Increasing Microbial Biofuel Production by In-silico Comparative Genomic Studies

Gautham Subramaniam Ramakrishnan, Manali Mukund Kamath, and Vidya Niranjan

Abstract—Algal biofuels may be a kind of viable alternative to fossil fuels; however, this technology must overcome a number of hurdles before it can be considered to use in the market and be broadly deployed. One of the major hurdles are the low fuel yields per unit of biomass. In this study, we aim to overcome this challenge by identifying several genes which are responsible for increased lipid production, from numerous sources that can potentially increase the lipid synthesis in the autotrophic alga, Chlamydomonas reinhardtii. Using in-silico comparative genomics, we have made a shortlist of a total of 17 genes which, if incorporated into the genome of Chlamydomonas reinhardtii and overexpressed, could increase lipid production.

Index Terms—Algal biofuels, Chlamydomonas reinhardtii, comparative genomics, lipid production.

I. INTRODUCTION

The continued use of petroleum products is now widely considered unsustainable owing to the depletion of fuel reserves and the contribution of these fuels to global warming [1]. Renewable, efficient and eco-friendly fuels are necessary for environmental and economic sustainability. Interest in a variety of such renewable biofuels has been rejuvenated due to the instability of petroleum fuel costs, a reliance on unstable petroleum resources, and the dangers of increasing atmospheric CO₂ levels.

Biofuels can be solids, liquids or gasses so long as they are derived directly from biological sources. The most common solid biofuel is lignified cellulose (wood) that can be burned for energy. Liquid and gaseous biofuels generally require more refining and include bioethanol, biodiesel and engine-combustible hydrocarbons as well as methane from anaerobic digestion. The aforementioned liquid biofuels offer significant potential to augment or replace petroleum gasoline for transportation purposes. Currently ethanol dominates the biofuel market and may be produced by a variety of methods, primarily heterotrophic fermentation of sugars purified from biomass feedstocks [2]. Biodiesel and other hydrotreated biofuels are derived mainly from vegetable oil feedstocks (lipids) [3].

Ethanol and biodiesel are primarily derived from plant sources, often food crops, because the established scale of food crops made them a convenient source of biomass necessary to produce biofuel on a commercial scale. However, an increasing demand for biofuel feedstocks has negatively impacted food markets, and raised a global “food vs. fuel” controversy. Furthermore, the land and fresh water requirements for growing crops and the long growth-to-harvest periods limit the expansion of plant based biofuel industries to the amount of arable land. In contrast, unicellular algae requiring smaller amounts of land that does not need to be arable, have faster growing cycles, contain a higher percentage of oil, and have been proposed to be a better solution to the food vs. fuel debate. Therefore, significant attention has been focused on algae as a next generation feedstock for biofuel production [4].

Photosynthetic algae, both microalgae and macroalgae (i.e., seaweeds), has been of considerable interest as a possible biofuel resource for decades. Researchers have been exploiting algae for biodiesel production due to their short life cycle, less labor required, less affection by venue, and easier scale up procedure [5]. Several species have biomass production rates that can surpass those of terrestrial plants, and many eukaryotic microalgae have the ability to store significant amounts of energy-rich compounds, such as triacylglycerol (TAG) and starch, which can be utilized for the production of several distinct biofuels, including biodiesel and ethanol [6]. Fatty acids are also important precursors to biofuel [7].

Microalgae are especially attractive as a source of fuel from an environmental standpoint because they consume carbon dioxide and can be grown on marginal land, using waste or salt water [6]. In addition, it may be possible to leverage the metabolic pathways of microalgae to produce a wide variety of biofuels (see Fig. 1).

Fig. 1. Microalgal metabolic pathways that can be leveraged for biofuel production.

Despite the immense potential, several technical barriers need to be overcome before microalgal biofuel can be
successfully brought into the market [8]. These include developing low-energy methods to harvest microalgal cells, difficulties in consistently producing biomass at a large scale in highly variable outdoor conditions, the presence of invasive species in large-scale ponds, low light penetrance in dense microalgal cultures and the lack of cost-effective bioenergy carrier extraction techniques of most microalga-derived biodiesel. The major drawback of microbial biofuel production, however, is the astonishingly low yield of fuel per unit of biomass [9]. In light of these problems, it seems genetic modification is likely to provide the key to unlock the feasibility of algal production strains.

Chlamydomonas reinhardtii is a green microalga (Fig. 2) whose lineage diverged from land plants over 1 billion years ago [10]. It has recently emerged as a model to test genetic engineering or cultivation strategies aiming at increasing lipid yields for biodiesel production [11].

Our work aims to provide some insight on the possible genetic manipulations of Chlamydomonas reinhardtii to obtain higher yields of biofuel using methods of in silico comparative genomics.

II. MATERIALS AND METHODS

A. Shortlist of Lipid Producing Genes

A selection of proteins involved in the regulation of lipid synthesis was shortlisted [12]. The shortlisted protein sequences were downloaded from the UniProt database [13]. The genes that were responsible for coding for these proteins were also deduced by study of available literature.

These genes, when expressed, were responsible either for the upregulation or downregulation of lipid synthesis. Hence, we conceived that manipulation of these genes could lead to an increased production of lipids. Only the genes that lead to an upregulation of lipid synthesis were selected for further study.

Based on homology, sources of the genes were narrowed down to 5 organisms, namely Arabidopsis thaliana, Chlamydomonas reinhardtii, Ostreococcus lucimarinus, Ostreococcus tauri and Volvox carteri.

B. Sequence Retrieval

The sequences of the genes encoding all the selected proteins were retrieved in FASTA format. Once these sequences were procured, the Chlamydomonas reinhardtii genome sequence was obtained from the JGI Genome Portal [14]. The 121-megabase (Mb) draft sequence of the Chlamydomonas nuclear genome has the following characteristics [10]:

- It was generated at 13x coverage by whole-genome, shotgun end-sequencing of plasmid and fosmid libraries, followed by assembly into ~1500 scaffolds.
- Half of the assembled genome is contained in 25 scaffolds, each longer than 1.63 Mb.
- The genome is unusually GC-rich (64%).
- The Chlamydomonas genome comprises of 17 linkages presumably corresponding to 17 chromosomes, consistent with electron microscopy of meiotic synaptonemal complexes [15].
- Seventy-four scaffolds, representing 78% of the draft genome, have been aligned with linkage groups.

The Chlamydomonas reinhardtii average gene properties were as follows:

- Gene density: 149 genes/ Mbp assembly
- Gene length: 3895 nt
- Transcript length: 1768 nt
- Exon length: 240 nt
- Intron length: 336 nt
- Exon frequency: 7.4 exons/gene

The protein and transcriptome data of this organism were also downloaded and stored [14]. The protein coding genes had the following features [14]:

- A reference set of 15,143 protein-coding gene predictions has been created.
- More than 300,000 ESTs have been generated.
- 8631 gene models (56%) are supported by mRNA or EST evidence.
- 35% have been edited for gene structure and/or annotated by manual curation (as of June 2007).
- Protein-coding genes have, on average, 8.3 exons per gene and are intron-rich relative to other unicellular eukaryotes.
- The average Chlamydomonas intron is longer (373 bp) than that of many eukaryotes and only 8% lack introns.

C. BLAST Using Bioedit

The next step involved the alignment of sequences using a standalone BLAST application called BioEdit [16]. As the genome is de novo and annotation of the protein is not complete, a BLAST [17] research of the listed proteins against the protein and transcriptome databases of C.
reinhardtii was performed.

Our objective was to map the homologous proteins responsible for lipid synthesis as well as to identify novel genes which are responsible for the lipid production. The result obtained gave us the percentage identity, alignment length, number of mismatches and the HSP (high-scoring segment pair) score of the said sequences.

The results were filtered to include only the proteins with HSP score greater than 500 and tabulated them. Only these proteins were chosen for the further study.

D. Screening the Candidate Genes

The resultant proteins and expression studies relevant to them were further studied. The results that were obtained from the sequence homology search against the protein and transcriptome databases of C. reinhardtii verified that the genes were transcribed and translated to proteins. This also ensured that regions with false positives, owing to the presence of pseudo genes, are not shortlisted. The number of favorable proteins was thus narrowed down to 34. Further detailed study was carried out and the function of each protein was found out along with the various genes involved in the upregulation of lipid synthesis. A total of 17 genes were screened and shortlisted. We proposed that incorporation of these genes into the genome of C. reinhardtii would help to achieve an increased production of lipid for biofuel, thus maximizing its marketability.

The final screening of the genes was conducted solely based on their functionality. The gene functions and their source organisms were identified as follows. The FadR (Fatty acid metabolism regulator protein), accA (acyl-CoA carboxylyl-transerase subunit alpha), accB (acyl-CoA carboxylase, BCCP subunit), accD (acyl-CoA carboxylase, beta carboxyl-transerase subunit), fabH (3-oxoacyl-[acyl-carrier-protein] synthase III) and fabI (enoyl-[acyl-carrier-protein] reductase, NADH-dependent) genes were shortlisted from Escherichia coli, str. K-12, substr. MG1655. This particular strain, a model organism, has a genome of length 4,641,652 base pairs. The genes accC (acyl-CoA carboxylase, biotin carboxylase subunit) and fabD (malonyl-CoA-[acyl-carrier-protein] transacylase) were catalogued from Escherichia coli str. K-12 substr. W3110, having a genome of length 4,646,332 base pairs. fabG (3-oxoacyl-[acyl-carrier-protein] reductase) gene was taken from Francisella tularensis subsp. mediasiatica FSC147 chromosome which consists of 1,893,886 base pairs. The gene fabZ (3R) hydroxymyristoyl acyl carrier protein dehydratase) was shortlisted from Lactococcus lactis, strain IO-1. The gene Gut1 (glycerol kinase) was listed from Saccharomyces cerevisiae (budding yeast) S288c, chromosome VIII. Similarly, Ptb (phosphate butyryltransferase) and buk (butyrate kinase) were catalogued from Bacillus megaterium WSH-002 chromosome and Bacillus megaterium QM B1551 chromosome respectively. adhE2 (Aldehyde-alcohol dehydrogenase) gene was taken from Azospirillum lipoferum str.4B plasmid AZO_p1. DGAT gene was listed from Mus musculus, chromosome 15. The genes ME (Malic Enzyme) and PEPC (phosphoenolpyruvate carboxylase) kinase were taken from Arabidopsis thaliana chromosome 5 and chromosome respectively.

E. Phylogenetic Analysis

The literature survey showed that Arabidopsis thaliana, Ostreococcus lucimarinus, Ostreococcus tauri and Volvox carteri were homologous to Chlamydomonas reinhardtii [12]. To confirm this, we carried out a phylogenetic analysis. The sequence of the protein Heteromeric ACC biotin carboxylase subunit (BCC) was retrieved for all these 5 organisms from GenBank. These sequences were analyzed using Clustal Omega, a new multiple sequence alignment program that uses seedeed guide trees and HMM profile-profile techniques to generate alignments [18].

III. RESULT AND DISCUSSION

In the BLAST search against the C. reinhardtii transcriptome, we obtained a total of 197 hits. Upon filtering based on the HSP value, this number reduced to 110. Similarly, in the search against the C. reinhardtii protein database, we recorded a total of 198 hits, which reduced to 117 upon filtering. The number of proteins is common to both the transcriptome and the protein database boiled down to 34. Upon further literature survey, we found that only 13 of these proteins had been studied for expression and there were 35 genes which could affect the lipid synthesis. Finally, we came up with a total of 17 genes that satisfied our searching criteria. They have been shown in Table I.

### TABLE I: FINAL LIST OF GENES ENHANCING LIPID YIELD

<table>
<thead>
<tr>
<th>GENE</th>
<th>SOURCE</th>
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<tbody>
<tr>
<td>FadR</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>accA</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>accB</td>
<td>Escherichia coli</td>
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<td>accC</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>accD</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>fabH</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>fabG</td>
<td>Francisella tularensis</td>
</tr>
<tr>
<td>fabZ</td>
<td>Lactococcus lactis</td>
</tr>
<tr>
<td>fabI</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>GUT1</td>
<td>Saccharomyces cerevisiae</td>
</tr>
<tr>
<td>Ptb</td>
<td>Bacillus megaterium</td>
</tr>
<tr>
<td>Buk</td>
<td>Bacillus megaterium</td>
</tr>
<tr>
<td>adhE2</td>
<td>Azospirillum lipoferum</td>
</tr>
<tr>
<td>DGAT</td>
<td>Mus musculus</td>
</tr>
<tr>
<td>me</td>
<td>Arabidopsis thaliana</td>
</tr>
<tr>
<td>PEPC</td>
<td>Arabidopsis thaliana</td>
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Although we found 35 genes responsible for regulation of lipid synthesis, only 17 of these had been proven to increase lipid production if overexpressed. As a result, only these 17 genes satisfied our searching criteria.

During the literature survey, we came across a study of homology in a paper by Merchant SS, et al. stating that O. lucimarinus, A. thaliana and O. tauri were homologs of C. reinhardtii, thus proving the validity of our work. Later, we obtained evidence that Volvox carteri, which is a species of colonial green algae [19], has also been studied for lipid production and is a homolog of C. reinhardtii [12]. Verification of this observation by a phylogenetic analysis between the said organisms and Chlamydomonas reinhardtii (Fig. 4) showed that Volvox carteri is indeed highly similar to C. reinhardtii. The presence of lipid producing genes in all
the mentioned organisms and their evident homology suggests that transformation of C. reinhardtii with these genes would be a feasible endeavor.

**Fig. 4. The phylogenetic tree obtained using clustal omega.**

**IV. CONCLUSION**

Microalgae has emerged as a popular feedstock for the production of biofuels and other products [20]. They are an extremely diverse group of organisms, many of which possess unique metabolic features that can be altered for the production of renewable biofuels [6]. These include:

- High efficiency of conversion during photosynthesis.
- Rapid rates of biomass production.
- The capability to produce a wide variety of biofuel feedstocks.
- The ability to thrive in diverse ecosystems

Although microalgae have long been considered a promising feedstock for the production of biofuels, studies have concluded that the economic viability of microagal biofuel production is in need of significant improvement. However, in contrast to previous efforts, we are now equipped with an arsenal of new genetic tools, genome sequences, and high-throughput analytical techniques that will equip and empower scientists to analyze and manipulate metabolic pathways with unrivaled precision [6].

To date, molecular genetic tools have been developed for only a few algae, including the green alga *Chlamydomonas reinhardtii*, and the diatoms *Phaeodactylum tricornutum* and *Thalassiosira pseudonana.* Among these, only C. reinhardtii has well established systems for recombinant protein expression from both the nuclear and chloroplast genomes, and even in this species only a handful of recombinant proteins have been expressed to any appreciable level [21].

Though expression studies have also been done for bacteria such as *E. coli* and there are molecular genetic tools available for the same, the prokaryotic genome of bacterial cells lacks certain advanced post translational modification systems that are seen in the higher eukaryotes. Additionally, microalgae such as C. reinhardtii are photosynthetic and are easier to cultivate. They can even be cultivated in natural, for instance, open pond systems with waste water as a source of nutrients [9]. It has recently been demonstrated that C. reinhardtii can be engineered to produce novel hydrocarbon molecules that are superior biofuels, demonstrating the potential of microalgae as a biofuel source.

**In silico** screening of genes for genetic engineering is a versatile methodology to aid us in creating mutants with high biofuel productivity. These methods at organism and functional level can reduce years of wet lab experimentation by narrowing down the huge number of genes and even pinpoint the most desirable ones. Optimizing microagal biofuel production using metabolic engineering tools requires an in-depth understanding of the structure-function relationship of genes involved in lipid biosynthetic pathway [12].

Our bioinformatics approach considers a wide range of data with various parameters and criteria. This enhances the quality and scope of the research we have conducted.

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**REFERENCES**


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