Transformation of *Ettlia oleoabundans*, a potential biofuel alga.

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By

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Abstract

The microalgae *Ettlia oleoabundans* produces up to 80% of its dry weight in triacylglycerols and is a prime candidate for biodiesel production. This study explores methods for transformation of *E. oleoabundans* by electroporation. Both the antibiotics hygromycin and spectinomycin were effective for transformant selection. First apparent successful production of *E. oleoabundans* protoplasts was achieved. Because this species produces nearly wall-less zoospores during its life cycle, synchronization was attempted using light/dark cycling and nitrate starvation to increase concentrations of zoospores to improve transformation.
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1 Introduction & Background

1.1 Energy and the environment

The comfortable world of “unlimited” cheap fossil fuels, in which many were ignorant of the changes in the atmosphere and the warming of the Earth and its oceans, has long past. Today, amidst climate change, global warming, and the dwindling supply of cheap petroleum fuels, human beings are looking to a brighter, cleaner future, one capable of sustaining itself.

1.2 The biofuel advantage

Biofuels are derived from recently living sources. Though a derivative of once living matter, fossil fuels are chemically produced deep within layers of the Earth under the high heat and pressure reached there, but not by the original organism and are, therefore, not a biofuel. Put more simply, biofuels can be produced repeatedly from growing organisms while fossil fuels cannot, making them a renewable energy source. Biofuels are not a new concept and have been used in different forms for millennia, the oldest, perhaps, being firewood and other forms of lignocellulosic material. Today many forms of biofuel are used, from wood to vegetable oils, to diesel-like biofuel (biodiesel) and many other combustibles. The potential advantages of using biofuels over the traditionally ubiquitous fossil fuels are numerous, and show exceptional promise for becoming a major sustainable energy source that, coupled with other energy forms, could entirely replace the use of fossil fuels on Earth.

1.3 Types of biofuels and their biological sources

The first, second, and third generation biofuels, as defined by the U.S. Department of Energy (2009), are reviewed herein. Such so-called first generation biofuels, those derived from conventional crop sources (sugars, starches, vegetable oils, and even animal fats) using conventional technologies, are as follows (U.S. Department of Energy, 2009):

- Solid Biofuels (straight biomass): solid biofuels are non-liquid and many are suitable for use as a fuel with minimal processing. Such fuels include wood (lignocellulose), sawdust (after compression into pellets), domestic bio-waste, agricultural wastes, and certain energy crops (such as corn, switchgrass, sorghum, wheat, poplar, soybean, canola, and jatropha). Solid biofuels are low cost and readily available but are usually limited to stationary applications such as domestic heating, cooking, and industry using traditional external combustion engines like
the steam engine. If combusted, they also produce considerable CO₂ and are likely at best carbon neutral.

- **Straight Vegetable Oils**: straight vegetable oils are a liquid biofuel pressed from any vegetable oil crop (such as canola or soybean) that can be used to run internal combustion engines for both transportation and stationary applications. Straight vegetable oil can be burned efficiently after simple filtration and viscosity reduction with minimal preheating; they are also at best carbon neutral.

- **Biodiesel**: biodiesel is a liquid biofuel composed of fatty acid methyl esters (FAMEs) derived from vegetable oils or animal fat by transesterification which produces 1 part glycerol (a sweet and highly viscous substance of very low toxicity used in pharmaceutical, food, and other domestic products) for every 10 parts fatty acid methyl ester. Chemically, biodiesel is most similar to petroleum mineral-diesel except that biodiesel contains neither mineral contaminants like sulfur nor the same ratio of fatty acid chains, as this varies with the ratios native to the source. Contemporary applications that use petrodiesel can also use biodiesel with little to no adaptation (depending on the fatty acid ratios of the biodiesel). If produced from cultivated crops, these are also at best carbon neutral.

- **Bioalcohols**: bioalcohols, a fluid biofuel, are alcohols biologically derived most commonly from sugar and starch sources by microbial fermentation. Common bioalcohols include ethanol, propanol, and butanol, and are presently used in both domestic heating and cooking applications and transportation. They are usually either carbon negative or neutral.

- **Biogas**: biogas is a gas derived from the anaerobic breakdown of organic matter. Of the two primary types, one is derived from the anaerobic digestion of manure, sewage, municipal waste, and energy crops, composed of methane and carbon dioxide (Evans, 2008). The other is derived from the gasification (bringing to a high temperature under controlled oxygen concentrations to break it down into gas) of wood (wood gas) or other dry biomasses (syngas), and is composed of varying quantities of nitrogen, carbon monoxide, hydrogen, and trace amounts of methane and oxygen (Evans, 2008). Natural gases derived from geological reservoirs have long been used for heating, cooking, electricity, and, less commonly, transportation. Biogas derived from anaerobic digestion of waste easily replaces natural gas where it is used in these applications as they are chemically similar. Biogas is also carbon
negative, and capturing methane for use as a fuel helps to reduce the greenhouse effect, as methane is about 21 times more potent a greenhouse gas relative to its CO$_2$ equivalent (U.S. Environmental Protection Agency, 2010).

So called “second generation” biofuels are those derived entirely from cellulosic non food crops using new and in-development biomass-to-liquid technologies. Techniques currently in development include the production of mixed alcohols, biohydrogen (from fermentation or the gasification of biomass; Demirbas, 2009), biomethanol, DMF (2,5-dimethylfuran: \((\text{CH}_3)_2\text{C}_4\text{H}_2\text{O}\)), Bio-DME (dimethylether, similar to biomethanol), wood diesel, and Fischer-Tropsch diesel (the production of hydrocarbons from biogas, carbon monoxide, and hydrogen with metal catalysts).

So called “third generation” biofuels are those derived entirely from microalgae. Different microalgae produce lipids for longer term energy storage, such as polyunsaturated fatty acids, hydrocarbons, and triacylglycerols (TAGs; Li et al., 2008). Algae are a low input, high output crop that have the ability to produce up to 70% of their dry weight in TAGs and thirty times more energy per acre than conventional land crops such as soybean (Gouveia and Oliveira, 2009; Chisti, 2007). Algal crops can be grown in all sorts of environments largely independent of climate and entirely independent of soil conditions. Extracted hydrocarbons or TAGs can be processed into a more usable form; hydrocarbons can be “cracked” like petroleum while TAGs can be transesterified to produce biodiesel.

1.4 Advantages of biofuels

There are many advantages of biofuels when compared with traditional fossil fuels. First and foremost biofuels are, unlike fossil fuels, renewable and sustainable if produced properly and have the potential to provide humans with relatively cheap and virtually limitless fuels. They are made from non-toxic, biodegradable sources and, when replacing fossil fuels, lead to a decrease in harmful carbon monoxide, hydrocarbons, and emissions of particulate matter and sulfur oxide (Gouveia and Oliveira, 2009). Biofuels have the potential to be entirely carbon neutral and even carbon negative, with the latter facilitating mitigation of atmospheric carbon dioxide. The carbon neutrality of any biofuel does not depend solely on the fuel itself, but rather, on its life cycle, the energy sources that go into the production, processing, and transportation of said fuel and whether or not they are carbon neutral. The more that industrial processes are optimized, the more efficient will be the environmental benefits.
Biofuels are cleaner than fossil fuels and inherently have fewer impurities, requiring little refinement. When compared with petrodiesel, biodiesel burns cleaner and more completely, with a significant reduction in unburned hydrocarbons (by 90%), a 75-90% reduction in aromatic carbon emissions, a 50% reduction in carbon monoxide emissions, and, as biodiesel is inherently very low in sulfur content, a 100% reduction in sulfur dioxide emissions (Peterson & Reece, 1996). Bioalcohols and biodiesels have a lubricating and cleaning effect on internal combustion engine parts, resulting in more efficient operation (Campbell, 2005). As cleaner burning fuels, biofuels are safer to use and more environmentally friendly for domestic needs, such as heating and cooking, electricity, and transportation.

Many biofuels, especially second and third generation biofuels, are chemically similar, if not identical, to contemporary fossil fuels, so little to no adaptation of contemporary technology is required. For more than two decades, automotive companies like GM, Ford Motors, Nissan Motors, and Honda Motors have produced flex-fuel vehicles adapted to running on bioethanol-gasoline mixes such as E100 (100% ethanol) and E85 (85% ethanol, 15% gasoline), and vehicles capable of running on compressed natural gas (CNG). Most diesel engines on the road are capable of running on biodiesel blends of up to 20% without any modification whatsoever (Campbell, 2005).

1.5 Disadvantages of biofuels

Biofuels are not without their disadvantages, some of which have already been mentioned. Social and economic disadvantages of biofuels center around what is referred to as the “food vs. fuel” debate, where food crops are grown for the production of biofuels instead of food, or where resources that could be used for the production of food crops (arable land, fresh water, and nutrients) are used for the production of energy crops (Elobeid and Hart, 2007). The ever expanding human population on Earth can not spare such resources for biofuels nor afford a rise in food prices as its supply decreases (Grunwald, 2008).

There are potential environmental disadvantages to biofuels. Poor practices in farming, forestry, or any other unsustainable use for the production of biofuels result in destruction of natural resources and potential loss of biodiversity. This disadvantage is not inherent in the fuel sources themselves, but in how industry produces them. Some biofuels have the potential to be more environmentally harmful because of how they must be chemically processed. For example, aldehyde pollution, formed by the oxidation of alcohols, presents an environmental disadvantage specifically to
bioalcohol fuels. Aldehydes are considered to be harmful to human health, classified as carcinogens and mutagens, and were found to be in slightly higher concentrations in ethanol fuel emissions compared to gasoline emissions (Hammel-Smith et al., 2002).

Certain biodiesels have longer carbon chains and few to no esters resulting in increased fuel viscosity, which at very lower temperatures can cause problems with fuel flow and ignition (Campbell, 2005). Though this is an issue with any diesel fuel, it is a difficulty faced by the transportation industry. The magnitude of the effect depends entirely on the feedstock used to produce the fuel, and the effects can be mitigated with the addition of fuel tank heating elements and other changes in injection and ignition designs. Even straight vegetable oil can be burned in an unmodified diesel engine but requires fuel heaters in colder climates.

1.6 Microalgae for biofuel production

As described in Section 1.3, biofuels derived from algae are “third generation” biofuels and are considered the “feedstock of the future for sustainable biodiesel production” because of the significantly higher growth rates and photosynthetic efficiencies over terrestrial plants; these are features inherent to microalgae due to their simple often unicellular structure (Gouveia and Marques, 2009; Li et al., 2008; Liu et al., 2008; Chisti, 2007; Usui and Ikenouchi, 1997). Microalgae are a low input, high output crop with the capability to produce up to 70% of their dry weight in oils (biofuel precursor) and twenty-three times more potential energy per acre compared to the highest oil yielding land crop, Palm oil (see Table 1; Gouveia and Marques, 2009; Chisti, 2007).

Additionally, due to the fact that algal cultures grow in an aqueous environment, they have more efficient access to water, CO₂, and other nutrients (Gouveia and Marques, 2009). Their environments can be completely controlled in photo bioreactors, making them easier to manage. This eliminates the need for arable land that could otherwise be used for food crops (Gouveia and Marques, 2009). Microalgae also do not all need fresh water and can instead use wastewater or seawater (depending on species), thereby eliminating competition for freshwater use in agriculture and for drinking water. This is of pivotal importance in parts of the world where drinking and agricultural water are scarce (Elobeid and Hart, 2007).

Examples of microalgal species currently being studied for their potential use as biofuel producers include species of *Chlorella* (Liu et al., 2008; Xiong et al., 2008; Xu et al., 2006; Illman et
al., 2000), *Dunaliella* (Takegi et al., 2006; Gordillo et al., 1998), *Nannochloris* sp. (Takagi et al., 2000), *Parietochloris incisa* (Bigogno et al., 2002), *Ettlia oleoabundans* (formerly *Neochloris* oleoabundans; Tornabene et al., 1983; Watanabe and Floyed, 1988; Deason et al., 1991), and *Botryococcus braunii* (Li and Qin, 2005; Metzger and Largeau, 2005), all of which have been reported to accumulate large quantities of lipids under the proper conditions (Li et al., 2008). Nonetheless, each species presents its own advantages and disadvantages, each one catering to different fuel types and/or different industrial applications (Table 2).

Table 1: Oil yield potential for common crops in liters per hectare. From Chisti (2007), as found in Gouveia and Oliveira (2009).

<table>
<thead>
<tr>
<th>Crop</th>
<th>Oil Yield (L ha(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>172</td>
</tr>
<tr>
<td>Soybean</td>
<td>446</td>
</tr>
<tr>
<td>Canola</td>
<td>1190</td>
</tr>
<tr>
<td>Jatropha</td>
<td>1892</td>
</tr>
<tr>
<td>Coconut</td>
<td>2689</td>
</tr>
<tr>
<td>Palm</td>
<td>5950</td>
</tr>
<tr>
<td>Microalgae (70% oil by wt in biomass)</td>
<td>136900</td>
</tr>
<tr>
<td>Microalgae (30% oil by wt in biomass)</td>
<td>58700</td>
</tr>
</tbody>
</table>

Table 2: Potential microalgae for biofuel production and their relevant properties (Gouveia and Marques, 2009; Li et al., 2008)

<table>
<thead>
<tr>
<th>Species</th>
<th>Habitat</th>
<th>Primary Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorella</td>
<td>freshwater</td>
<td>fatty acids</td>
</tr>
<tr>
<td><em>Dunaliella</em></td>
<td>marine</td>
<td>fatty acids</td>
</tr>
<tr>
<td><em>Nannochloris</em> sp.</td>
<td>marine</td>
<td>fatty acids</td>
</tr>
<tr>
<td><em>Parietochloris incisa</em></td>
<td>freshwater</td>
<td>polyunsaturated fatty acids</td>
</tr>
<tr>
<td><em>Ettlia oleoabundans</em></td>
<td>freshwater</td>
<td>triacylglycerols</td>
</tr>
<tr>
<td><em>Botryococcus braunii</em></td>
<td>freshwater</td>
<td>hydrocarbons</td>
</tr>
</tbody>
</table>
Though *P. incisa* is rich in polyunsaturated fatty acids, the lipids are not ideal for conversion to biodiesel (Li et al., 2008). On the other hand, *B. braunii* produces primarily large hydrocarbon molecules, botryocenes, that are ideal for liquid biofuel production by liquefaction or pyrolysis, but like the polyunsaturated fatty acids are not suitable for the production of biodiesel (Li et al., 2008). Both *Nannochloris* and *Dunaliella* are marine microalgae and are naturally already adapted for biofuel production in coastal regions using seawater. These two species, however, would not be suited to inland production using freshwater (Li et al., 2008). *E. oleoabundans*, however, is a freshwater microalgae which produces up to 80% triacylglycerols of its total lipid content, most of which are saturated fatty acids ranging in lengths of 16-20 hydrocarbons making this species ideal for the production of biodiesel (Li et al., 2008; Tornabene et al., 1983).

### 1.7 *Ettlia oleoabundans*: culture & oil production

The freshwater microalgae *Ettlia oleoabundans* has been reported to produce more than half its dry weight in oil (56%; Gouveia and Marques, 2009), 80% of which are triacylglycerols of 16-20 hydrocarbons in length (Li et al., 2008; Tornabene et al., 1983). Table 3 contains a typical fatty acid profile for *E. oleoabundans*. Unfortunately, such high productivities have only been achieved after about 6 days in nitrate deficient medium, which is problematic since nitrate sufficient medium is necessary to achieve maximal biomass productivity (Gouveia and Marques, 2009; Pruvost et al., 2009; Li et al., 2008;).

*E. oleoabundans* reproduces by releasing flagellated, asexual spores called zoospores from the cytoplasmic space (Deason et al., 1991; Komarek, 1989). *Ettlia's* zoospores posses a very fine cell wall that cannot be observed without the aid of electron microscopy or staining (Deason et al. 1991). Figure 1 shows common morphologies of an *E. oleoabundans* culture.

### 1.8 Challenges in producing transportation fuels from algae

As promising as the use of microalgae for biofuel production is, especially with the species described in section 1.6, their use in an optimized industrial process is not without challenges. *Chlorella, Dunaliella, Nannochloris* sp., *P. incisa, B. braunii*, and *E. oleoabundans* all produce a high percentage of their total biomass as a biofuel precursor. Unfortunately, however, this is not always achieved because such production is only performed when cells are under stress typically in the form of nutrient starvation, which also results in poor overall productivity (Li et al., 2008).
Figure 1: *Ettlia* morphologies including (A) normal cells, (B) large cell (no zoospores; yellow arrow), and (C) cell containing zoospores (yellow arrow).

Table 3: Fatty acid profile for *Ettlia oleoabundans*.
(fatty acids not listed were not detected; Gouveia and Marques 2009)

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th><em>Ettlia oleoabundans</em> (% w w⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0 (myristic)</td>
<td>0.43</td>
</tr>
<tr>
<td>16:0 (palmitic)</td>
<td>19.35</td>
</tr>
<tr>
<td>16:1 (palmitoleic)</td>
<td>1.85</td>
</tr>
<tr>
<td>16:2</td>
<td>1.74</td>
</tr>
<tr>
<td>16:3</td>
<td>0.96</td>
</tr>
<tr>
<td>16:4</td>
<td>7.24</td>
</tr>
<tr>
<td>18:0 (stearic)</td>
<td>0.98</td>
</tr>
<tr>
<td>18:1 (oleic)</td>
<td>20.29</td>
</tr>
<tr>
<td>18:2 (linoleic)</td>
<td>12.99</td>
</tr>
<tr>
<td>18:3 (α-linoleic)</td>
<td>17.43</td>
</tr>
<tr>
<td>18:4</td>
<td>2.10</td>
</tr>
<tr>
<td>Saturated</td>
<td>20.76</td>
</tr>
<tr>
<td>Unsaturated</td>
<td>64.60</td>
</tr>
</tbody>
</table>

Additional challenges include producing final fuel products, which meet the proper specifications for their intended application (such as combustion in a diesel engine) and are resistant to degeneration in storage. Specifically, biodiesel must have the proper cetane number, flash and solidifying points, and overall viscosity across ambient and operating temperatures, and lipids from any
plant source must be transesterified to produce fatty acid methyl esters (aka biodiesel), which posses the necessary properties (Pruvost et al., 2009; Miao and Wu, 2006). The ability of these fuels to remain in longer term storage is a factor in how sensitive they are to oxidation. Unlike phospholipids and glycolipids, which function as structural components in cells, triacylglycerols (TAGs) provide long term energy storage in cells, are neutral, and are preferred for their high percentage of fatty acid and absence of phosphate (Pruvost et al., 2009; Miao and Wu, 2006).

As with any nascent industrial process, the production of biofuels with algae remain inhibited by the high cost of production. Lowering these costs will require the optimization of the industrial process but will also require selection of algal species and nutrient components for cost effective production. A few promising species for production are known and have been noted herein (Section 1.6). To overcome the biological limitations increasing production cost, researchers are turning to genetic transformation and manipulation of these species.

1.9 Genetic transformation of microalgae

Genetic transformation has been explored in a collection of species of microalgae, the most commonly investigated being Chlamydomonas reinhardtii (Coll, 2006). The ultimate goal is the modification of metabolic pathways to increase growth rates and/or lipid production rates. Other species that have been successfully transformed include Chlorella spp., Dunaliella salina, Euglena gracilis, Haematococcus pluvialis, Volvox carteri, the diatoms Cyclotella cryptica, Navicula saprophila, Phaeodactylum tricornutum, and Thalassiosira weissflogii, the dinoflagellates Amphidinium klebsii and Symbiodinium microadriaticum, and the red algae Cyanidioschyzon merolae and Porphyridium spp. (Coll, 2006).

Reported methods that have yielded successful transformation include Agrobacterium tumefaciens, glass bead agitation, electroporation, and microparticle bombardment. The latter method has most commonly been used for chloroplast transformations while the two prior methods are most commonly used for nuclear transformations (Coll, 2006). Glass bead agitation has been a popular method for transforming microalgae because of its simplicity and easy reproducibility, requiring no specialized or expensive equipment (Coll, 2006). One of the great challenges of transforming microalgae using the first two techniques (glass bead agitation, about 100 transformants per million; electroporation, about 1,000 transformants per million) has been the need to eliminate as much of the cell wall for the cells to be transformed. Given the great diversity in cell wall chemistry, it is
challenging to produce protoplasts for many species, thus the genetic transformation of microalgae has not been simple or straightforward (Coll, 2006). One method other than enzymatic digestion to remove cell walls (the traditional means of producing protoplasts) is to grow cultures in ammonium deficient medium to prevent the formation of intact cell walls, as was done with *C. reinhardtii* (Coll, 2006; Kindle, 1998; Nelson and Lefevre, 1995). Cells containing recalcitrant cell walls have required the use of other methods such as glass bead agitation with a vortex for several minutes, but this has not produced or increased the number of transformants (Coll, 2006; Kindle, 1990). Microparticle bombardment (the gene gun) can circumvent the cell wall problem, as protoplasts do not need to be generated to achieve decent transformation efficiencies (about 100 transformants per million) (Coll, 2006), however, expensive specialized equipment is required.

Though its use has been little reported, transformation of microalgae with *A. tumefaciens* was accomplished with *C. reinhardtii* (about 350 transformants per million) (Kumar et al., 2004). This method appears to be very promising as a means of penetrating the cell wall: it can possibly transform walled microalgae (vs. protoplasts), provide long term exogenous DNA replication (18 months without selection was reported), strong T-DNA integration (T-DNA: the transferred DNA; in this case, the Tumor Inducing (Ti) Plasmid of *A. tumefaciens* and exogenous inserts), and yield reasonably good transformation efficiencies (Coll, 2006).

Electroporation also has been reported as a reliable and reproducible method for transforming algal species and produces the highest transformation efficiencies (about 1,000 transfected per million cells; Coll, 2006). Electroporation was thus selected as the initial transformation method to be explored.

### 2 Hypothesis & Objectives

The overall goal of this project is to develop a simple reproducible method for the transformation of *E. oleoabundans*. The specific objectives of this project are to:

1. Determine if the algae are susceptible to an antibiotic to enable selection.
2. Construct antibiotic resistance plasmids to enable selection of successful transformants.
3. Optimize electroporation.
4. Select culture conditions to optimize transformation.
3 Methods

3.1 Microalgae cultures

Cultures of *Ettlia oleoabundans* (*E. oleoabundans*; UTEX1185) were maintained in 125 mL shake flasks containing 20 mL of Bolds Basal Medium with 3-fold Nitrogen and Vitamins (3N-BBM+V, see Appendix: Formulations; Bischoff and Bold, 1963) and 5 mL of inoculum. To ensure sufficient aeration and prevent settling, culture flasks were kept on a shaker at 100 rpm. Cultures were illuminated with continuous cool-white fluorescent light (about 30 μmol m$^{-2}$s$^{-1}$) and maintained at approximately 25 °C. Algae were subcultured weekly into fresh media (20 mL BBM + 5 mL old culture) to keep them in a constant state of exponential growth.

Larger volumes of *E. oleoabundans* were grown in upright 1,200 mL Roux bottles (BellCo Glass Part # 5630-12000) containing 500mL to 800mL 3N-BBM+V and 15-25mL of mid log phase algal inoculum (starting optical density of about 0.1 at 540nm), outfitted with a gas inlet, gas outlet, and culture sampling port (Figure 2). Water-saturated air was pumped through a sterilizing filter on the gas inlet, down a glass conduit, and into the culture at the base of the Roux bottle to provide the culture with CO$_2$ for growth. Air was exhausted from the bottle through the glass conduit outlet at the top of the bottle. A second glass tubing at the base of the Roux bottle running through the stopper at the top was connected to a flexible tube that was either connected to a valve or clamped off, making a culture sampling port. Cultures were illuminated with continuous cool-white fluorescent light (approximately 60 μmol m$^{-2}$ s$^{-1}$; Figure 2).

Light/dark synchronized *E. oleoabundans* cultures were grown in upright 1,200 mL Roux bottles containing 500mL to 800mL 3N-BBM+V and 15-25mL of mid log phase algal inoculum (starting optical density of about 0.1 at 540nm), outfitted with a gas inlet, gas outlet, and culture sampling port (Figure 2), enclosed in an fan cooled, near-light-tight cardboard box and maintained on a 12 hour light/dark cycle controlled with a General Electric 24-Hour Two-Outlet Mechanical Timer (SKU: 06669; cool-white fluorescent light; approximately 60 μmol m$^{-2}$ s$^{-1}$; Figure 3). Cultures were entrained for 4-12 days to establish synchronized reproductive cycles. The enclosing box was either fully ventilated (exhaust flap open; 28 °C at all times) or partially ventilated (exhaust flap closed; 28 °C in dark, 32 °C; Figure 3).
3.2 Antibiotic bioassay

Antibiotics against *Ettlia oleoabundans* were tested to find one effective at killing *Ettlia* cells for later transformation selection. Tests were conducted by subculturing 1 mL of log phase in *E. oleoabundans* culture into 15 mL of 3N-BBM+V + antibiotic at specific concentrations in 2 cm diameter test tubes. Antibiotics tested include spectinomycin (Sigma Aldrich, St. Louis MO: S4014) at 5 μg/mL, 10 μg/mL, 25 μg/mL, 50 μg/mL, 100 μg/mL, and 200 μg/mL hygromycin (Phytotechnology Labs, Shawnee Mission, KS: H385) at 1 μg/mL, 5 μg/mL, 15 μg/mL and 400 μg/mL. Culture tubes were maintained on a shaker (100 rpm) at about 25 °C at an angle using slanted racks, and illuminated with continuous cool-white fluorescent light at about 30 μmol m⁻² s⁻¹. Optical density (OD) readings were taken at 540 nm daily for up to seven days as a measurement of cell culture density. Growth curves were plotted as OD using average values for each antibiotic concentration. Effective concentrations were determined by comparing the changes in OD for cultures ± antibiotic.
3.3 Transformation vector preparation

The 35s-GFP, and 35s-GUS cassettes from pGreen (Hellens et al., 2000) plasmids were ligated into the multiple cloning site of the pCAMBIA 1300 plasmid (a gift from Dr. Argelia Lorence, Arkansas Biosciences Institute, Jonesboro, AR). *E. coli* DHSα cells in -80 °C cryostasis containing
either pGreen 35s-GFP or 35s-GUS cassettes were thawed in LB media (Bertani, 2004; Appendix: Formulations) and cultured at 37 °C overnight. The plasmids were extracted from DHSα cells using the standard alkaline lysis protocol and then ethanol precipitated (http://preuss.bsd.uchicago.edu/protocols/Alkaline.html). Presence of the plasmids was verified by loading 1 μL of each sample onto a 1% agarose electrophoretic gel run for 60 min at 90 V. Each cassette was spliced out of its pGreen backbone with EcoRV (blunt cut) restriction enzyme (New England Biolabs, Inc., cat# R0195S) in 30 μL digest volumes (1 μL of 20 units/μL EcoRV, 3 μL DNA sample, 3 μL NEBuffer 3, 4 μL BSA, and 28 μL reagent grade diH2O) incubated for 90 min at 37 °C and then isolated by a 1% agarose electrophoretic gel extraction followed by phenol chloroform extraction and ethanol precipitation. GFP and GUS restriction maps are shown in Figure 4.

The pCAMBIA 1300 plasmid was digested with PvuII (blunt cut) restriction enzyme (New England Biolabs, Inc., cat# R0151S) in 30 μL digest volumes (1 μL of 5 units/μL PvuII, 4 μL DNA sample, 3 μL NEBuffer 3, 4 μL BSA, and 27 μL reagent grade diH2O) incubated for 90 min at 37 °C, dephosphorylated with shrimp alkaline phosphatase (SAP) (1 μL of 1 unit/μL SAP and 10x SAP Buffer), incubated for 15 min at 37 °C, heat shocked at 65 °C for 15 minutes, and then isolated by a 1% agarose electrophoretic gel extraction followed by phenol chloroform extraction and ethanol precipitation. See Figure 5 for the restriction map of the pCAMBIA 1300 plasmid.

### 35S-GFP

![35S-GFP Diagram](image)

### 35s-GUS

![35s-GUS Diagram](image)

Figure 4: Restriction maps for 35s-GFP & 35s-GUS EcoRV. The pGreen backbone is not included; map was generated using NEBCutter 2.0 (Vincze et al., 2003)
The 35s-GFP and 35s-GUS inserts were ligated into open, dephosphorylated pCAMBIA 1300 backbones at an approximate 3:1 molar ratio in 20 μL working volumes with T4 DNA ligase (1 μL T4 DNA Ligase, 10x T4 DNA Ligase Buffer, and reagent grade DI H2O), and incubated overnight at 15 °C.

Ligands were transformed into heat competent E. coli and cultured in LB + kanamycin (kanamycin resistance is conveyed by pCAMBIA 1300) overnight at 37° C and subsequently streaked onto LB Agar (1.5% w/v) + kanamycin plates for transformant selection. Surviving colonies were then picked and cultured again in LB + 100 μg/mL kanamycin overnight at 37 °C. The constructs were isolated from the cells by standard alkaline lysis protocol (Appendix: Protocols) and ethanol extraction (70% ethanol extraction followed by 100% ethanol extraction). Because the kanamycin resistance gene is part of the pCAMBIA 1300 backbone, selection with kanamycin does not ensure proper ligation of the inserts into the backbone. Therefore, construct digests with EcoRV were run on 1% agarose electrophoretic gels for 60 min at 90 V alongside a pCAMBIA 1300 PvuII digest, and 35s-GFP and 35s-GUS EcoRV digests to verify that proper constructs were present. Once verified, cells were prepared in glycerol solutions and put into cryogenic stasis with liquid nitrogen and stored at -80 °C.
3.4 Life cycle synchronization of *Ettlia* to improve transformation

To optimize transformation efficiency, *Ettlia oleoabundans* cells in culture were subjected to a light/dark cycle or nitrate starvation in an attempt to synchronize their life cycles. Zoospores (youngest stage) have extremely thin cell walls as reported for *E. oleoabundans* by Watanabe and Floyd (1988). Selection of zoospores could eliminate the need for cell wall digestion and enhance transformation. Synchronized cultures under light/dark cycles were maintained as previously described in Section 3.1 using the culture systems shown in Figure 2 and 3.

Cells grown under 24 hour 12:12 light/dark (L/D) cycle were sampled hourly over a 24 hour period after the cells had been entrained either 4 or 10 days at 28 °C or 32 °C, respectively. Using a phase contrast microscope counts of total number of cells, number of large cells, and number of cells containing zoospores were conducted for each sample during a 24 hour L/D cycle to determine when the cells released zoospores.

In nitrate starved cultures, *E. oleoabundans* was grown until nitrate in the medium was depleted. Medium nitrate content was indirectly determined by monitoring culture density at OD540nm. Nitrate depletion is marked by a halt in growth, as previously found by Pruvost et al. (2009). Following nitrogen starvation by 48 hours, cultures were re-suspended in N replete media and growth was monitored hourly. When a sudden change in OD (540nm) was observed, cultures were harvested quickly by centrifugation at 5,000 x g for 5 minutes, resuspended in 1mL of medium, and electroporated as described in Section 3.7.

3.5 Protoplast formation

*Ettlia oleoabundans* cells in log phase growth were subjected to cell wall digestion with 1% cellulase (w/v), 1% macerase (w/v), and 1% pectinase (w/v) in 0.4M D-sorbitol (Sigma Aldrich, St. Louis MO: S1876) for 24 hours at room temperature under gentle agitation. Following digestion, cell samples were centrifuged at 100xg for 10 minutes, washed with 0.4M D-sorbitol, and were stained with 1 volume of 0.1% calcofluor (fluorescent brightener 28; excitation 365nm; emission 435nm; Sigma Aldrich, St. Louis MO: F3543) for 1 hour. After staining, cells were centrifuged at 100 x g and washed twice in 0.4M D-sorbitol, and assayed for successful protoplast formation under UV fluorescent microscopy.
3.6 Electroporation optimization

Electroporation is a relatively inexact science but parameters for field strength (V/cm) and capacitance (μF) can be determined to optimize the transformation process. “Dry” or “blank” electroporation of 1 mL of algal cells in BBM media was tested with field strengths of 250, 350, 450, 550, 650, 750, and 850 V/cm at capacities of 10, 50, or 60 μF. Cell viability was assayed by measuring growth at 540nm for each sample over time after electroporation and comparing their growth curves to growth curves of two cultures that were not electroporated; one represents 100% cell viability and another is diluted 2 fold to represent 50% cell viability. Parameter combinations that resulted in cell viabilities near 50% were determined to be more optimal (Life Technologies; manufacturer's recommendation).

3.7 Transformation by electroporation and selection

Culture samples were taken in log phase growth, concentrated by centrifugation at 4,000 x g for 10 minutes to a factor of about 5 and electroporated with 600, 800, and 1000 V/cm at 10 μF with 2.5μg/mL DNA. Light/dark synchronized samples were taken from the synchronized culture at the transition from light to dark (12th hour in 24 hour cycle), 30 min after the transition, and 1 hour after the transition; these were all times where zoospore concentration appeared to be highest. Nitrate starved synchronized samples were taken at first sign of reproduction. Both types of synchronized cultures were then electroporated as previously described. Electroporated samples and one non-electroporated sample (control) were then cultured for approximately 4 days on BBM plates (75% of sample on plates with 20 μg/mL hygromycin; 25% of sample volume on control plates). Cultures were deemed to be successfully transformed if they showed hygromycin resistance conveyed by the hygromycin resistance gene (HYG) in the pCAMBIA 1300 backbone of each construct. Surviving colonies were assayed for GUS and GFP (excitation 395 nm; emission 509 nm) activity.

3.8 Statistical analysis

Experiments were run in triplicate when applicable. Mean values were plotted for each triplicate in cartesian coordinate figures. Error bars presented in these figures represent Standard Error.
4 Results & Discussion

In the development of a transformation protocol for *E. oleoabundans*, use of electroporation was explored. Different approaches to achieving successful transformation and increasing the efficiency of transformation included protoplast formation, and reproductive cycle synchronization.

4.1 Transformant selection antibiotics

Before developing any transformation method, a means of selecting for transformants had to be established. The effect of two antibiotics at different concentrations were tested on growth of *E. oleoabundans*, spectinomycin and hygromycin in liquid culture. Hygromycin inhibited growth, even at 1 μg/mL (Figure 6). There was significant bleaching of cells, which should make it easy to select for transformed colonies on plates (Figure 6D and E). Spectinomycin was also found to be effective at inhibiting cell growth of *E. oleoabundans* (Figure 6F), however, hygromycin was chosen for further work because its effective inhibition at extremely low concentrations and we had ready access to the hygromycin resistant gene (HYG) in the pCAMBIA 1300 plasmid (Figure 5).

4.2 Transformation vector verification

Transformation vectors were constructed from a pCAMBIA 1300 backbone and either a 35s-GFP or 35s-GUS insert ligated into the pCAMBIA multiple cloning site. All restriction digests were blunt cuts, so the cut pCAMBIA 1300 backbones were dephosphorylated prior to ligation. Figure 7 shows the reverse digestion of the newly ligated pCAMBIA 35s-GFP and pCAMBIA 35s-GUS constructs, with bands accounting for pCAMBIA 1300 and either 35s-GFP or 35s-GUS, indicating successful vector construction.

4.3 Formation of protoplasts

Commonly, the approach to transforming microalgae, and higher plant cells, by electroporation is by formation of protoplasts (Coll, 2006). *E. oleoabundans* cells in log phase growth were subjected to cell wall digestion with 1% cellulase (w/v), 1% macerase (w/v), and 1% pectinase (w/v). Following digestion, cell walls were stained with calcofluor (fluorescent brightener 28; excitation 365nm; emission 435nm; Sigma Aldrich, St. Louis MO: F3543) to assay for successful protoplast formation. Cells stained in absence of enzyme show fluorescent activity surrounding the cell indicating the presence of cellulose while cells stained after 24 hours in the presence of enzyme show little to no
Figure 6: Effect of antibiotics on *Ettlia* growth. Hygromycin at (A) 400, (B) 15, 5, and (C) 1 μg/mL vs. 0 μg/mL; (D) 400 μg/mL (3 right) and 0 μg/mL (3 left) day 1; (E) 400 μg/mL (3 right) and 0 μg/mL (3 left) day 4. Spectinomycin at (F) 200, 100, and 50 μg/mL vs. 0 μg/mL.
fluorescent activity (Figure 8). Figure 8 shows calcofluor staining revealed probable protoplast formation where blue rings (colored by computer) surrounding cells are absent after enzymatic digestion.

Figure 7: Reverse digest of pCAMBIA35s-GFP and pCAMBIA35s-GUS constructs for verification of proper ligation. Lane 1: HyperLadder 100; lane 2: pCAMBIA 1300 PvuII Digest (8,636bp); lane 3: 35s-GFP EcoRV digest (1,394bp); lane 4: 35s-GUS EcoRV digest (2,501bp); lane 5: pCAMBIA 1300 35s-GUS digest with EcoRV; lane 6: pCAMBIA 1300 35s-GFP digest with EcoRV.

Figure 8: *E. oleoabundans* cells stained with calcofluor in (A) absence of enzyme and (B) presence of enzyme. Blue ring around cell (yellow arrow) in A indicative of cellulose, while in B the arrow shows no visible blue ring, which indicates a probable protoplast. Large fluorescent structures are calcofluor crystals or stained cell aggregates.
4.4 Electroporation tests on *E. oleoabundans*

Electroporation tests were conducted to determine optimal parameters for capacitance (μF) and field strength (V/cm) to achieve high transformation efficiencies. Parameters that result in approximately 50% cell viability post electroporation usually yield greater transformation efficiencies (Life Technologies). Log phase cells were electroporated using field strengths of 250, 350, 450, 550, 650, 750 or 850 V/cm at capacities of 10, 50, and 60 μF to determine which combination would yield a percent viability around 50%. Figure 9 shows the effect of electroporation of *E. oleoabundans* at 850 V/cm and 10 μF on subsequent cell growth (measured at OD540nm) against non electroporated culture (representing 100% cell viability) and non electroporated culture diluted two-fold (representing 50% cell viability). Cultures electroporated with 850V/cm at 10 μF produced the lowest cell viabilities relative to cultures electroporated with every other combination (not shown here for simplicity). Electroporating log phase growth *E. oleoabundans* with 850V/cm at 10 μF, though closer to the 50% cell culture than every other combination, does not produce cell viabilities near 50%, which indicates the strength and integrity of the cell wall of this species.

![](image)

Figure 9: The effect of electroporation with 850V/cm at 10 μF on cell viability and subsequent growth of *E. oleoabundans* relative to 100% and 50% cell cultures.
4.5 *Ettlia oleoabundans* reproductive cycle synchronization by 1:1 light/dark cycle

Given the apparent resilience of mature *E. oleoabundans* cells to electroporation, a creative means of acquiring cell wall deficient clones was explored. Though little is known about the reproductive habits of *E. oleoabundans*, it is known that the species does produce zoospores which have extremely thin cell walls (Watanabe and Floyed, 1988; Komarek, 1989). Algae can be synchronized with a light/dark cycle to release zoospores more homogeneously. Cultures of *E. oleoabundans* were, therefore, grown to mid log phase on a 12:12 hour light/dark cycle and were sampled hourly for 24 hours. At each sample point, total cell counts the following were counted in triplicate: total cells, large cells, and cells containing putative zoospores. Figure 10 shows the percentage of large cells and cells with zoospores at each hour for a 24 hour L/D period.

The data in Figure 10A represents sampling after 4 days in the light/dark cycle. There was no meaningful change in culture density at any time during the 24 hour period, but there was a distinct increase in the percentage of large cells and cells containing zoospores around the 12th hour of the cycle, at the transition from light to dark. Thus the second trial, represented in Figure 10B, was conducted after 10 days to lengthen the entrainment period in the light dark cycle. Samples were taken every half hour from 9 hours to 13.5 hours. There was an increase in culture density over the preceding hours at about 0.5 hours after the transition to the dark part of the cycle (12.5 hours), accompanied by about a 0.6% increase in the number of cells containing putative zoospores. However, the total average cell count only increased from about 175 cells grid\(^{-1}\) to about 227 cells grid\(^{-1}\) (about 23% increase) and the percentage of these cells which contain zoospores only reached 0.87%. This suggested that, while some synchronized reproduction may be occurring, it only represents a small percentage of the total population during a given 24 hour period. Nonetheless, the higher than normal percentage of zoospores in culture during the shift into the dark could yield greater transformation efficiencies than with normal log phase growth cultures, and transformation was tested using cells harvested at 12.5 hours. Unfortunately, no transformants were obtained.

4.6 *Ettlia oleoabundans* reproductive cycle synchronization by nitrate starvation

Nitrate starvation was also used to synchronize *E. oleoabundans* in order to improve transformation efficiencies. *E. oleoabundans* cultures were grown until nitrate in the medium
Figure 10: Synchronization of E. oleoabundans reproduction with 12:12 hour light/dark cycle. Red and blue curves, respectively, indicate the percentage of total cells containing putative zoospores and those cells that are larger than average, but without apparent zoospores (left y axis); yellow curve depicts total average cell count (right y axis). (A) cells sampled hourly for 24 hours after 4 days on cycle (exhaust flap closed; 28 °C in dark, 32 °C in light); (B) cells sampled every half hour after 10 days on cycle (exhaust flap open; 28 °C).
was depleted and were then resuspended in nitrate replete medium after 48 hours. Growth was monitored hourly after nitrate repletion. Unfortunately, the time at which growth begins after nitrate repletion has not yet been determined, and transformation of cells at this point has not yet been attempted. However, data gathered during this experiment do indicate that nitrate starvation may induce synchronization, as no apparent growth takes place for more than 24 hours after nitrate repletion (Figure 11), yet 62 hours later cultures achieved higher cell densities of 0.894 OD.

Figure 11: Growth measured at OD 540nm for nitrate starved cells after nitrate repletion.

5 Conclusions & Future Work

Attempts to develop a method for the genetic transformation of *E. oleoabundans* used electroporation and not other approaches (such as glass bead agitation), mainly because of the tough cell wall in this species. As shown in Figure 7, protoplasts were apparently formed by enzymatic digestion, with at least partial digestion of the cell wall, the first successful apparent production of protoplasts in this species. However, transformation of *Ettlia* protoplasts by electroporation did not yield transformants. Life cycle synchronization was attempted with a 24 hour 1:1 light/dark cycle and nitrate starvation to increase the concentration of zoospores in culture and take advantage of their thin cells walls; it remains to be determined if this synchronization method will result in transformation. As shown in Figure 10, the 24 hour 1:1 light/dark cycle synchronization results in only a 23% increase in total culture density. Although the increase in cell density takes place at the same time during the 24 hour cycle (from the 12th-13th hours), which suggests there may be a light/dark synchronizing effect, the percentage increase indicates that only a small percentage of the population may undergo reproduction.
Though transformation of nitrate depletion synchronized cells has not been completed to date, data presented in Figure 10 gathered during this experiment do indicate that nitrate starvation may induce synchronization, as no apparent growth takes place for more than 24 hours after nitrate repletion.

Future studies could explore the use of different light/dark cycles. For example a 16:4, 24 hour light dark cycle or even cycles longer than 24 hours could be used to produce populations with very high zoospore concentrations, and these may be more amenable to transformation. Though less conventional, the use of a freeze-thaw technique, which has been used in the transformation of other organisms such as Agrobacterium tumefaciens (Hellens et al., 1999; An et al., 1988), could also be a simple and effective means of transforming Ettlia. As was shown with C. reinhardtii, (Kumar et al., 2004) A. tumefaciens may also prove an effective means of transforming E. oleoabundans with cell walls. Experimenting with A. tumefaciens could be accomplished using the pCAMBIA 35s-GFP and 35s-GUS plasmids, as they possess a second origin of replication for this species. While not used directly as a method of transformation, it has been reported that sonication can be used to pass macromolecules into cell walled microalgae where electroporation cannot (Azencott et al., 2007). Either direct sonication of cells in the presence of exogenous DNA or sonication of cells followed by electroporation in the presence of exogenous DNA could prove a unique and reproducible transformation methodology. Finally, though it has not been reported as a method of transformation in other algal species, chemical transformation of Ettlia protoplasts with PEG or commercially available kits requires very little DNA and no specialized equipment.

The effort this report entails represents the first of many in an ongoing project to help bring clean and sustainable liquid fuels to market by working to reduce the prohibitive costs the nascent algal biofuels industry faces. The work presented herein has focused on establishing an efficient and reproducible method for the transformation of Ettlia oleoabundans, which could be used to engineer the species' metabolism and thereby increase biofuel productivity. This project will be continued with the aim of not only developing a transformation method for Ettlia but also other potential biofuel algal species.
References


Appendix: Formulations

Bold's Basal Medium with 3-fold Nitrogen and Vitamins (3N-BBM+V; modified; Bischoff and Bold, 1963)

<table>
<thead>
<tr>
<th>Stock solutions in g/L water</th>
<th>for 1L final medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) 25.0 g NaNO₃</td>
<td>30.0mL</td>
</tr>
<tr>
<td>(2) 2.5 g CaCl₂·2H₂O</td>
<td>10.0mL</td>
</tr>
<tr>
<td>(3) 7.5 g MgSO₄·7H₂O</td>
<td>10.0mL</td>
</tr>
<tr>
<td>(4) 7.5 g K₂HPO₄·3H₂O</td>
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</tr>
<tr>
<td>(5) 17.5 g KH₂PO₄</td>
<td>10.0mL</td>
</tr>
<tr>
<td>(6) 2.5 g NaCl</td>
<td>10.0mL</td>
</tr>
<tr>
<td>(7) trace element solution (below)</td>
<td>6.0mL</td>
</tr>
<tr>
<td>(8) vitamin B₁ (see below)</td>
<td>1.0mL</td>
</tr>
<tr>
<td>(9) vitamin B₁₂ (see below)</td>
<td>1.0mL</td>
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</table>

Make up to 1 litre with distilled water. Autoclave at 15 psi for 15 min.

Trace element solution (number 7 above)
Add to 1L of distilled water 0.75 g Na₂EDTA and the minerals below in exactly the following sequence:

- FeCl₃·6H₂O 97.0 mg
- MnCl₂·4H₂O 41.0 mg
- ZnCl₂·6H₂O 5.0 mg
- CoCl₂·6H₂O 2.0 mg
- Na₂MoO₄·2H₂O 4.0 mg

Vitamin B₁ (number 8 above)
0.12 g Thiaminhydrochloride in 100 mL distilled water. Filter sterile.

Vitamin B₁₂ (number 9 above)
0.1 g cyanocobalamin in 100 mL distilled water, take 1 ml of this solution and add 99 mL distilled water. Filter sterile.
# Appendix: Data

**E. oleoabundans** light/dark cycle cell count data

Table 4: Light/dark cycle cell counts from hour 15.5-19.5 in 24 cycle after 10 days.

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<th>Count Types</th>
<th>330</th>
<th>4</th>
<th>430</th>
<th>5</th>
<th>530</th>
<th>6pm (light-dark)</th>
<th>630</th>
<th>7</th>
<th>730</th>
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<td>16.5</td>
<td>17</td>
<td>17.5</td>
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<td>18.5</td>
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<td>0</td>
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<td>0</td>
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<td>0</td>
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<tr>
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<tr>
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