Project Title: Flue gas carbon dioxide capture with rapid growth algae to produce biodiesel and other renewable fuels

Contract Number: RD3-2    Milestone Number: 3    Report Date: 15 December 2010

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Congressional District: 6th Congressional District

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MILESTONE 3 REPORT

EXECUTIVE SUMMARY

In meeting Milestone #3 objectives, our activities have focused on the large scale cultivation of the algae species Dunaliella tertiolecta in order to accumulate enough algae (and subsequently algae oil) for conversion into biodiesel. We have grown, harvested and dried 7141 grams (on a dry matter basis) of Dunaliella tertiolecta for this phase of the project.

The cultivation of Dunaliella tertiolecta was scaled up from small, 1.5 liter photo bioreactors to 55 gallon aquariums (typically containing 190 – 200 liters) and also to 275 gallon totes which were maintained with a liquid level of approximately 300 liters. This technique allowed for a more realistic evaluation of the algae growth, carbon sequestration and accumulation of sufficient biomass from which lipids could be extracted.

Outdoor lighting was used for growth in one aquarium and 2 totes. These growth vessels were maintained outdoors under natural light with an average mid-summer photoperiod of 15 hours light and 9 hours dark (per one 24 hour period). Indoor lighting was employed for three aquariums and one tote. These growth containers were maintained indoors under artificial light with a 24 hour continuous light photoperiod. All growth aquariums received approximately 200 ml/minute of air that contained 5.6% carbon dioxide during periods of light and only air during periods of darkness. The totes received the same mixtures during the same periods only at slightly higher flow rates of 300 ml/minute.

It was noted that the size of the Dunaliella tertiolecta cells decreased as their growth rate increased. During fast growth the cells would divide into four, eight or sixteen new cells. This rapid division resulted in smaller cells with a typical diameter of 3 to 5 microns. Prior to the rapid division (fast growth), the cells typically divided into two new cells that were larger with typical diameters in the 8 – 12 micron range.
The average growth rate for the Dunaliella tertiolecta is 0.0790 grams per liter per day (on a dry matter basis). The carbon dioxide utilization can vary widely due to gas flow rates, tank volume and other factors; a typical example indicates a carbon dioxide capture efficiency of approximately 85%.

It was found that harvesting the algae when the optical density is at 0.8 yields a higher percentage of neutral lipids (9.65%) than when the algae is harvested at a lower optical density such as 0.4 (6.49% neutral lipid). Neutral lipid is desirable for conversion into biodiesel.

The dried Dunaliella tertiolecta algae is currently being pulverized with a small grain mill (burr mill type). This mill grinds the dried algae into particles ranging from 1 to 10 microns in size (see Pictures 5-7 in Appendix A).

A dried sample of Dunaliella tertiolecta algae was analyzed for composition by Minnesota Valley Testing Labs (New Ulm, MN). The results are shown in Table 1. The fat content of 4.02% is slightly low compared to the samples analyzed at SarTec on other occasions which typically ranged between 6% and 10%. The somewhat high optical density for this sample most likely indicates that more carbon than normal was being converted into starch (a storage product) instead of lipids at this stage of growth.

TECHNICAL PROGRESS

M3 Goal 1: Onsite demonstration of cultivation techniques to RDF administration
Algae cultivation and harvesting techniques have been demonstrated to an RDF administration representative (Mark Ritter).

M3 Goal 2: Provide sample of algae biomass
Samples of algae paste (1) post centrifugation harvesting and (2) post drying and grinding have been sent to an RDF administration representative (Mark Ritter).

M3 Goal 3: Algae composition analysis
An elemental analysis of a dried sample of Dunaliella tertiolecta was performed by Minnesota Valley Testing Labs (New Ulm, MN). The sample was harvested from aquariums ST31-29P, -29L, -29M and -29Q on the 2nd of August, 2010. The optical densities of the algae cultures were 0.99, 1.52, 1.35 and 1.00 respectively. The fat content of 4.02% is slightly low compared to the samples analyzed at SarTec on other occasions which typically ranged between 6% and 10%. The somewhat high optical density for this sample most likely indicates that additional carbon was converted into starch (a storage product) at this stage of growth instead of lipids. When the growth rate is rapid (at lower optical densities), more of the carbon goes into the cell membrane as lipids. Table 1 shows the elemental analysis composition results.
Table 1. Analysis of Dunaliella tertiolecta (centrifuged & dried), harvested 8-2-2010.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>10.10 %</td>
</tr>
<tr>
<td>Ash</td>
<td>7.70 %</td>
</tr>
<tr>
<td>Calcium</td>
<td>1100 ppm</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>33.98 %</td>
</tr>
<tr>
<td>Copper</td>
<td>275 ppm</td>
</tr>
<tr>
<td>Fat (ethyl ether)</td>
<td>4.02 %</td>
</tr>
<tr>
<td>Fiber, crude</td>
<td>4.24 %</td>
</tr>
<tr>
<td>Iron</td>
<td>7520 ppm</td>
</tr>
<tr>
<td>Magnesium</td>
<td>3000 ppm</td>
</tr>
<tr>
<td>Manganese</td>
<td>92 ppm</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>9600 ppm</td>
</tr>
<tr>
<td>Potassium</td>
<td>11 ppm</td>
</tr>
<tr>
<td>Protein</td>
<td>44.20 %</td>
</tr>
<tr>
<td>Sodium</td>
<td>9000 ppm</td>
</tr>
<tr>
<td>Zinc</td>
<td>113 ppm</td>
</tr>
</tbody>
</table>

Figure 1. Lipid profile of *Dunaliella tertiolecta* separated and identified by GC-MS. Peak 1: Hexadecadienoic acid methyl esters. Peak 2: Hexadecatrienoic acid methyl esters. Peak 3: Hexadecanoic acid methyl esters. Peak 4: Octadecadienoic acid methyl ester. Peak 5: Octadecatrienoic acid methyl ester
As detailed in the Milestone #2 report, lipid was extracted from the Dunaliella tertiolecta algae (and four other algae species) and prepared for gas chromatography-mass spectrometry analysis. The results are shown in Figure 1. The Dunaliella tertiolecta algae lipids analyzed are primarily comprised of fatty acids that contain sixteen and eighteen length carbon chains with varying degrees of saturation (Hexadecanoic acid and Octadecadienoic acid methyl esters). These observations are in agreement with published results on algae lipid profiles. The extraction methodology, sample preparation and GC-MS analysis are described in Appendix B.

M3 Goal 4: Preliminary results of algae culture tests and preservation processes

We have grown, harvested and dried 7141 grams (on a dry matter basis) of Dunaliella tertiolecta for this phase of the project. This gives us enough algae biomass to extract a sufficient quantity of algae oil for the next phase of the project (biodiesel production with the Mcgyan process). The cultivation of Dunaliella tertiolecta was scaled up from small, 1.5 liter photo bioreactors to 55 gallon aquariums (typically containing 190 – 200 liters) and also to 275 gallon totes which were maintained with a liquid level of approximately 300 liters. This technique allowed for a more realistic evaluation of the algae growth, carbon sequestration and accumulation of sufficient biomass from which lipids could be extracted.

Outdoor lighting was used for growth in one aquarium and 2 totes. These growth vessels were maintained outdoors under natural light with an average mid-summer photoperiod of 15 hours light and 9 hours dark (per one 24 hour period). Indoor lighting was employed for three aquariums and one tote (Appendix A pictures 1-2). These growth containers were maintained indoors under artificial light with a 24 hour continuous light photoperiod. All growth aquariums received approximately 200 ml/minute of air that contained 5.6% carbon dioxide during periods of light and only air during periods of darkness. The totes received the same mixtures during the same periods only at slightly higher flow rates of 300 ml/minute. Initially the algae are maintained in glass slant tubes containing a gelatinous agar and nutrient mixture. Algae grown in slant tubes are used to inoculate small (250 – 300 ml) flasks (the flasks receive a small, continuous flow of carbon dioxide during the lighted part of their photoperiod -15 hrs light/9 hours dark). After the optical density increases the algae cultures are transferred to larger flasks and finally, actively growing algae from the larger flasks are used to inoculate the aquariums and totes.

The indoor, fluorescent lighting used in this project has significantly less intensity than sunlight. During sunny, winter days the typical light readings measured are 30,000 – 33,000 Lux (1) or 700 – 800 umol/sq. meter/second (2). During the summer, outdoor light readings can vary widely but 1000 – 2000 umol/sq. meter/second are typical. The indoor fluorescent lights located around the aquariums emit 13,460 Lux on average and when measuring light from inside the aquariums readings are approximately 4300 Lux. If the output of the fluorescent bulbs is measured with the FIELDSCOUT quantum light meter, readings of 280 – 300 umol/sq. meter/second are observed.

It was noted that the size of the Dunaliella tertiolecta cells decreased as their growth rate increased. During fast growth the cells would divide into four, eight or sixteen new cells. This rapid division resulted in smaller cells with a typical diameter of 3 to 5 microns (Appendix A picture 3). Prior to the rapid division (fast growth), the cells typically divided into two new cells that were larger with typical diameters in the 8 – 12 micron range.
**Productivity**

Dunaliella tertiolecta algae were grown in aquariums both outside and inside for the purpose of comparing the growth rates under natural light and artificial light. The outdoor aquarium was treated identically to the indoor aquariums with the exception that it only received natural sunlight whereas the indoor aquariums received artificial light with a 24 hour photoperiod. The outdoor aquarium was also subject to uncontrollable variables such as temperature fluctuations, rain and variations in light intensity (clouds, etc…).

Dunaliella tertiolecta was harvested from an indoor aquarium at three specific optical densities (O.D. = 0.4, 0.6 and 0.8) to compare the lipid content and determine whether there is any benefit to harvesting earlier or later in the growth cycle. The results are presented in Table 2. A higher neutral lipid content (9.65%) was found for the algae harvested at O.D. = 0.8

**Table 2.** Analysis of algae residue after soxhlet extraction of lipid sampled at different optical densities.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Dried Algae Mass (g)</th>
<th>Lipid Mass (g)</th>
<th>Neutral Lipid (%)</th>
<th>Optical Density</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST29-153</td>
<td>80.29</td>
<td>5.21</td>
<td>6.49</td>
<td>0.40</td>
</tr>
<tr>
<td>ST29-150</td>
<td>82.00</td>
<td>5.49</td>
<td>6.69</td>
<td>0.60</td>
</tr>
<tr>
<td>ST29-152</td>
<td>81.23</td>
<td>7.84</td>
<td>9.65</td>
<td>0.80</td>
</tr>
</tbody>
</table>

Exponential growth was measured in the Dunaliella tertiolecta aquariums. This high growth typically occurs when the optical density of the algae solution is between 0.20 and 0.50. Data was collected from the outdoor aquarium (ST31-29F) on 5-18 and 5-19-2010 at 1 – 2 hour intervals during conditions of full sunlight. The short-term growth rate under this lighting condition varied from 0.543 to 0.814 grams per liter per day (dry matter basis – highlighted yellow in Table 3). This exceeds the best short-term growth rates measured in the indoor aquariums which were grams per liter per day (dry matter basis). This result demonstrates that there is good potential for rapid algae growth outdoors when conditions are favorable. The results of this experiment are shown in Table 3 and Figure 2.

**Table 3.** Experimental growth rates for Dunaliella tertiolecta in an outdoor aquarium.

<table>
<thead>
<tr>
<th>Day</th>
<th>Optical Density</th>
<th>Growth Rate (g/l day)</th>
<th>Cumulative Growth Rate (g/l day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50.00</td>
<td>0.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>51.00</td>
<td>0.68</td>
<td>0.0651</td>
<td>0.0651</td>
</tr>
<tr>
<td>51.13</td>
<td>0.78</td>
<td>0.2895</td>
<td>0.3546</td>
</tr>
<tr>
<td>51.19</td>
<td>0.84</td>
<td>0.3341</td>
<td>0.6887</td>
</tr>
<tr>
<td>51.23</td>
<td>0.86</td>
<td>0.1810</td>
<td>0.8697</td>
</tr>
<tr>
<td>51.27</td>
<td>0.94</td>
<td>0.7238</td>
<td>1.5935</td>
</tr>
<tr>
<td>51.31</td>
<td>1.00</td>
<td>0.5429</td>
<td>2.1364</td>
</tr>
<tr>
<td>51.35</td>
<td>1.09</td>
<td>0.8143</td>
<td>2.9507</td>
</tr>
</tbody>
</table>
Carbon sequestration

A contributing factor in choosing Dunaliella tertiolecta as the species to focus on in this research was that a large percentage of the carbon sequestered by Dunaliella tertiolecta algae is incorporated into the cell membrane of this species (D. tertiolecta doesn’t have a cell wall). With most other species, about 40% - 50% of the carbon is incorporated into the cell wall structure of the algae. From the perspective of harvesting lipids from algae, having the carbon stored in cell wall structures is less desirable because it is more difficult to extract the lipids from algae species with cell walls as opposed to cell membranes. The overall average growth rate for Dunaliella tertiolecta in these experiments (which is the sum of growth achieved over the duration of the experiment divided by the total days) was found to be 0.0790 (gramps per liter per day).

The potential for sequestering carbon dioxide by Dunaliella tertiolecta is shown in Table 4. The data is based on average growth rates in indoor vessels employing fluorescent lighting.

Table 4. Average carbon dioxide capture by Dunaliella tertiolecta algae.

<table>
<thead>
<tr>
<th>Species</th>
<th>CO₂ Capture Based on Harvest (tons/acre year)</th>
<th>CO₂ Capture Based on Optical Density (tons/acre year)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dunaliella tertiolecta</td>
<td>42.12</td>
<td>61.77</td>
</tr>
</tbody>
</table>

Note: Based on: 365 days/year production, water depth =0.30 meters and 100% capture.
**Carbon budget**

An example of carbon dioxide capture efficiency can be demonstrated with the results from outdoor aquarium ST31-29F which had the optical density increase from 0.25 to 0.73 over a period of three days (July 13 – 16, 2010). This equates to a growth rate of 0.0601 grams per liter per day (on a dry matter basis). The liquid volume in this vessel was 190.9 liters and the gas flow was set at 200 ml/minute for 900 minutes/day with 5.6% carbon dioxide. Carbon dioxide weighs 1.98 grams/liter. It takes approximately 1.6 grams of carbon dioxide to grow 1 gram of algae (dry matter). The total carbon dioxide input was 21.35 grams/day and the amount used by the algae was 18.36 grams; which results in an 85.98% capture efficiency. This number will vary, but it implies that part of the carbon dioxide input passes right through to the atmosphere.

**Harvesting, preservation and pulverizing**

Algae cultures that have an OD450 (3) reading of 0.8 or greater are pumped out of their vessel and into a holding tank after which the solution is fed by gravity into a solids-retaining centrifugal separator (Alfa- Laval model 104B). The flow rate of liquid through the centrifuge is 1.0 – 1.5 liters/minute; higher flow rates reduce capture of algae cells. Typically, if the inflow has an optical density of 1.32 the outflow will be approximately 0.12. The captured algae paste (Appendix A picture 4) is then removed, weighed, dried in a food dehydrator (for 18 – 20 hours at 135 degrees F) and re-weighed; after which the percent dry matter is calculated. This new drying procedure was implemented on 2/19/2010 to help minimize changes that could potentially take place in the lipid content of the algae paste if it was stored in a refrigerator prior to drying. Dried algae specimens were then pulverized in a coffee grinder. Initially, each batch of dried algae was further pulverized in a ball mill (rock tumbler) for 5 days. After 5 days, ground, dried algae were collected and weighed for record. This particular process was slow. With larger quantities of algae to pulverize, a small grain mill (burr mill type, Country Living Products, Stanwood, WA) is currently being used. This mill grinds the dried algae into particles ranging from 1 to 10 microns in size (see pictures 5-7 in Appendix A).
MILESTONES
We have completed Milestone 3 and have started progress on Milestone 4.
The primary goals for Milestone 4 are:
M4 Goal 1: Develop algae oil extraction process
M4 Goal 2: Harvest algae biomass and extract algae oil
M4 Goal 3: Produce biodiesel from extracted algae oil through the Mcglyan process
M4 Goal 4: Optimize Mcglyan reactor operating parameters
M4 Goal 5: Test algae biodiesel for ASTM 6751 specifications

PROJECT STATUS
The project is on budget and ahead of schedule.

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PROJECT FUNDING PROVIDED BY CUSTOMERS OF XCEL ENERGY THROUGH A GRANT FROM THE RENEWABLE DEVELOPMENT FUND.
APPENDIX A

Picture 1. A photograph of Dunaliella tertiolecta algae grown with natural light outside (aquarium and tote).
Picture 2. A photograph of Dunaliella tertiolecta algae grown with artificial light inside (aquariums).
Picture 3. A microscope photograph of Dunaliella tertiolecta algae cells (green) and 3 micrometer diameter zirconia beads (clear/white). This shows the approximate size of the algae cells to be 3 microns in diameter.
**Picture 4.** Dunaliella tertiolecta algae paste post harvesting with the centrifuge (not dried yet).
Picture 5. A photograph of the mill used for pulverizing the dried Dunaliella tertiolecta algae.
Picture 6. A photograph of the pulverized Dunaliella tertiolecta algae post mill grinding.
Picture 7. A microscope photograph of pulverized Dunaliella tertiolecta algae post mill grinding, approximate size range 1-10 micrometers.
APPENDIX B

Dunaliella tertiolecta lipid extraction and preparation procedure for GC-MS

Extraction methodology
There are a significant number of methodologies for lipid extraction. In exploring for the ideal extraction method, we have tested the well-known Bligh and Dyer extraction as well as the Folch extraction which employs chloroform and methanol on wet algae paste. These methods have the benefit of allowing direct extraction of lipid from a wet sample, however, they call for a large quantity of extraction solution and create a significant amount of waste solvent. Since our goal is to be able to process large quantities of algae in an economical and environment-friendly way, our exploration eventually led us to the Soxhlet extraction method which utilizes hexane as the extraction solvent. Soxhlet extraction involves refluxing a solvent to repeatedly wash and extract the sample, then the lipid is obtained by evaporating off the solvent. The benefits of using this extraction method include: (1) the extraction solvent (hexane) can be recycled and reused, (2) hexane is easier to handle than the more toxic chloroform and thus more environment friendly, and (3) the lipids obtained with this extraction method are neutral lipids that are ready for conversion into biodiesel via the Mcgyan process. Samples of algae were harvested and prepared for lipid extraction.

Lipid extraction
For Soxhlet extraction, eighty grams of ground algae were placed in a Whatman cellulose extraction thimble and setup in a Soxhlet extractor. A one liter round bottom filled with 800ml of hexane and some boiling stones was then attached to the bottom of the extractor. This was followed by attaching a water cooling coil tube to the top of the extractor with cold water running through to help condense the rising hexane vapor. Lastly, heat was applied and the extraction was allowed to run until the extracting hexane appeared colorless (typically 48 hours). The lipid containing hexane solution was filtered through activated carbon to remove pigments from the algae and then the hexane was removed using a rotary evaporator. This resulted in neutral algae lipids being obtained that were transparent and ranged from light yellow to amber in color.

Sample preparation for GC analysis
HCl catalyzed methanolysis of algae lipid was performed to prepare the sample for GC-MS (Gas Chromatography Mass Spectrometry) analysis. Approximately 4-5 mg of algal lipid was added to a small Teflon capped vial (2 mL) with 0.2 mL methanol in it. Next, 0.8 mL of 5% acetyl chloride in methanol was added to the vial. The vial was tightly capped and heated in a sand bath at 60 °C for 6 hours. After cooling, the contents of the vial were transferred to a test tube containing 1 mL saturated NaHCO3 and 2 mL pentane. The two layers were agitated by pipette until gas evolution ceased. The pentane solution was carefully decanted by pipette and transferred to another test tube and dried over a molecular sieve (4 Å). The pentane was evaporated by drying with a stream of air and the remaining residue was taken up in ~1 mL of HPLC grade methanol and analyzed by GC-FID (Flame Ionization Detector) and GC-MS.

Gas chromatography-mass spectrometry (GC-MS) analysis
The gas chromatograph electron impact mass spectrometer is a powerful and extremely useful instrument that combines two techniques to allow a single method of analyzing mixtures of chemicals. Through gas chromatography, the components of a mixture were first separated; subsequently the separated components were characterized and identified individually by mass
spectrometry. By combining these two techniques, this instrument not only allows qualitative and quantitative evaluation, but also identification of components in a mixture solution. Pertinent to this project, this instrument allows efficient identification of specific fatty acids contained in the various species of algae oil. In the future, this instrument will aid in qualitative and quantitative evaluation of the different chain length FAMEs in the biodiesel produced from the algae oil.

An Agilent 6890 gas chromatograph electron impact mass spectrometer was used for this analysis. One µL of sample solution was injected in splitless mode at a flow rate of 1.0 mL/min with helium as the carrier gas onto a 5% phenyl-methylpolysiloxane column (DB-5; 30m x .25 mm i.d.; 0.25 µm film thickness). The elution temperature program had an initial temperature of 50 ºC and then linearly ramped to 180 ºC at 15 ºC min⁻¹, then to 230 ºC at 2 ºC min⁻¹, and finally to 310 ºC at 30 ºC min⁻¹. The final temperature was held for 13.67 minutes (total run time = 50 min). Mass spectra were acquired using HP6890 MS software and peak identification was aided with the NIST MS library. The observed mass range was set from 37 to 800 amu to remove any solvent contribution.
APPENDIX C

References/Notes

(1) Milwaukee Instruments model SM700 submersible Luxmeter (light meter).

(2) Spectrum Technologies Inc. FIELDSCOUT quantum light meter (measures in umol/sq. meter/second).

(3) OD450 is the optical density of the sample measured at 450 nM wavelength using a spectrophotometer.