilities in China produce algae for their nutritional value, not for biofuel [3]. This is because microalgal biofuel companies can currently only produce microalgal oil at a price that is at least five times more expensive than palm oil (\$0.66/L) and that according to estimates needs to be reduced to as low as \$0.48/L to be competitive with petrodiesel [4,5]. To achieve this, the microalgal oil industry must improve many technical and biological aspects of production.

One of the most important biological aspects of microalgal lipid production is the strain used for cultivation. Selection of a suitable strain has downstream effects on nearly every level of production, including growth conditions (pH, CO<sub>2</sub>, light intensity, salinity), harvesting method, oil extraction and if cost-effective enough, the quantity and quality of the biodiesel produced. The overarching importance and impact of the producer strain has driven research into more sophisticated methods of selecting, evaluating and identifying microalgal strains with suitable characteristics. However, many of the approaches which are used in crop plants (forward genetics, segregation, crossing & hybrid production) are not developed in algae, as defined sexual cycles are difficult to induce for most microalgae (with the exception of *Chlamydomonas*). Therefore, recent years have seen tremendous interest in genetically-modified (GM) species as well as improvement of non-GM species for lipid production. In addition, microalgae can be adapted to various conditions (e.g. salinity tolerance), but these traits may not be stable unless continuously selected for. This review discusses the various traits that are desirable for microalgal oil production with a focus on lipids as feedstock for biodiesel. It also highlights the importance of strain selection and evaluation and the various GM and non-GM methods for improving lipid productivity.

## 2. Collection, Isolation and Screening of Microalgae for Oil Production

### 2.1. Collection and isolation of microalgae

Microalgae are found in nearly all natural waters, including freshwater, brackish water, marine ecosystem, and also in extreme environments such as volcanic waters. Nevertheless, collection of microalgae for lipid production must focus on locations with the greatest likelihood of providing strains that are suitable for oil production in an outdoor setting. Firstly, microalgal species should be collected from the local area, or at least an area with similar climatic and ecological conditions as in the intended production area. This is because native strains are likely to be already acclimatized to local conditions and have a competitive advantage over foreign species. Furthermore, the sampling should focus on the aquatic environments that are exposed to fluctuating and/or occasional adverse conditions such as tidal pools and estuaries. These locations naturally select for microalgae that are robust, fast-growing, and have survival mechanisms (e.g. accumulation of storage lipids) to cope with changing conditions [6]. This is likely to increase the chances of finding a strain that is most suitable for high oil production.

After samples have been collected from the environment, individual microalgal strains can be isolated and purified using a range of techniques. Traditional techniques such as micromanipulation and serial dilution to individual cells can be time and energy intensive, but are usually successful in isolating pure cultures, although they may fail to isolate rare strains. Antibiotic selection and enrichment of microalgae from mixed cultures can be used to select for strains with desirable traits, such as a high growth rate and pH- or salinity-tolerance. Automated processes involving flow cytometry and robotics have been developed for rapid isolation of microalgal strains [7,8,9]. The use of high-throughput fluorescence assisted cell sorting (FACS) can distinguish different microalgal species by relying on the species' different chlorophyll auto-fluorescence and green autofluorescence properties. Microalgal cells can also be stained with sub-lethal doses of lipid-staining Nile Red reagent prior to cell sorting and this can help isolate the cells with a high lipid content [10]. However, high lipid-containing microalgal strains (e.g. *Botryococcus braunii*) often display slow growth and this may result in a low overall lipid productivity. Once isolated, a pure culture should be preserved by slow propagation in stock cultures or cryopreservation to prevent loss of competitiveness by genetic drift [11].

### 2.2. Screening criteria

Two of the most important criteria when screening microalgae for oil production are the lipid productivity (depends on growth rate and lipid contents) and composition. A fast-growing highly oleaginous microalgal strain would translate directly to an overall increased productivity. However many fast-growing strains have low lipid contents, but their lipid biosynthesis is highly inducible and, therefore, under appropriate conditions their lipid productivity can be quite high [12]. For biodiesel production, the qualitative and quantitative composition of a strains' triacylglycerides (TAG), the fraction of the lipids that are suitable for biodiesel production, affects the quality of the biodiesel produced and its potential to meet the biodiesel standards. The lipid content of different microalgal

species can vary from 2% to 75% [13], and range from 10% to 30% on average [14]. To be considered potentially suitable for commercial use, a microalgal strain should have a base lipid content of at least 20–30% (% of dry weight, DW). In addition, its fatty acid (FA) content should consist of a mix of saturated and monounsaturated short chain FA, and as little polyunsaturated FA (PUFA) as possible. More importantly, these numbers should be achieved not only in the laboratory, but also in medium- to large-scale outdoor operations that closely mimic an industrial production setting. Many microalgal strains may achieve a high lipid productivity in the laboratory, but fail to do so in the variable outdoor conditions. Thus, it is important that laboratory screening is followed up by outdoor evaluation to determine the suitability of a strain for oil or biodiesel production.

Although most published studies have focused on a single species [11], an increasing number of multi-strain comparative studies evaluating the lipid content and composition in outdoor conditions is becoming available [15,16,17]. These studies often consist of a first round of laboratory screening for comparatively assessing the growth rate, the lipid productivity and the FA composition of several species prior to testing the best performers in larger scale outdoor photobioreactors or raceway ponds. The use of Nile Red staining of microalgal lipids combined with flow cytometry is a powerful tool in identifying the algae with a high lipid content [18]. In the case for biodiesel, several microalgal species (e.g. *Chlorella* sp., *Nannochloropsis* sp., *Tetraselmis* sp.) so far tested, possess the suitable lipid productivity and FA composition for producing biodiesel to conform to most fuel standards, but no single species appears capable of meeting all requirements for a top grade biodiesel [16]. Attaining a good grade of biodiesel may require mixing lipids from different species [19].

Another important criterion for selecting microalgae for oil production is the ease of harvest. Harvesting costs can contribute up to 20–30% of the total cultivation costs [5]. Therefore, microalgal oil production must use cost-effective harvesting methods such as settling and flocculation to keep the cost of production of the biodiesel to a minimum [20]. Some of the microalgae that have been identified as having a high lipid content have been harvested using low-cost methods. Microalgae such as *Tetraselmis*, *Chlorella* and *Scenedesmus* can settle naturally under suitable conditions, while species such as *Nannochloropsis* can be harvested using various flotation or flocculation techniques [21]. Nevertheless, it may be useful to specifically select a microalga that is easy to harvest.

Screening for strains with a high tolerance to extreme environmental conditions (e.g. a high pH and/or salinity) may be useful. In an outdoor setting, particularly in open ponds, contamination by grazers and other undesirable microalgae can be a difficult problem. A high-tolerance microalga would not only better withstand the variable environmental conditions, but its culture environment could be deliberately altered to reduce the potential for contamination. A certain level of salinity tolerance is necessary also for a freshwater strain because evaporation of freshwater increases salinity over time. Finally, the ease of extraction of the oil from different strains can be quite different. For example, *Nannochloropsis* sp. is generally regarded as one of the highest TAG-accumulating algae [15,16], but its tough cell walls can make extraction difficult. Table 1 summarizes some of the desirable traits in a microalga intended for biodiesel production. Although a “perfect” microalga does not exist, the species and strain selection must consider the issues relating to cultivation, harvesting and extraction.

**Table 1.** Desirable traits of a microalga intended for oil production.

| <b>Selection consideration</b> | <b>Desirable traits</b>   |
|--------------------------------|---|
| Initial screening              | Local strain<br>Rapid growth<br>High extractable oil contents<br>High saturated fatty acids, low unsaturated fatty acids<br>Recoverable by settling or foam flotation   |
| Outdoor cultivation            | Rapid and dominant growth<br>Salinity tolerance<br>High/low temperature tolerance<br>Ability to control grazers<br>High light tolerance<br>Shear resistance   |
| Harvesting                     | Cells that autoflocculate or settle at time of harvesting (this may coincide with nutrient depletion/lipid accumulation)<br>Cells amenable to foam flotation  |
| Extraction                     | Cells amenable to easy extraction<br>High lipid recovery  |
| Added benefits                 | Rapid and synchronized lipid production (high lipid inducibility)<br>Utility of the microalgal cake after oil extraction (e.g. high protein contents for food/feed; presence of omega-3 fatty acids, antioxidants, sterols, carotenoids, astaxanthins and other pigments) for added value |

### 3. Microalgal Strain Improvements: Non-GM Metabolic Engineering and Genome Editing

No matter how robust a selection and screening process, it is rare to find an alga that meets all the main criteria for commercial oil production, in particular the criteria relating to large-scale operations. For example, many microalgae that are easy to harvest (e.g. *Tetraselmis*, *Dunaliella*) do not have as high a lipid content as *Nannochloropsis*, which is more difficult to harvest and to extract oil from. Nevertheless, microalgae are excellent candidates for molecular improvement, be it via GM or non-GM methods. Firstly, they have short life cycles (hours to days) which reduce development time. Secondly, their small size and unicellular nature excludes the need for large breeding programs and reduces cost. Thirdly, ultraviolet (UV) light and chemical mutagens can be easily applied to microalgae. Fourthly, microalgae can be selected and screened using traditional screening methods (e.g. antibiotics) as well as automated high-throughput techniques.

The above mentioned non-GM method of mutagenesis followed by high-throughput selection are commonly used for improving microalgal strains. The advantages of the non-GM methods are that they require little or no knowledge of the biochemistry and genetics of the microalgal strain being improved and avoid the regulatory complications associated with the use of GM strains outdoors. In combination with the above noted methods, the use of Nile Red as a fluorescence probe for detecting neutral lipids is common [22]. Correlations between the Nile Red fluorescence signal and the TAG content have been established for some microalgal species [23]. Some of the traditional improvement strategies of mutagenesis (e.g. the use of antibiotics and herbicides for selection) and

subsequent selection of mutants using time-consuming analyses (e.g. gas chromatography, thin layer chromatography) were slow. Such studies typically achieved a yield improvement of between 10% to 40% and were limited mostly to two to three rounds of mutation-selection (Table 2). More recently, Nile Red-staining combined with high-throughput FACS has allowed to accurately sort through millions of cells and select individual cells with a high lipid content. FACS has enabled isolation of cells with lipid levels of  $\geq 60\%$  DW, in some cases without mutagenesis (Table 2).

In addition to UV light, chemical mutagens (ethyl methane sulfonate, EMS; nitrosomethylguanidine, NTG; *N*-methyl-*N*-nitrosourea, MNU), have been successfully used with various microalgal species (Table 2). In some of these studies, selection just for the high lipid cells produced mutants with reduced growth rates [10], emphasizing the importance of a growth selection step to ensure that strains maintain a high growth rate while producing a high level of lipids [24,25]. A further side effect of repeated mutation-selection has been a change in the FA content. An elevation of the PUFAs has been found in mutants relative to wild-types [26–29]. Rapid automated screening combined with conventional mutagenesis make this non-GM improvement approach attractive. These approaches combined with advances in transcriptomics could in the future help reveal potential targets for genetic engineering. For example, in the future it may be possible to develop non-lethal fluorescent *in situ* hybridization probes, specific to certain transcripts, that enable cell sorting-assisted selection of high-expressing mutants for a particular gene in microalgae.

**Table 2.** Some microalgae mutation studies.

| Species                            | Mutagen      | Selection       | Yield increase                                    | Reference |
|------------------------------------|--------------|-----------------|---|-----------|
| <i>Phaeodactylum tricornerutum</i> | UV           | survival        | 37–44% EPA <sup>a</sup>                           | [26]      |
| <i>Pavlova lutheri</i>             | UV           | survival        | 10–20% TFA <sup>b</sup>                           | [29]      |
| <i>Nannochloropsis oculata</i>     | MNU          | quinalofop      | 17–20% TFA <sup>a</sup> , 5–18% EPA <sup>a</sup>  | [28]      |
| <i>Nannochloropsis oculata</i>     | EMS          | antibiotics     | 14–22% TFA <sup>a</sup> , 12–29% EPA <sup>a</sup> | [27]      |
| <i>Nannochloropsis</i> sp.         | none         | FACS            | 300% FS   | [45]      |
| <i>Nannochloropsis</i> sp.         | EMS          | survival        | 13–26% TLC <sup>d</sup>                           | [24]      |
| <i>Haematococcus pluvialis</i>     | UV, EMS, NTG | survival        | 23–59% total carotenoid <sup>c</sup>              | [46]      |
| <i>Schizochytrium</i> sp.          | UV, NTG      | selective media | 35% TFA <sup>b</sup>                              | [47]      |
| <i>Tetraselmis suecica</i>         | none         | FACS            | 400% FS   | [23]      |
| <i>Tetraselmis suecica</i>         | UV           | FACS            | 114–123% FS                                       | [48]      |
| <i>Chlorella sorokiniana</i>       | UV           | plate reader    | 30–40% TLC <sup>b</sup>                           | [25]      |
| <i>Isochrysis galbana</i>          | UV           | FACS            | 60% TLC <sup>b</sup>                              | [44]      |

TFA: total fatty acid; TLC: total lipid content; FS: fluorescence signal; a, b, and c: % DW; d: mg/(L day)

Genome editing has recently emerged as a promising technology that may be exempt from the GM status for regulatory approvals for some countries. The well-known CRISPR/Cas9 system has recently been adapted for gene editing in the marine diatom *Phaeodactylum tricornerutum* where it can efficiently generate stable targeted gene mutations [30]. Possible useful applications towards higher oil contents may include mutations in genes of the  $\beta$ -oxidation fatty acid degradation pathway.

#### 4. Microalgal Strain Improvement: GM-Metabolic Engineering

Genetically-modified microalgae are attracting a lot of interest, with a focus on developing new highly efficient strains. Unlike random mutagenesis followed by screening, developing transgenic microalgae requires a comprehensive knowledge of genomics, transcriptomics and the metabolic pathways for identifying the target genes for engineering. In addition, tools are required for gene manipulation, including selectable markers, vectors and techniques for systemic insertion in screening libraries [11]. The list of fully sequenced microalgal genomes in public databases (Phytozome, Joint Genome Institute, NCBI) continues to grow. This provides a valuable tool for annotating transcriptomic data and identifying the key genes in various metabolic pathways.

While genomic data provide us with what an organism is potentially capable of doing, transcriptomics, metabolomics and proteomics reveal what pathways are currently active/suppressed with respect to a specific situation (for a review on this topic including TAG biosynthesis pathway figures, see [31]). As the cost of sequencing reduces, an increasing amount of transcriptomic data is becoming available. For the production of biofuels, pathways that are linked to lipid accumulation are of particular interest. These pathways have been studied in species such as *Dunaliella tertiolecta* [32], *Haematococcus pluvialis* [33], *Phaeodactylum tricornutum* [34], *Neochloris oleabundans* [35], *Chlorella vulgaris* [36], and *Chlamydomonas reinhardtii* [37,38]. These studies have successfully reconstructed pathways for FA, TAG, starch biosynthesis, FA  $\beta$ -oxidation, TAG catabolism, and starch degradation. These pathways exhibited differential expression during lipid accumulating conditions such as nutrient starvation. Genes involved in the basic metabolic pathways such as ribosome biogenesis, the peptide metabolic processes and RNA processing were upregulated during the stationary phase after nutrient depletion, suggesting an enhanced basal metabolism is required to cope with depleting nutrients [38]. On the other hand, genes related to photosynthesis were down-regulated during nutrient starvation [38]. This was followed by upregulation of lipid metabolism and membrane related genes during the lipid accumulation phase [38], pointing to possible lipid reshuffling during this stage. Examination of transcript abundance during different stages of lipid accumulation revealed multiple carbon fixation pathways, suggesting that a buildup of enzyme precursors may play a more important role in lipid biosynthesis than the actual enzyme levels themselves [34].

While transcriptome studies do not directly contribute to strain improvement, they identify the key pathways and genes that could be the targets of genetic engineering. Genes such as ACCase (acetyl-CoA carboxylase), DGAT (diacylglycerol acyltransferase) and CiS (citrate synthase) have been identified this way and manipulated to increase lipid production. The overexpression of an ACCase-encoding gene in the diatoms *Cyclotella cryptica* and *Navicula sapuvila* resulted in an increased enzymatic activity, although no increase in lipid content was detected [39]. The silencing of a CiS-encoding gene in *C. reinhardtii* increased TAG production by 169% [40], while the overexpression of DGAT2 in *P. tricornutum* increased its neutral lipid content by 35% [41]. Aside from lipid-related pathways, the improvement of the microalgal photosystems has also been the focus of much interest. This is because of the ~ 43% of the solar energy captured via photosynthesis only 4–8% is converted into biomass [1]. This may be improved, for example, by reducing the total light-capture antenna size to minimize the energy loss in a culture by self-shading and non-

photochemical quenching. This has been achieved by reducing the levels of light harvesting complex (LHC) I and LHC II mRNAs and proteins [42] and also by reducing the size of the photosystem II (PSII) antenna [43]. In both cases the growth rates of the transgenic algae were significantly increased, with the transgenic strains achieving higher cell densities when grown in large-scale bioreactors.

## 5. Conclusion

Advances in metabolic engineering and microalgae breeding by strain selection and improvement represent the tip of the iceberg with regard to the overall effort required for making microalgal oil production economically viable. Compared to commercial land crops, barely any effort has gone into metabolic engineering, selection and breeding of microalgal species. Similarly, compared to the petroleum industry, production of algal fuels has had an extremely short developmental history. Therefore, there is much scope for improving all aspects of production of microalgal oils.

## Conflict of Interest

All authors declare no conflicts of interest in this review.

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