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Cellulosic-Derived Biofuels Program in Kentucky - Part II Final Report

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Executive Summary

A systematic and comprehensive approach was developed to provide the necessary foundation to initiate a commercial biofuels industry in central Kentucky. The overall concept was to utilize locally produced biomass to provide an energy source to grow algae in a heterotrophic environment. The heterotrophic algae could then be harvested and intercellular lipids could be extracted for conversion to bio-based diesel and jet fuels. This is a continuation and expansion of the foundation work provided in Contract SP4701-09-C-0038 "Cellulosic-Derived Biofuels Program in Kentucky" submitted to the Defense Logistics Agency in September, 2012 (Addendum 1). A schematic of the process of going from biomass to bio-derived renewable fuel is described in Figure 1.

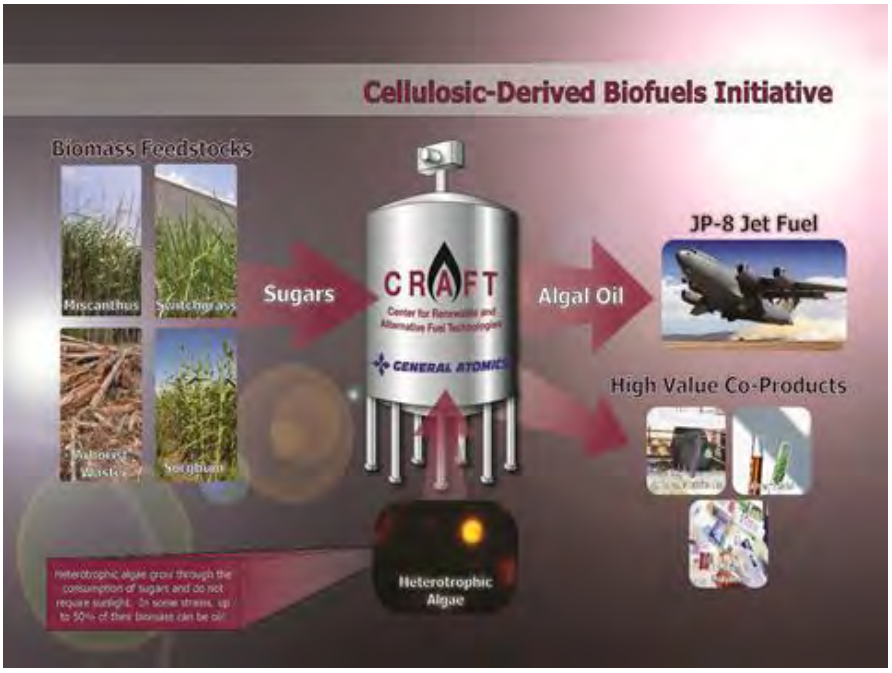

Figure 1. Illustration shows the process of going from biomass to renewable fuel using heterotrophic algae.

The tasks undertaken in this contract involved: biomass logistics, saccharification of biomass into fermentable sugars, conversion of the biomass sugars into algal oil, environmental impacts of the growing biomass to renewable fuel, economics of production of switchgrass and that of a commercial facility. As proof of principle, a lab-scale integrated biomass to algal oil process was demonstrated. Furthermore, evaluation co-products streams of the processes as a valuable economic input were evaluated. Most tasks were undertaken to develop and apply the principles in a scaled up pilot facility for rapid deployment. However, some tasks were expanded to develop technologies that are more fundamentally experimental with long-term potentials. Utilization of ionic liquids in biomass saccharification and lignin conversion along with supercritical methanol transesterification are two examples of high risk technologies that have long term potential for transformative implications to a commercial enterprise.

Some key findings of the report are:

- Switchgrass as a primary biomass source can be stored in round bales secured with net wrap outdoors on a well-drained gravel lot unprotected without loss of fermentable sugars.
- Amount of land on post-mined sites may not be as available to grow bioenergy crops in eastern Kentucky as originally thought.
- Rapid sugar analysis for biomass sugars was developed and utilized in the development and optimization of a pre-treatment/enzymatic saccharification process.
- Sugars generated (primarily glucose and xylose) support algae growth and oil accumulation.
- Growth of heterotrophic algae for oil accumulation was characterized.
- Development of harvesting and dewatering process were developed primarily by flocculation.
- Extraction of algae oil was accomplished by a combination of cell lysis (ball mill and sonication) and organic solvents (hexane and isopropyl alcohol).
- Process of taking switchgrass as a biomass source and conversion into algal oil for use in the manufacture of transportation fuel was demonstrated in San Diego, CA at General Atomics Research Laboratories and at Eastern Kentucky University at the Center for Renewable and Alternative Fuel Technologies in Richmond, KY.
- Ionic liquids show promise for use in saccharification of biomass and utilization and the degradation of lignin co-products.
- De-oiled algae meal shows promise as an excellent feed supplement for the aquaculture industry.
- Incentive for local production of switchgrass appears to be about $60/ton.
- Cost of algal oil from a commercial facility producing 50 million gallons/year are estimated to be from $7.89 to $10.28/gal with current technologies not including value of co-products.

Task B1: Biomass Logistics

Task B1.01 Storage Methods of Kentucky Biomass

EKU CRAFT: Phase I-A included establishment of biomass for the project. Most biomass crops are harvested once a year to be utilized as a carbon and energy source for algae over an entire year. Evaluation of different methods of storage will be carried out to maintain the highest quality of carbohydrates for algae. This is an important component in the overall efficiency of the biomass to algal oil process.

Switchgrass (Panicum virgatum) that is utilized for bioenergy production is typically harvested once per year. For commercial applications, storage methods need to be low cost without significant loss of biomass quality. This study was designed to determine the effects of three storage methods of switchgrass harvested in round bales on the quantities of fermentable sugars (glucose and xylose) over a one year period. Additional information was collected on changes in dry matter and ash content during the storage period. Quiescent switchgrass was harvested in February 2012. Mowing, windrowing and baling were done on the same day. Baling was done with a 4' x 4' (1.2 x 1.2 m) round baler with bales secured using nylon net wrapping. Bales weighed an average of 638 lbs (290 kg). Round bales were moved and stacked in a 3-2-1 pyramidal configuration and either stored inside undercover in a hoop structure or outside in a well-drained graveled lot. Bales stacked outside were either left uncovered or protected with a water proof tarp. Switchgrass samples were collected with a 2' (.6 m) hay probe monthly for 13 consecutive months. Probed samples were divided into thirds representing the outer portion of the bale, middle 1/3 and center of the bale. A composite sample from the entire bale was also collected. Switchgrass samples were processed in a Wiley mill with a 2 mm screen. Samples were pre-treated in with NaOH at 90^{o} C for 1 hr. Samples were triple washed and pH adjusted to 5.0 with citric acid and temperature brought up to 50^{o} C. Novozyme Cellic® CTec^{2} and HTec^{2} were added (.088 µl/gm fiber) and saccharification continued for 72 hrs. To terminate enzymatic saccharification samples were heated to 90^{o} C for 1 hour and supernatant was harvested and stored frozen. Thawed and filtered supernatant samples were analyzed in triplicate (n=468) for glucose and xylose using a Thermo-Dionex 3000 UPLC-CAD. The samples were analyzed after validated calibration curves were generated with sugar standards. Overall, there were no significant differences in the glucose or xylose concentrations between the three storage methods (outside-uncovered, outside with tarp covering or stored inside; Figure 2). However a highly significant interaction existed between storage method and month of storage for glucose ($p=.001$; Figure 3) but not xylose ($p=.313$). There was no significant difference ($p>.05$) in the sample location (outer 1/3, middle 1/3 or middle of bale) for glucose or xylose concentrations. Moisture content tended to be different ($p=.075$), with bales stored covered with a tarp having lower moisture content than those stored outside uncovered. Ash content averaged 3.08% and no significant differences were found between the storage methods or between the different months. From this study, it can be concluded that switchgrass harvested and baled in round bales secured with net wrapping can be stored outside on a well-drained gravel lot for one year with minimal loss in fermentable sugar quality. A detailed report on the storage methods on fermentable sugars is located in Addendum 2.

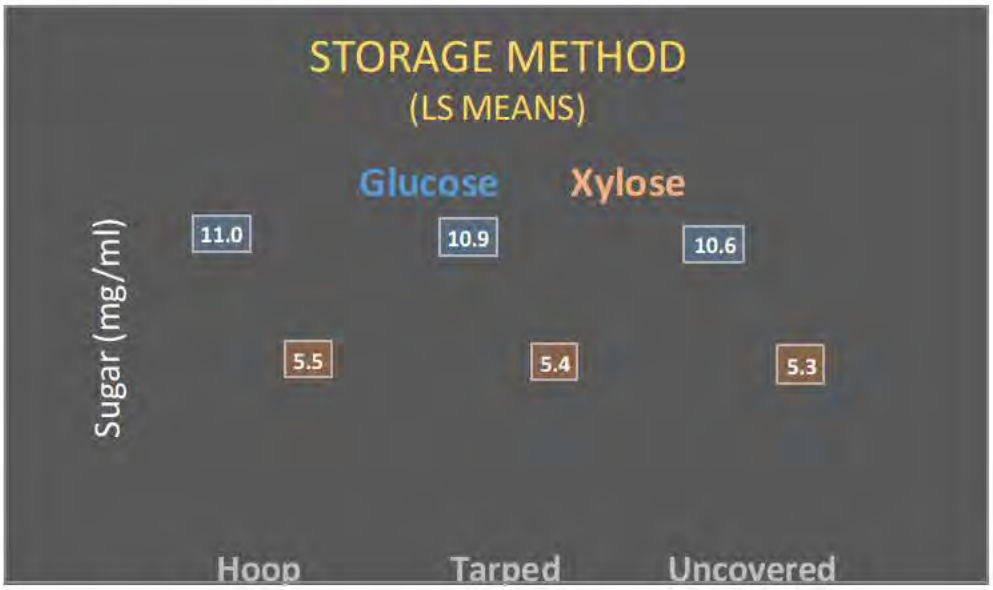

Figure 2. Concentration of Fermentable Sugars Based on Storage Methods of Switchgrass .

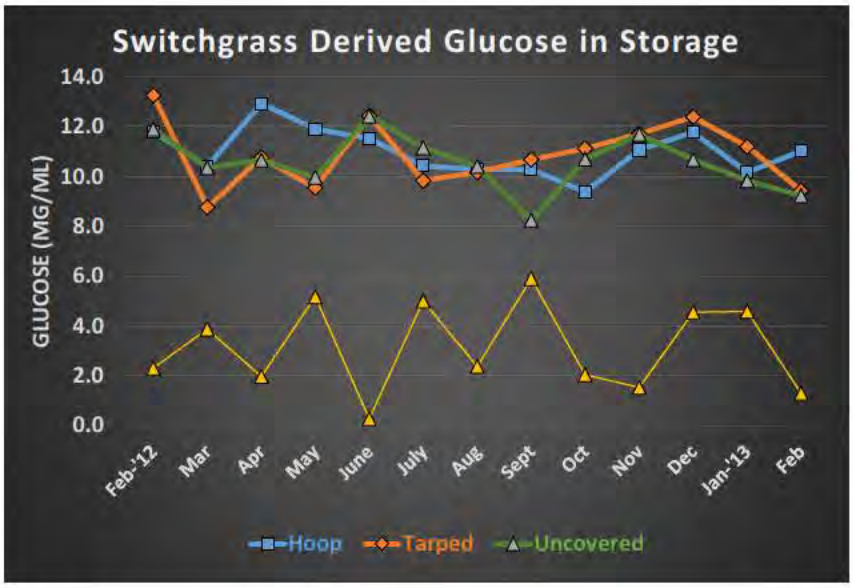

Figure 3. Effect of Storage method on fermentable sugars over a one year storage period.

Task B1.02 Post-Mine sites and Utility Right of Way Potential for Biomass Production

Kentucky State University: Kentucky State University will collaborate with EKU CRAFT to: 1) determine the total area of post-mining sites in Kentucky that are capable of producing biomass for biofuel facilities, 2) determine costs of transporting biofuel feedstock from post-mine areas to the proposed Biofuel Facility in Winchester, Kentucky and 3) assess the amount of cropland that could be converted to biofuel feedstock to support the needs of the Biofuel Facility by utilizing the rights-of-way for feedstock production.

Eastern Kentucky offers a large area of previously and currently mined land, it is crucial that this land first be analyzed for slope breaks to determine its potential for biomass production along with transport distances in an economic feasibility study. Pike County, KY was utilized as a model area to investigate the potential for biomass production for alternative energy since most of the county has been mapped with ArcGIS. Using slope analysis tools within ArcGIS is crucial to effectively find these tracts of land and for awareness into how maximum biomass production can occur or provide insight for potential sites for future cellulosic biofuel refineries (Figure 4). Furthermore it is apparent that the production of another form of biomass, such as sustainable woody biomass might be better suited for the rugged mine area environment.

The area of five selected surface mine sites were analyzed to determine potential to produce and harvest switch grass for biofuel feedstock. The key objective of this study was to analyze slope break and distinguish areas within these five mine sites for which slope was $\leq 20\%$ and identify tracts of land within the permitted mine boundary that were larger than .81 ha (2 acres). The five sites totaled 6818 ha of land and after the sites were analyzed for slope using ArcGIS 10.1 it became apparent that 185 ha met the slope criteria for potential switch grass production. From the five analyzed mine sites the average for the calculated permit boundary is 1364 ha while the average for harvestable land greater than .81 continuous hectares is 37 ha ($p=0.0048$). This corresponds to approximately 2.72% of land from the five permitted mine sites as potential area for growing biomass (Figure 5). Our study realizes the desperate need for accurate production estimates for further economic and feasibility analyses. A summary of the full report is in Addendum 03.

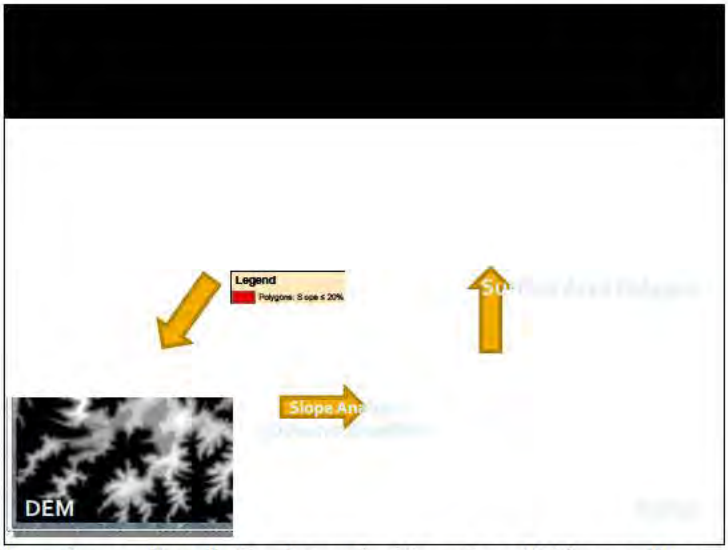

Figure 4. Depiction of area of study for determination of available area for production of biomass on mine sites in Eastern Kentucky.

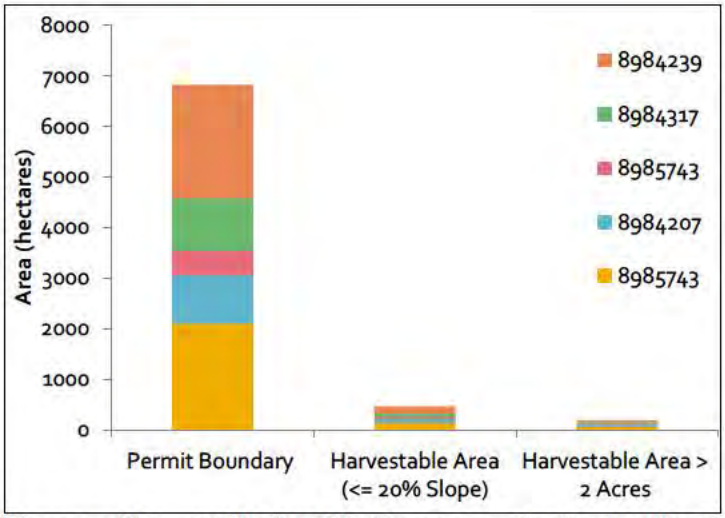

Figure 5. Available area of harvestable land for biomass production in Pike County, Kentucky

Task B2: Saccharification of Biomass

Task B2.01 Biomass Screening: Rapid Characterization Using Advanced Chemical Instrumentation

EKU CRAFT: The EKU Department of Agriculture and the EKU Department of Chemistry will collaborate on the investigation of energy crop and pre-treatment factors (both physical and chemical) that may influence crop management and post-harvest handling, as well as the ability of the material to supply simple carbohydrates for algae growth and oil production. Advanced technologies in analytical instrumentation, including Direct Analysis in Real Time (DART™) Mass Spectrometry and Scanning Electron Microscopy (SEM) will be utilized to rapidly screen biomass and evaluate relative efficiencies to supply carbohydrates following various pre-treatments.

Development of Biomass Sugar Analysis

A Direct Analysis in Real Time Mass Spectrometry (DART-MS) method for quantitation of six carbon sugars in saccharification matrix (solution used for the enzyme hydrolysis of switchgrass) was developed and validated. The DART ion source was used to produce ammonium adducts of the spiked glucose molecular ion and the d_2-glucose (internal standard) that were detected by scanning with a linear ion trap mass spectrometer. Calibration curves were obtained over a linear range of 10 to 3000 µM with correlation coefficients better than 0.997 and method recoveries were 94.9% to 103.0%. Matrix effects were observed and managed with matrix-matching standards for generating calibration curves. Limits of detection and quantitation were 5.84×10^{-6} M and 1.95×10^{-5} M, respectively. These results indicate this method could be implemented for quantitation of glucose generated from saccharification samples. Addendum 04 describes the full procedure for DART-MS analysis.

Since DART can be considered a complex thermal desorption ionization process, an optimization study of the helium gas temperature and introduction into the ionization region was performed. It was observed these parameters have a significant effect on the overall signal intensity as well as the signal-to-noise ratios in DART mass spectra. Using these optimized parameters, a set of different glucose concentrations (ranging from 10 to 3000 mM) were analyzed and used to determine a linear dynamic range (with the use of an internal standard). The analysis of the samples was done with minimal sample preparation and found to be reproducible over time (Addendum 05).

Ionic liquids (ILs) are being investigated as a novel way to obtain sugars from biomass (Task B2.03) and have the potential to dissolve the lignin byproduct from biomass (Task B7.01). Therefore, analysis of the ionic liquids (ILs) solvent containing either imidazolium or phosphonium cations combined with different types of inorganic and organic anions was performed with the DART-MS. Ionic liquids were directly inserted into the ionization source using a glass probe without dissolution into organic solvents. Mass spectra of the ILs were collected in both positive and negative mode with a linear ion-trap instrument (Figure 6). The intact cation of the compound was typically the dominant peak in positive mass spectra and cluster ion formation was present. Some individual anions were not readily observed in the negative mass spectra (based on the type of anion); however, the mass difference of adjacent cluster ions equal the mass of a complete IL and the anion mass could be verified by subtracting the known cation mass. The degree and intensity of the cluster ion formations was found to be dependent on the nature of the specific ILs as well as the DART temperature gas stream. From these results it appears that DART-MS can be used to analyze solutions that contain ILs (Addendum 06).

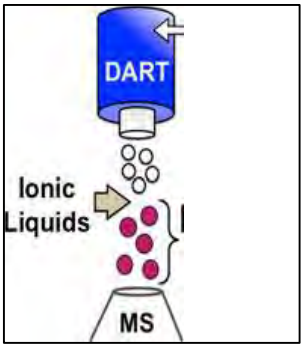

Figure 6. Schematic of Ionic Liquids in the DART-MS analysis.

Characterization of Biomass during Pre-Treatment and Saccharification

A comprehensive evaluation of four different pre-treatment procedures with seven different biomass sources that would be available in Kentucky was conducted. The pre-treatment was followed by the same enzymatic saccharification protocol utilizing commercially available enzymes from Novozyme described in Task B1.01 and Addendum 1. Pre-treatment protocols were selected based on having potentially low energy inputs with few environmental impacts. Pre-treatment with NaOH produced significantly greater concentration of biomass sugars compared to ammonia (NH_3), calcium hydroxide ($Ca(OH)_2$), or strong acid (sulfuric acid: H_2SO_4). All pre-treatments were superior to the water control group. The NaOH pre-treatment worked best at the 10% (wt/vol) loading. Sodium hydroxide pre-treatment also had the fewest environmental impacts compared to ammonia and strong acid hydrolysis. Addendum 07 is a detailed description of the results from the pre-treatment studies.

Scanning Electron Micrographs of Switchgrass and Woody Biomass during Pre-Treatment and Enzymatic Saccharification

To evaluate the physical changes occurring during pre-treatment and enzymatic saccharification of biomass that has potential for biofuels and biochemical processes, a series of micrographs were taken of switchgrass and woody biomass. Pre-treatment and enzymatic saccharification protocols are described in Addendum 1. Scans were obtained at multiple magnifications between 50 and 2000 x during the pre-treatment and enzymatic saccharification. In addition, physical changes were observed during the pre-treatment and enzymatic saccharification and compared to untreated biomass. Over time, an obvious disruption of the lignocellulosic fibers was monitored that appears to be related to the release of both glucose and xylose. However, quantitative evaluation of the physical changes were difficult to obtain. Addendum 08 contains a brief report with the SEM micrographs of the treated biomass.

Task B2.02 Biomass Conversion and Separation Studies

Joint EKU CRAFT/General Atomics: Saccharification, the conversion of cellulose and hemicelluloses into soluble simple carbohydrates, is an essential step in the utilization of biomass feedstocks for fuel production. EKU will investigate the utilization of cellulosic materials, and the development of enzymatic and/or stoichiometric strategies to facilitate the liberation of carbohydrates for ultimate utilization by heterotrophic algae. General Atomics will build upon acid hydrolysis processes for treatments of biomass to liberate stored carbohydrates. Methodologies evaluated in this task will also undergo economic feasibility determination as the sugar feedstock cost is a critical component in the overall cost of bringing algae derived fuels to commercialization. In particular, optimization of the procedure will involve identifying vital control points and applying fundamental strategies in processing, pre-treatment, and enzyme applications during saccharification.

Two approaches were undertaken for the biomass conversion to fermentable sugars. General Atomics, as part of their subcontract scaled up existing technology using strong acid hydrolysis. Since this is existing technology, strong acid hydrolysis holds greater promise to commercialization in a more rapid time frame. Eastern Kentucky University considered

with environmental impacts (see Task B5) with strong acid hydrolysis embarked on developing less harsh saccharification processes with either a weak base pre-treatment followed by enzymatic saccharification or utilization of ionic liquids.

Task B2.03 Development of Metalloporphyrin-Ionic Liquid Complexes for Degradation of Biomass

EKU CRAFT: The goal of this task will be to synthesize biomimetic (i.e. mimic enzyme function) iron porphyrin complexes and study their reactivity using a range of ionic liquid solvents. Ionic liquids are gaining wide recognition as environmentally friendly solvents for various biochemical and chemical reactions. Ionic Liquids have recently been shown to dissolve cellulose at standard temperature and pressure. These novel metalloporphyrin-ionic liquid complexes would function as both catalyst and solvent and have the potential to greatly improve the efficient production of carbohydrates from biomass that is environmentally sound.

To determine if enzymes could be utilized for biomass saccharification and would be effective in different ionic liquids, three different enzymes were evaluated in three different ionic liquids. Three enzymes evaluated were commercially available cellulase from Novozyme, and two thermostable cellulases, malate dehydrogenase and isocitrate dehydrogenase purified from both *E. coli* and *Thermus thermophilus*. The three ionic liquids evaluated were: 1-Allyl-3-methyl imidazolium chloride[2] (AMIM Cl), dimethyl imidazolium bromide and XS (xylene sulphonate). All three enzymes tested showed nearly as much activity in the presence of AMIM Cl as in its absence. Dimethyl imidazolium bromide and XS (xylene sulphonate) inactivated all enzyme at all concentrations tested. These results indicate that cellulosic enzymes remain active in the presence of at least one ionic liquid, AMIM Cl, and show promise of developing more environmentally favorable and less energy intense saccharification process for utilization of biomass. Addendum 09 is a more complete description of the used of ionic liquids during enzymatic saccharification.

As previous described we have demonstrated that some enzymes retain activity in the presence of select ionic liquids (Addendum 9). Recent work has focused on the study of enzymes from thermophilic sources since many industrial processes, such as biomass conversion, require higher temperatures in throughput processing. Thermophilic enzymes showed the most stability at high concentrations of AAIM Cl and the least with BMIM XS. We had thought ionic liquids would have less of an effect on thermostable enzymes, but the kinetics of inactivation using AMIM Cl were roughly the same for both mesophilic and thermophilic enzymes. We know that ionic strength itself is not affecting enzyme performance because even at high concentrations of potassium chloride, the enzyme maintained as much activity as without it. When compared with mesophilic enzymes, the thermophilic enzymes were able to withstand higher concentrations of the ionic liquids without hindering their activity. Our investigation into other enzymes and ionic liquids is continuing. In some preliminary experiments, consistent with the work reported above, we have shown cellulase maintains activity in the presence of AAIM Cl and is easily inactivated with the Br and XS derivatives tested so far (Addendum 10).

Task B2.04 –Biomass Conversion Process Scale-Up

General Atomics: The cellulose-to-sugar conversion technology selected under Phase I-A and Tasks B2.02 and B2.03 will be scaled-up from laboratory scale to a size sufficient to demonstrate larger scale production (10 Kg dry biomass) pilot scale viability. A teaming partner (e.g., from the ethanol production industry) familiar with sugar production from cellulosic materials may be contracted to aid in process scale-up and follow-on testing. Replicate testing will be performed to confirm throughput and conversion efficiency utilizing feedstock in the biomass sources identified in Phase I-A demonstrating the capabilities of supporting an eventual commercial-scale facility. The results of this task will feed into the Pilot Plant Design task (Task B8.01).

General Atomics successfully hydrolyzed biomass sugars from switchgrass with a scaled-up production of 10 kg from the initial 0.5 kg. Acid hydrolysis involved sulfuric acid (70-77% wt %) decrystallization at room temperature followed by hydrolysis at 90^0 C for one hour. Calcium hydroxide ($Ca(OH)_2$ was used in the neutralization of the H_2SO_4. Experiments were conducted with switchgrass processed into pellet or chopped into 100 mm lengths. The maximum sugar concentrations in the hydrolyzate were 18.3 g/L glucose, 30.6 g/L xylose, 1 g/lL cellobiose with a total sugar concentration of 49.9 g/L. This represents approximately 29% of the theoretical yield for glucose and 64% for xylose. Complete details of the procedures, experiments conducted and results are in Addendum-GA (pages 2-23).

Task B3: Carbohydrate to Oil Conversion Process Development

Research on the efficient conversion of carbohydrates extracted from biomass (Task 1B-2.02) into usable oil by algae will be the primary focus along with technologies for the harvest of the algae and extraction of the algal oil.

Task B3.01 Algae Growth Studies from Carbohydrates obtained from Biomass

Joint EKU CRAFT/General Atomics: Identification and understanding the growth requirements of different strains of heterotrophic algae that can effectively and efficiently convert carbohydrates derived from different biomass sources into algal oil is important in optimally utilizing many sources of biomass as an algae feedstock. Biomass feedstocks, comprised primarily of cellulose, hemicelluloses and lignin, are complex raw materials. Selection of robust strains of algae that are able to convert C6 (glucose) and C5 carbohydrates from hemicelluloses, in addition to other by-products of the hydrolysis process employed, to usable oil is critical. Various algal strains identified in Phase I-A will be employed to understand carbon utilization patterns and oil yield to maximize process efficiency with respect to biomass growth, oil yield and process kinetics. To achieve these goals, algal biomass growth will be optimized using advanced statistical and bioprocessing technologies including nutrition optimization, novel feeding regimens and process control.

General Atomics conducted a series of experiments to determine growth characteristics of *Chlorella p* (strain: GA 0137) with sugars obtained from switchgrass hydrolysate. Optimum growth rate over a 6 day period was a mixture of switchgrass sugars and glucose

(50:50). However, in subsequent experiments, undiluted switchgrass sugar with urea as a nitrogen source produced the most non-polar lipids (visible through Nile Red staining) and consumed the largest amount of sugar compared to other N sources and combinations of sugars. In additional experiments with commercially purchased switchgrass hydrolysate from Sweetwater Energy the algae, *Rhodoturula g.* (strain: GA2012) outperformed *Chlorella p.* (strain: GA0137) in growth characteristics. This may be due to GA2012 utilizing the pentoses better than GA0137. Additional studies will be needed to evaluate lipid accumulation. For complete details see Addendum-GA (Pages 24-50).

During the growth phase of algae incubation a nitrogen source is needed for protein synthesis for optimize production. A stress is often introduced. Deprivation of nitrogen is often used as a stressor to trigger algal cells to form oil. A two stage system where the first stage has a media that maximizes growth and cell numbers and a second stage 2 that provides a stress (N deprivation) for enhances oil production. A study was conducted to evaluate water sources (DI *vs* Tap Water), protein sources (urea *vs* yeast extract) along with two levels of aeration in a two stage system. Tap water significantly improved algae grow and low-cost urea can be substituted for a N source without significant impacts. Second stage oil accumulation studies were conducted to evaluate the best media combinations for lipid production. Second Stage Media that was devoid of nitrogen sources, potasium and phosphate buffers had fewer cells, but higher oil content. Evaluation of *Schizochytrium sp* was undertaken to compare growth and oil accumulation to *Chlorella*. Schizochytrium did not perform as well as Chlorella with the same media that was optimized in the above mentioned experiments. Further experiments were conducted to determine the appropriate media utilizing switchgrass hydrolyzate with and without media salts in combination with Stage 1 and Stage 2 of fermentation. Algae (GA1036) can be grown without additional media salts, although a lower concentrations than glucose controls. For complete details of the series of algae growth and oil accumulation experiments see Addendum-GA (pages 36-50).

Eastern Kentucky University conducted a series of experiments to validate techniques in for the growth of heterotrophic algae at different glucose concentrations and verification of the ability of algae to grow on sugars obtained from biomass produced in Task B2.02. Additional experiments were conducted to determine which genes are involved in the sequestering of oil in algae.

Algae Growth on Biomass Sugars:

To determine if heterotrophic algae could be grown on biomass derived sugars a series of experiments were conducted. In the first experiment a comparison was made evaluating growing algae with either glucose (control) or 100% biomass sugars. In experiment I, algae growth was inhibited compared to 100% glucose controls. This inhibition was speculated to be due to the inclusion of sodium azide as a preservative for the biomass sugars. In the second experiment a step wise increment of biomass sugars (without sodium azide) were used to replace glucose at 0% (control), 25%, 50% and 75%. Replacing glucose at the 25%, 50% and 75% concentration significantly increased the number of algae cells compared to controls (p=.041). These series of experiments support

the use of biomass sugars as an energy source for the growth of heterotrophic algae. Addendum 11 is a full description of the growth of heterotrophic algae on biomass sugars.

Genes Involved in the Accumulation of Oil in *Chlorella p.*

Single celled microalgae produce triacylglycerol (TAG) when grown in unfavorable conditions. TAG is a form of stored energy that can be employed to make a usable form of biofuel through chemical processing. *Chlorella protothecoides* was chosen for this project because it can be grown in many different abiotic conditions, has a rapid reproduction rate, and can easily be cultured in a laboratory. This allows for a large number of experiments to be done in a short time. Our study seeks to identify the genes that control lipid production and regulation in *Chlorella Protothecoides*. In order to produce lipids at an optimal level, the algae were grown in nitrogen deficient media (lipid producing conditions). To determine if these conditions enhanced lipid production, flow cytometry and QPCR were run. This data was then compared to previous algae samples in which next generation sequencing was performed and the upregulation and downregulation of certain genes was confirmed through the use of Cofactor (Figure 7). The attainment of this data can contribute to an increased production of biofuels. These results could also potentially aid in the ultimate decrease of the world's dependence on fossil fuels as an energy source Addendum 12 is a full description of the determination of genes involved in oil accumulation in heterotrophic algae.

Figure 7. Lipid biosynthesis pathway. Illustration the specific pathway used to produce lipids. Words in red are identified as enzymes that are being differentially expressed. Words highlighted in yellow are ones that are being upregulated.

Task B3.02 – Algae Bio-Oil Production Process Scale-Up

General Atomics: The algae strain(s) identified under Phase I-A as optimal for bio-oil production from carbohydrates will be utilized for heterotrophic bio-oil production (Task B1-3.01). The process equipment will be increased in scale to a size sufficient to prove pilot scale viability. Testing will be performed on the larger-scale equipment to optimize such parameters as nutrient requirements, air flow requirements, and growth rates, using the data obtained in Phase I-A as initial targets. A high cell density, non-sterile environment will be evaluated for larger-scale operations as a means to reduce cost, although confirmation of this operating scenario will be required during Phase I-B. The results of this task will feed into the Pilot Plant Design task.

Algae production was scaled-up in order to provide sufficient algal biomass for further downstream processing (harvesting, drying & oil extraction) and to demonstrate proof of principle for technologies and procedures developed in Task B3.01. A 200 liter Scale fermenter was developed from a plastic drum fitted with a head plate that consisted of a 1 hp motor/mixer, pH probe, dissolved oxygen probe, aeration and sampling port. Seed algae (GA0137) from 5 L bioreactors were used to inoculate the disinfected 200 L bioreactor. Algae did not utilize the switchgrass sugars as effectively as the lab-scale system, but was able to harvest approximately 1 kg of dry matter algae from the 200 L system. Five hundred liter scale-up fermenter system was initiated. Two trials were conducted at the 500 L scale. Both trials were terminated early, due to contamination. Additional trials with alternative sterilization methods of the fermenter and media will be needed. For complete details of the 200 L and 500 L scale-up trials see Addendum-GA (Pages 51 – 68).

Task B4: Algae Biomass Harvesting & Oil Extraction

Task B4.01 – Algae Harvest and Bio-Oil extraction

General Atomics: Although many identified strains of algae have high oil content, the ability to remove excess water after harvesting algae and extraction of the oil economically with minimal environmental impact is important to the total process. Novel methods that will investigate different processes/methods of drying and oil extraction will be evaluated to determine their engineering and economic viability at commercial scale if implemented.

General Atomics evaluated two methods of dewatering and extraction of oils from heterotrophic algae grown in Task B3.02; Flocculation and supercritical methanol transesterification. Flocculation is a well-recognized process used in many industrial processes that can potentially be transferred more rapidly for the concentration of heterotrophic algae on a commercial scale. Supercritical methanol transesterification is a more experimental process that will require more detailed investigation and evaluation before it can be deployed at a larger scale. However, it has potential to improve the efficiency and reduce the costs associated with the harvesting, dewatering and oil extraction processes.

Eastern Kentucky University conducted a series of experiments to evaluate different solvents and organic solvent ratios on the algal oil profiles.

Flocculation Experiments: A series of experiments were conducted to determine if heterotrophic algae would auto-flocculate evaluating: two flocculants (SB-260 and SB-A201), flocculent concentrations and four different algae concentrations. Additional experiments were conducted to determine the optimum pH, effects of addition of chitosan (derived from crab shells) and different combinations of the two flocculants on effectiveness of removal of algae from the media. In summary, pH (range 4-14) had no significant impact on flocculation over short durations (3 min). Addition of chitosan was completely ineffective at improving flocculation. The combination of the two flocculants at a pH range of 7 to 11 was effective in removing over 80% of the algae (Figure 8). For complete details of the flocculation experiments please see Addendum-GA (pages 69-81).

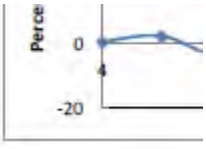

Figure 8. Influence of pH on flocculation of GA0137 culture (100%,)D 40) with SB-260 (150 ppm), SB-260 (150 ppm) with SB-A201 (150 ppm) and SB-260 (150 ppm) with SB-A201 (300 ppm).

Supercritical Methanol Transesterification Experiments: The purpose of these experiments was to explore some process conditions for supercritical methanol transesterification for future evaluation and process development. A Parr autoclave reactor was utilized for these experiments. The supercritical methanol transesterification process is outlined in Figure 9.

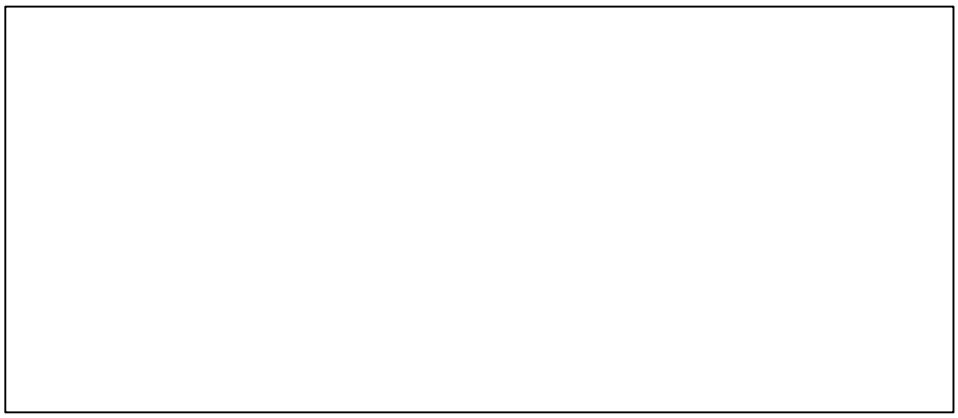

Figure 9. Overview of supercritical reaction solution analysis.

Optimum yield (51.6%) occurred at 250^0 C when water was added to the algae biomass in addition to methanol. These conditions were slightly lower than what is considered supercritical for methanol (270^0 C). Yields without water at 250^0 C without water and 270^0 C with water were 23.6% and 31.0%, respectively. A complete description of the supercritical methanol transesterification experiments are in Addendum-GA (pages 82-94).

Organic Solvent Extraction of Algal Oils (EKU):

A preliminary experiment was conducted to determine the optimum amount of time and ball size to mill dried algae before solvent extraction (Table 1). Each test consisted of 100 grams of dried heterotrophic algae. Five tests were completed: 10 minute ball mill with 10mm ball and hexane, 2 hour milling with 10mm ball, 30 minute milling with 30mm ball, 30 minute milling with 30mm ball and hexane, 30 minute milling with 30mm ball and reduced hexane/IPA. Addendum 13 describes the results from the ball mill extraction study.

Table 1. Summary of the Ball Mill and Solvent Extraction Studies

Ball Mill Conditions		100 gms Algae		Extracted Volume (ml)	Visual Appearance
Ball Size (mm)	Time (min)	Hexane	IPA		
		Solvent: Algae Ratio			
10	10	5	3	5	Green
10	120	5	3	20	Opaque
30	30	5	3	27	Dark
30	30	2.5	1.5	20	Dark
30	30	5	0	15	Golden

Using the DART mass spectrometry system, lipid profiles were performed from the extractions presented in Addendum 13. It appears the 30 minute of milling using 30 mm balls and 120 minute milling with 10 mm ball yielded the greatest amount of TAG. The isopropyl alcohol (IPA) is needed to extract the TAG and reducing the hexane and IPA reduced the amount TAG extracted. Figure 10 is an example of the lipid profile from the

30 minute ball milling with 30 mm balls using hexane and IPA as solvents. Addendum 12 contains the details of the lipid profiles from all extraction procedures.

Figure 10. Lipid profile from a sample generated with a 30 min ball mill with 30mm balls using hexane and IPA as solvents. Analysis performed with the DART-MS system.

Task B4.02 – Bio-Oil Recovery/Collection Process Scale-up

General Atomics: The bio-oil recovery/collection technologies selected under Phase I-A will be increased in scale to a size sufficient to prove commercial viability. Testing will be performed on the larger-scale equipment to optimize such parameters as oil collection efficiency, oil composition and/quality, throughput, and energy usage. The test results will be compared to those of Phase I-A. The resulting bio-solids will also be evaluated in Task B7 and compared to Phase I-A results to confirm suitability for use as animal feed, other high-value applications, or recycle to recovery additional nutrients. The results of this task will feed into the Pilot Plant Design task (Task B1-8.01).

This task was performed in conjunction with Task B4.01–Algae Harvest and Bio-Oil extraction and is presented in detail in Addendum-GA (pages 69-81).

Task B5: Environmental Analysis

Task B5.01 – EKU: Initial data will be collected for a Life-Cycle Analysis of the entire process starting from biomass production to the renewable diesel product. This information is necessary for verification of the sustainability of the process.

A parametric analysis to assess the sensitivity of input parameters to environmental impacts was conducted to grow heterotrophic algae using sugars derived from biomass. Input data was customized for central Kentucky. SimaPro 7.3.2 was used for the software analysis that incorporated Ecoinvent 2.2 and U.S. LCI unit process databases.

Environmental Assessment modeling was conducted using IMPACT 2002+ (v 2.1). Separate environmental impact assessments were made for different stages in the overall processes going from production of switchgrass, conversion to cellulosic sugars, growth of heterotrophic algae (two stages – growth & oil production), harvesting/dewatering, oil extraction and conversion to renewable fuel. The environmental impacts were then integrated to form a single impact assessment for the entire process. Electricity contributed the largest environmental impact at 25%. Sodium hydroxide used in the pre-treatment process prior to enzymatic saccharification contributed an additional 15.3% of the environmental impacts. Pumps and conveying equipment a total of 15.8% and other chemicals contributed 12.5%. Urea as a nitrogen fertilizer used for production of switchgrass and as a non-protein nitrogen source for growth of heterotrophic algae contributed 6.5% of the environmental impacts. Switchgrass production as the biomass for cellulosic sugars contributed 5% of the environmental impacts (Figure 11).

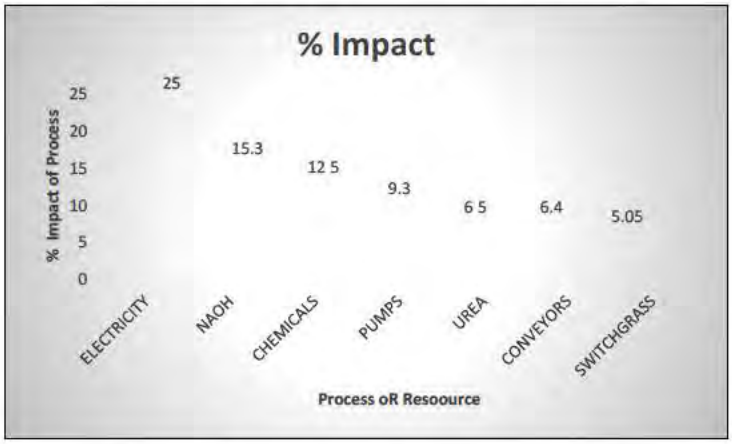

Figure 11. Distribution of Inputs and processes in the conversion of biomass to biodiesel in their contribution to the environmental impact assessment. Only Impacts >5 are shown.

Of the total impact categories, human health was affected the greatest with 37% of the total (Figure 12). This was followed by resources (31%), climate change (24%) and ecosystem quality (8%). A detailed environmental impact assessment is presented in Addendum 14.

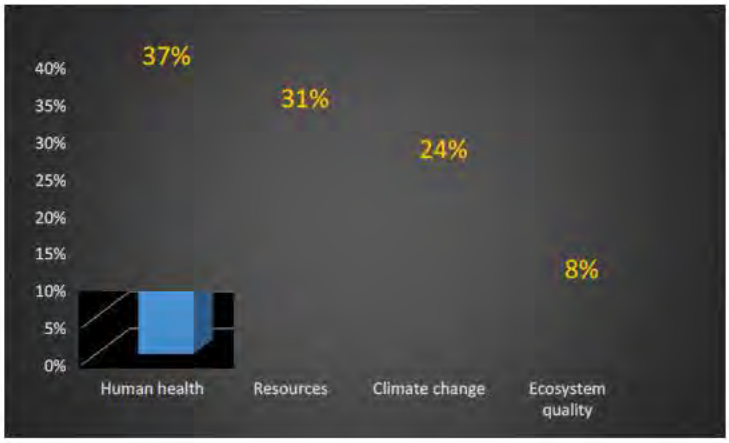

Figure 12. Relative impacts on human health, ecosystem quality and resources for conversion of algae oil to biodiesel.

Task B6: Lab Scale Integrated System for Biomass to Algal Oil

Task B6.01 – Biomass to Algal Oil Process – Laboratory Scale Model – General Atomics: Development of a laboratory scale process for biomass through the entire process to production of algal oil. The technologies selected under Phase I-A will be incorporated in a laboratory scale integrated model to a size sufficient to demonstrate viability of a complete system. A teaming partner (e.g., from the ethanol production industry) familiar with carbohydrate production from cellulosic materials will be consulted to aid in process scale-up and follow-on testing. Replicate testing will be performed to confirm throughput and conversion efficiency of feedstocks from biomass sources identified in Phase I-A, to demonstrate the feasibility of an eventual commercial-scale facility. The results of this task will feed into the Pilot Plant Design task (Task B1-8.01).

An integrated lab-scale Biomass to Algal Oil system has been successfully demonstrated by General Atomics at their headquarters in San Diego and transferred to Eastern Kentucky University and re-demonstrated. Figure 13 depict the acid hydrolysis of 10 kg of switchgrass into biomass sugars that were utilized as energy feedstock to grow heterotrophic algae in a 200 liter fermenter controlled by an Applicon bioreactor (Figure 14). Algae was flocculated (Figure 15), dried, processed in a ball mill and extracted using a hexane/isopropyl alcohol mixture as described in Task B4.01 (Organic Solvent Extraction).

Figure 13. Photos of various hydrolysis process steps including a) pelletized switchgrass feed, b) decrystallization solution, c) hydrolysis step, d) lignin after separation from acidic hydrolyzate, e) gypsum after separation from neutralized hydrolyzate, and f)

Figure 14. 200 Liter fermenters at General Atomics and EKU controlled by Applicon Bioreactors.

Figure 15. Algal biomass in process of being flocculated and ready for drying oven.

Task B7: Co-product Utilization

Effective use of Co-products from Biomass and Algae Processes: EKU CRAFT: Efficient utilization of products from saccharification of biomass (primarily lignin) and the remnants algae after oil removal will ultimately improve the economic viability of the entire process but also reduce the impact the biomass to algal oil process has on the environment.

Task B7.01 Development of Metalloporphyrin-Ionic Liquid Complexes for Degradation of Lignin

EKU CRAFT: The goal of this task will be to utilize ionic liquids (as described in Task B2.03) to dissolve lignin with the reactivity of the metalloporphyrin complex could overcome many of the challenges to lignin utilization. These novel metalloporphyrin-ionic liquid complexes would function as both catalyst and solvent and have the potential to greatly improve the efficient production of bio-products from lignin.

Ionic liquids tagged with porphyrins (Addendum 15), Co(Salen) complex (Addendum 16) and metallopophyrins (Addendum 17) have been evaluated as a catalyst for the oxidation of lignin. Methodology for the synthesis of many of these ionic liquids have been developed at Eastern Kentucky University. Purified lignin and lignin from switchgrass have been demonstrated to dissolve in two ionic liquids (Figure 16).

Figure 16. Dissolution of Lignin obtained from switchgrass in Ionic Liquids at 100°C

Veratryl alcohol (VA) was used to evaluate the effectiveness of different ionic liquids as a catalyst for breaking the linkages between sub-units of lignin because VA carbon and oxygen linkages are similar to natural lignin. The effect of ionic liquids and water as solvents for reactions catalyzed by the metallopophyrin FeT1239 was the most effective ionic liquid (50% greater conversion) on the oxidation of veratryl alcohol (Addendum 16). These results show a potential for the use of specific ionic liquids under the right conditions to be able to oxidize lignin to be able to harvest its vast chemical potential.

Task B7.02 Algae Biomass Meal for KY Aquaculture Feed

Kentucky State University: Algal meal (spent algal biomass after oil extraction) has the potential as a high protein nutrient source for the aquaculture industry. The objective of this task is to evaluate the replacement of a commercial ration composed of fishmeal, soybean meal & corn meal, with algae meal for the diets of an omnivore, the channel catfish.

Large quantities of de-oiled algae meal (AM) would be generated in the commercial production of the biomass to algae oil for biofuels process. A target production goal would be 50% of the algae biomass as algal oil. This would leave the remaining 50% as de-oiled algae meal. In commercial farm-raised fish, feed costs represents a significant cost of production and typically cost 2 to 3 times as much as livestock feed. Many species of fish utilize algae as a feed source in the wild. This study was designed to determine if de-oiled algae meal (AM) could be effectively utilized as a feed supplement for farm-raised channel catfish during an 8-week feeding trial. The algae meal (AM) was from *Chorella sp.* That had been grown heterotrophically and the lipid extracted with organic solvents.

Diets were formulated to contain 28% protein and 6% lipid. The Control Diet contained no algae meal and the four Experimental Diets contained either10%, 20%, or 40% AM. An additional diet contained 40% AM with 2% supplemental lysine. The feeding trial was conducted using juvenile channel catfish (5.7 ±1.4 g) stocked ainto fifteen 37.7-L aquaria recirculating system at 10 fish/tank. There were three replicate tanks per dietary treatment. Fish were fed to apparent satiation twice daily at 0900 and 1400.

After 8 weeks channel catfish fed diets 10% AM, 40% AM, and 40% AM+LYS were significantly larger ($P \leq 0.05$) than fish fed the Control 9% AM) fish fed the 20% AM diet were not different from the Control. Feed conversion ratio was significantly lower (more efficient; $P \leq 0.05$) in fish fed the 40% AM+LYS than in fish fed any other diet. These data indicate that channel catfish can efficiently utilize algae meal at levels up to at least 40% of the total diet and at that level lysine supplementation significantly improved feed conversion efficiencies.

These data indicate that channel catfish readily accept and can efficiently utilize AM at levels up to at least 40% of the total diet and at that level lysine supplementation significantly improves feed conversion efficiencies. As this byproduct may be in ample supply due to the demand for biofuels and microalgae as future feedstock, this data suggests that AM may be a suitable ingredient for aquaculture feed (Addendum 18).

Task B8: Pilot Plant Engineering

Task B8.01 –Pilot Plant Design – General Atomics: A final design package (FDP) will be prepared for the selected pilot scale plant size. The FDP will be of sufficient detail with the necessary data to include a complete bid package that can be utilized by candidate construction companies for the development of proposals to construct the pilot and demonstration plants. The FDP will include process descriptions, process specifications, process flow diagrams (PFD), piping and instrumentation diagrams (P&IDs), equipment sizing calculations, equipment arrangement drawings,

data sheets, and electrical drawings. Also included in this task will be a plan for transition from pilot to demonstration plant scale to final commercial scale.

Figure 17 shows the block flow diagram for the full-scale production facility, with a baseline production capability of 50M gal/yr biodiesel. The process has 7 distinct unit operations: (1) cellulosic biomass storage and handling, (2) conversion of cellulosic biomass to simple sugars, (3) conversion of sugar to algal oil (or fermentation), (4) harvesting/dewatering, (5) drying, (6) oil extraction, and (7) fuel production (or transesterification). A full description of each of the seven unit operations along with flow diagrams and mass balances are provided in Addendum GA (pages 99 to 120).

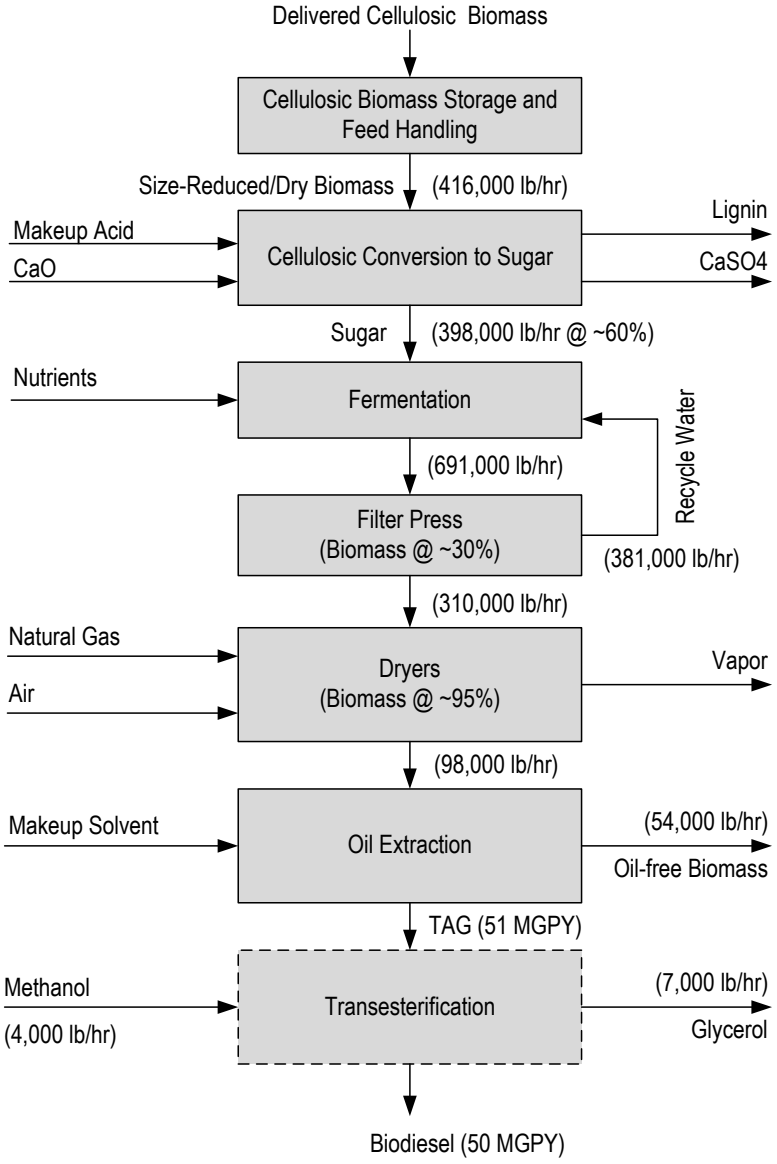

Figure 17. Block flow diagram for biomass to biodiesel for a commercial plant producing 50 million gallons per year.

Task B9: Economics and Cost Analysis of Production

Task B9.01 Economics of alternative biomass storage

EKU CRAFT: As different storage methods are being evaluated in Task B1.01, the cost of the different methodologies will need to be fully evaluated relative to the cost vs benefits. ***Note:*** *This task was amended to evaluate the supply response for local producers to grow the dedicated bioenergy crop – switchgrass.*

A review of 18 recent studies that provided estimates of the cost of switchgrass at the farm gate found a range of $31 to $94 per ton (85% dry matter), with a simple average of $53 per ton (Epplin, 2009). The variation is a reflection of different assumptions about a small number of factors that affect cost in a major way. These are primarily the opportunity cost of land, the extent to which the cost of the nutrients removed from the soil by switchgrass are included, the yield of switchgrass, whether the yield varies with land or soil quality, machinery cost, and fertilizer prices (O'Connor, 2010). This report examines how these factors are likely to affect the supply of switchgrass in central Kentucky. In particular, we focus on how the yield of switchgrass, machine costs, the price of hay, and the opportunity cost of land influence the supply of switchgrass in the context of converting land from hay production to switchgrass production.

The premises underlying the baseline budget are summarized in the following:

- Switchgrass is an alternative crop for land which is currently producing either grass-legume hay or grass hay.
- A stand of switchgrass is assumed to last at least 10 years without deterioration in years 3-10.
- There is a probability of .25 that reseeding is required in year two. The reseed is assumed to last 9 years.

The return to land is a metric that is often used by farmers in making decisions about land use. The data demonstrate the importance of both yield and machine cost in influencing the price needed to achieve a required return to land.

An analysis of the breakeven price of switchgrass and return to land compared to hay was conducted at two different machinery costs at different yield projections. When machine costs are high, the breakeven price for switchgrass is $30-$36 per ton when hay is $60/ton, $37-$42 per ton when hay is $70/ton, $44-$49 when hay is $80/ton. However, the breakeven price for switchgrass does not provide a positive return per acre for any of the four levels of yield. The same is true when machine costs are low and for the price of hay at $60 or $70/ton. At a price of $80/ton, the return to land ranges from $10 to $44 per acre, and the breakeven price for switchgrass varies from $42 to $48/ton.

A profit target for a farmer will involve both a return on land and a return on the funds invested in conversion. In some cases, the land return requirement determines the supply price while in other cases the rate of return does so. For grass-legume hay conversion, the per acre return requirement is the constraint when the price of hay is $60/ton while the rate

of return requirement is the constraint when he price of hay is $80/ton. When hay is $70/ton, the land return is dominant for lower yielding land. For grass hay conversion, the return per acre is the operative constraint except when the price of hay is $80/ton and machine costs are low.

The supply price decreases as the productivity of land increases. Supply price is higher for higher machine costs but the effect is less for higher yields. When meeting the return per acre requirement is dominant, there is no difference in the supply price between conversion from grass-legume hay and grass hay. When meeting a specified rate of return is the dominant factor, then the supply price is lower for converting from grass hay.

The economics of converting from one crop (hay) to another (switchgrass) was evaluated. Other things equal, the returns from conversion increase as the price of switchgrass increases. The gains from conversion decrease as the price of hay increases. As production moves from lower yielding land to higher yielding land, the gain in return per acre and the rate of return on investment increase. This would suggest that switchgrass would be more attractive compared to grass-legume hay on more productive land rather than less productive land. This conclusion does depend on the assumption that the yield of switchgrass improves as the yield of grass-legume hay improves.

Yield has a significant influence on the profitability of growing switchgrass. On low yielding land with high machine costs, it is profitable to convert from both grass-legume and grass hay to switchgrass if the price of switchgrass is $60/ton. On high yielding land with high machine costs, conversion is profitable at $50/ton when the price of hay is $60 or $70 per ton.

When the price of switchgrass is $60, it will be supplied from land of all four levels of productivity, the two machine costs, and the three prices of hay. At the other end, a price of $40/ton brings forth supply only when machine cost is low and yield is superior. As price increases, producers with lower yields and higher machine costs become suppliers. A detailed analysis of the supply response is in Addendum 19.

Task B9.02 Influence of Plant Size on the economics of algal oil production

EKU CRAFT and General Atomics: As the design of the pilot plant are near completion, estimates of the economics of the design will be necessary (Coordinated with Task B8.01 & B9.01).

An initial cost model was prepared to allow estimation of the algal oil production costs for a full–scale facility with a production rate of 50M gal/yr biodiesel fuel. Accounting for yield of the transesterification process (95% efficiency assumed), the algal oil production requirement is approximately 51M gal/yr as triglyceride (TAG) for an eventual yield of 50M gal/yr of biodiesel. The cost model incorporates vendor-supplied cost data along with General Atomics (GA) estimates and industry standard cost factors to yield estimated capital and operating costs for each unit operation. The baseline technologies used in the model are described in Table 2.

Table 2. Baseline Technologies for Economic analysis.

Algae Feedstocks	Fresh water microalgae
Algae Growth	Heterotrophic method
Carbon Source	Lignocellulosic sugar
Fermentation	2-stage fermentation method
Harvesting/Dewatering	Belt press and direct dryer
Lipids Extraction	Dry extraction using mechanical disruption followed by dry biomass extraction with hexane solvent
Conversion of Lipids to Biodiesel	Base-catalyzed transesterification

Lipids stored in algae are neutral; therefore, they all are capable to be converted to biodiesel. The cost data for the process equipment for the conversion of lignocellulosic biomass to sugar are currently under investigation. For cost estimate purposes, the cost of lignocellulosic sugar directly taken from literature was used. The cellulosic sugar cost ranges from $0.04 to $0.10 per pound (note that commercial food grade sugar costs about $0.22 per pound at the time of the analysis). As a result, the estimated algal oil production costs range from $7.89/gal to $10.28/gal TAG, respectively, of which oil-free biomass co-product credit is $1.37. Figures 18 and 19 show the distribution of the costs for the two cases of sugar cost, respectively. The analysis suggests that the fermentation operating cost distribution of 55% - 69% is dominant in the overall production cost; the algal biomass drying cost is dominant in the harvesting and oil extraction costs. The sugar cost contributes 17% to 34% to the overall production cost of algal oil. The model assumes that all sugars (i.e., pentose and hexose sugars) are consumed and converted to lipids by algae. Also, lipids extracted from algae are 100% non-polar; therefore, are usable for biodiesel conversion. The cost estimates are based on 2009 dollars the project is 100% equity financed with a 20-year straight line depreciation of assets. A full description of the cost model, assumptions and calculations are in Addendum-GA (pages 95-147). These estimates are based on the engineering material balance described in Task B8.

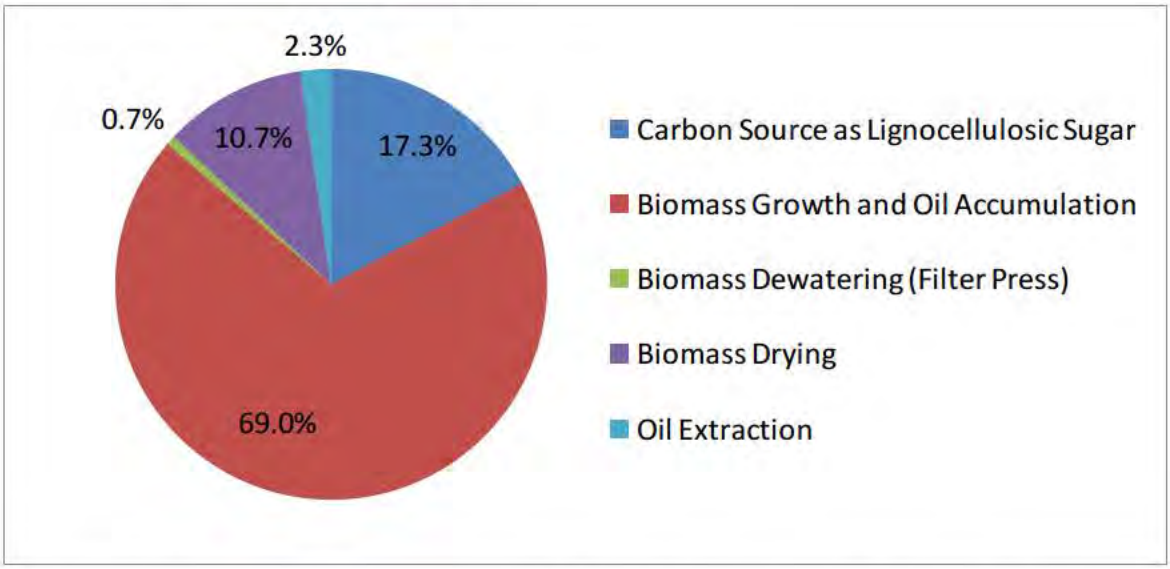

Figure 18. Cost Distribution per Gal Algal Oil for Case of $0.04/lb Sugar (Total = $9.25/gal Algal Oil without Co-products).

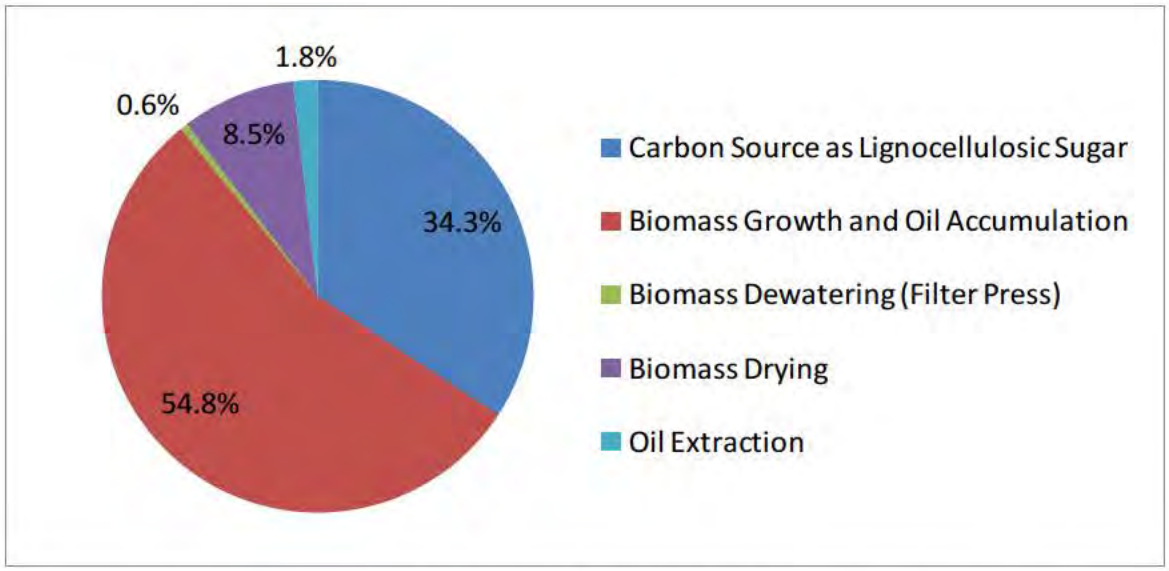

Figure 19. Cost Distribution per Gal Algal Oil for Case of $0.10/lb Sugar (Total = $11.65/Algal Oil without Co-products).

The cost estimate is done only for the 4 major blocks: fermentation, algal biomass harvesting, algal biomass drying, algal oil extraction. As stated earlier, the process conversion of lignocellulosic biomass to sugar is not included in this study. Sugar material is considered as a chemical component and bought into the plant for use with a cost range from $0.04 to $0.10/lb from literatures. Also, the technology for the process of converting algal oil to biodiesel is well developed and established; therefore, no projections are made in the cost analysis. This section presents the cost summaries for the 50MGPY algal oil production facility.

The major equipment list, specification and cost including installation and delivery for each unit and total cost are listed in Addendum-GA (pages 95-98; 121-147). The total capital investment and its component costs are provided in Table 3.

Table 3. Cost Evaluation Summary for a 50 M gal/year biomass to biodiesel processing plant.

Item	From	To
Total Capital Investment (Million $)	-	$1,239
Operating Cost (Million $)	$465	$588
Cost Basis Annual Production Rate (Mton/yr algal oil)	-	51
Unit Processing Cost without coproduct credits ($/gal algal oil)	$9.13	$11.53
Unit Processing Cost with coproduct credits ($/gal algal oil)	$7.89	$10.28