

Final Report

Project Title: Research for Developing Renewable Biofuels from Algae

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Task A. Expansion of knowledge related to lipid production and secretion in algae

A.1 Lipid biosynthesis in target algal species

- Systems biology approaches are being used in combination with recent advances in *Chlorella* and *Chlamydomonas* genomics to address lipid accumulation in response to defined nutrient regimes. The UNL Algal Group continues screening additional species of *Chlorella* and other naturally occurring algae for those with optimal triglyceride production.
- Of the strains examined by the DOE's Aquatic Species Program, green algae, several species of *Chlorella* represent the largest group from which oleaginous candidates have been identified.

A.1.1. Lipid profiling

- Neutral lipid accumulation is routinely monitored by Nile red and BODIPY staining using high throughput strategies to screen for naturally occurring algae that accumulate triglyceride. These strategies complement those using spectrofluorometry to quantify lipid accumulation.
- Neutral lipid accumulation is routinely monitored by high performance thin-layer chromatography (HPTLC) and high performance liquid chromatography (HPLC) of lipid extracts in conjunction with A.1.
- Carbon portioning experiments have been completed and the data currently are being analyzed and prepared for publication.
- Methods in the Black lab were developed to identify and quantify triacylglycerol (TAG), major membrane lipids [diacylglycerol trimethylhomoserine, phosphatidylethanolamine and chloroplast glycolipids], biosynthetic intermediates such as diacylglycerol, phosphatidic acid and lysophospholipids and different species of acyl-coenzyme A (acyl CoA).

- The Black and DiRusso laboratories developed HPLC, LC-MS and GC-MS methods for the separation, quantification, and stable isotopic analysis of galactolipids (mono-, di-, and tri-galactosyldiacylglyceride), sulfolipids (sulfoquinovosyldiacylglyceride), and phospholipids (phosphatidylglycerol, phosphatidylinositol, phosphatidylserine, phosphatidylcholine, and phosphatidylethanolamine).
- The Black and DiRusso laboratories, in collaboration with Yongfeng Lu (Professor, Department of Electrical Engineering, University of Nebraska), developed enhanced Raman Spectroscopy and Coherent Anti-Stokes Raman Scattering (CARS) microscopy to develop novel methods to define TAG accumulation. This group has been able to define this process to develop probes that have more broad use, including for use in our bioreactors (see below). These efforts have considerably advanced our analytical repertoire and have been extended using CARS microscopy, which is allowing us to visualize lipid accumulation in three dimensions in living cells.
- The Black and DiRusso laboratories completed experiments analyzing changes in galactolipid content and labeling ($^{13}\text{CO}_2$) during stationary phase growth with or without a nitrogen source. The results demonstrate that galactolipid content decreases dramatically overall during nitrogen starvation, but that the extent of degradation varies greatly between specific galactolipid pools. The monogalactosyldiacylglyceride (MGDG) pool is reduced by nine fold, the digalactosyldiacylglyceride (DGDG) pool by four fold, and the trigalactosyldiacylglyceride (TGDG) pool by less than 15%. Additionally, the change in analyte profiles is specific to galactolipid type and provides evidence for equilibrium between DGDG and TGDG pools and disequilibrium between these pools and the MGDG pool. This is consistent with the spatial separation of these pools into different chloroplast membranes. Pulse-chase labeling from two days of growth in $^{13}\text{CO}_2$ followed by two days of incubation in unlabeled CO_2 demonstrate a surprisingly high degree of *de novo* fatty acid synthesis during nitrogen starvation and incorporation into the DGDG and TGDG pools to a much greater extent than the MGDG pool. Labeling decreases in the DGDG and TGDG pools while remaining the same in the MGDG pool during incubation on the unlabeled CO_2 . This data, coupled with the decrease in MGDG content compared to the nitrogen replete condition, suggests the redistribution of specific fatty acids from the MGDG pool out of the galactolipid pool, likely into triglycerides. This data will be compared to the analyses of triglyceride and membrane lipid pools to provide a complete picture of fatty acid redistribution through membranes and into triglycerides and reveal the exact contribution of photosynthetic activity to the final triglyceride content.
- The Black, Cahoon and DiRusso laboratories developed metabolic profiling strategies that can be used for comprehensive evaluation of the microalgal lipidome, correlating changes in lipid composition with changes in gene expression. LC-MS/MS methods for lipid profiling have been developed for use with a Shimadzu Ultra-high Pressure Liquid Chromatography (UPLC) system in combination with molecular detection by a QTRAP4000 mass spectrometer. These groups are employing LC-MS/MS methods for profiling major lipids and lipid metabolites, including triacylglycerol (TAG), glycerolipids, and fatty acyl-CoAs. These methods monitor metabolic pathways responsible for incorporation of fatty acids into storage lipid (TAG) and membrane

lipids (glycerolipids). For biofuel applications, the goal is to identify metabolic bottlenecks that limit the incorporation of specific fatty acids into TAGs.

- The Black laboratory developed a reliable MRM (multiple-reaction monitoring) method for high-throughput profiling of algal glycerolipid compositions. MRM methods take advantage of characteristic MS/MS fragmentation patterns to give a profile of molecular species selected by the investigator for monitoring. In order to increase speed and sample throughput, a method based on direct sample infusion was utilized to profile the major glycerolipid classes (PG, PS, PC, PE, PI and galactolipids MGDG, DGDG and TGDG) in the neutral lipid fraction of a Bligh-Dyer extract. Each class was profiled in a 5-minute infusion that monitored 40 to 50 molecular species (typically C30 to C40 species containing 0-8 double bonds). Quantitation was based on peak area ratios of each molecular species relative to an added internal standard and was done using MultiQuant software (ABSciex). In addition, a calculation step was added to correct for the small proportion of molecules that are two mass units heavier due to the naturally occurring distribution of ^{13}C isotopes. M+2 isotope species overlap glycerolipid species containing one less double bond. The correction for isotope distributions was critical in obtaining accurate quantitation of low-abundant species that are close in mass to highly abundant species. Certain classes of glycerolipids, if not highly abundant, will require an additional purification/enrichment step in order to give sufficiently high signals for accurate quantitation. Additional purification steps to enrich the glycerolipid fraction and reduce possible interference by other molecules present in the neutral lipid fraction such as diacylglycerides and triacylglycerides may also improve the accuracy of quantitation by this method. While this method cannot distinguish glycerolipid molecules of identical mass, which differ in their fatty acid constituents (for example, PG-18:0/18:2 and PG-18:1/18:3), it proved to be an efficient way to quantitate the overall abundance of each membrane lipid classes and characterize changes in fatty acid chain length and unsaturation.
- The Black laboratory established methods for the quantification and regiochemical determination of intact triacylglycerol (TAG) using ESI-LC-MS/MS. In response to the need for a high throughput means to quantify intact TAGs, the Black laboratory developed a method using Electro-Spray Ionization LC-MS/MS. Extracts are hydrogenated in a non-enzymatic process to produce saturated intact lipids, greatly reducing the complexity of the samples and allowing the use of multiple resonance monitoring (MRM). This results in a high-throughput absolute quantification of intact TAGs to the picogram including regiochemistry. The number and positioning of fatty acyl group double bonds is lost as a consequence, however the method is much more sensitive and greatly reduced the preparation time. Validation was done using commercially available oils and authentic standards. Tests using extracts of *Chlamydomonas reinhardtii* compared favorably to GC-MS and literature values.
- Acyl-CoAs are central intermediates in the formation of plant and algal oils, supplying the precursors for triacylglycerol synthesis. Acyl-CoA pools are typically very low in plants and algae, as these intermediates do not accumulate to high levels but are either rapidly incorporated into other molecules such as triacylglycerides, or are degraded by β -oxidation. When engineering plant or algal oil to contain a desired fatty acid composition, in particular if the incorporation of unusual fatty acids is desired (for

example short chain C8:0-C12:0 fatty acids for jet fuel applications), a buildup of these fatty acids in the acyl-CoA pool may indicate a metabolic bottleneck that limits the efficiency of incorporation into triacylglycerol. The Cahoon laboratory established methods for acyl-CoA measurements suitable for very small amounts of plant or algal biomass, typically 2-10 mg freshweight (fw) of tissue. The extraction methods developed are highly efficient for use with small amounts of plant tissue and algal biomass. Efforts are continuing to optimize extraction methods and will explore pre-column solid phase extraction steps to remove interfering substances that contribute to column degradation and the need for periodic column regeneration. Using the current approach, the Cahoon laboratory can detect fmol amounts of C8:0-C20:0 acyl-CoAs and can reliably profile total acyl CoA pools in which total acyl-CoAs are found in the range of 1-3 pmol/mgfw, and individual acyl-CoA species can be detected at levels of .01-0.1 pmol/mgfw.

- The Cahoon laboratory addressed the accumulation of short and medium-chain fatty acids (C8-C14), which are desirable components of biofuels intended for jet fuel applications. Metabolic engineering of plant and algal oils is now underway to achieve high levels of triacylglycerol (TAG) containing these fatty acids. This group has developed LC-MS methods to assess incorporation of medium chain fatty acids into TAG and phospholipids using both MRM and neutral loss scanning as methods of detection.
- The Cahoon laboratory developed advanced methods to identify and quantify plant and algal oils contain 16- and 18-carbon fatty acids in Q2 of 2012. Typical components of high (C14:0)-engineered seed oil include tri-C14:0, C14:0/C18:3/C14:0, C14:0/C18:3/C18:3, and C14:0/C18:3/C16:0. Using ESI-MS/MS, product ion profiles for individual triacylglycerol (TAG) species were obtained by direct infusion of purified transgenic Camelina seed oil (high C14:0), and optimal instrument voltages and potentials for the production of characteristic product ion fragments were obtained. This information was used to set up MRM (multiple reaction monitoring) methods for detection of the TAG species present in both plant and algal oil, and used in combination with chromatographic separation methods optimized previously for C8-C20 TAG species. Detection of C14:0 TAG was also done using a neutral loss scan (loss of C14:0 fatty acid), which provided a simplified profile of C14:0 containing TAG species and confirmed the MRM chromatographic profile. Retention times for each triacylglycerol species were established. The LC-MS method allows for a qualitative assessment of the relative amounts of triC14:0 compared to triacylglycerol species containing one or two C14:0 fatty acids, and also provides information on intact TAG structures which is useful in identifying potential bottlenecks in the pathway toward synthesis of tri-C14:0 TAG species.

A.2. Fatty acid biosynthesis in microalgae

- Bioinformatic data indicate that algae including *Chlorella* and *Chlamydomonas* have specific acyl-ACP-thioesterases that release fatty acids from acyl carrier protein (ACP) for export to the cytoplasm.
- Several specific acyl-ACP-thioesterases are being targeted for overexpression in the Cahoon laboratory to increase fatty acid pools that are subsequently activated to acyl CoA, which are then trafficked to other metabolic processes, including the synthesis of storage lipids (e.g., TAG) without compromising cell growth and fitness.

A.3. Targeting the acyl CoA synthetases to promote fatty acid secretion

- The DiRusso and Black laboratories have addressed the role of the acyl CoA synthetases and the question of fatty acid secretion; RNAi constructs have been generated in *Chlamydomonas*. This group has shown phenotypic changes in response to N depletion as well as overall growth characteristics. As expected, there are free fatty acids in the growth media, but levels are very low; current efforts are directed towards optimizing growth conditions and to define changes in acyl CoA synthetase activities with the goal of increasing secreted fatty acids.

A.4.1. Improved algal oil compositions for biofuels

- The Cahoon laboratory has preliminarily addressed Δ^{12} oleoyl CoA desaturase in *C. reinhardtii* using RNAi and microRNA (miRNA) techniques to enhance oil composition.

A.4.2. Another determinant of oil quality for biofuels is the carbon chain-lengths of the component fatty acids.

- These funds have provided the foundation of information required for continued studies for producing fatty acids with altered chain lengths in both *C. reinhardtii* and *Chlorella* species following experiments detailed above that are directed to further our understanding the lipid biosynthetic pathways. One possible strategy being addressed by the Cahoon laboratory will use a variant of acyl-ACP thioesterase of the fatty acid thioesterase B class in targeted algal strains.

Task B. Creation of molecular tools for genetic and biochemical manipulation of algae

B.1. Develop protocols for stable genetic transformation of *Chlorella* and related algal species.

- Three different approaches for DNA delivery continue to be tested: microprojectile bombardment of walled cells, electroporation or glass bead vortexing of spheroplasts in the presence of polyethylene glycol, and *Agrobacterium tumefaciens*-mediated gene transfer. These efforts spanned several labs including those of DiRusso, Cerutti and Black.

B.2. Define gene regulatory networks that control lipid accumulation in *Chlamydomonas* using transcriptomics analysis of cells grown phototrophically and subjected to nitrogen deprivation, in order to identify transcription factors and miRNAs involved in gene regulation of metabolic components (with an emphasis on the control of lipid biosynthesis and competing pathways). Some of the questions being asked include: What mRNAs encoding metabolic enzymes are induced/repressed and in which pathways do they participate? Is there coordinated regulation of certain metabolic modules? Are mRNAs encoding certain transcription factors up regulated prior to the expression of structural genes in specific biosynthetic pathway? Can network analysis be used for the identification of specific regulatory factors? And what small RNAs (e.g., microRNAs, siRNAs, etc.) are produced and what role do they play (via their action on target transcripts) in the regulation of lipid production?

- The Cerutti and Weeks laboratories are interested in examining the gene regulatory networks that control lipid accumulation in the unicellular green alga *C. reinhardtii* under nitrogen starvation. They have carried out a pilot transcriptomic analysis on cells grown photoautotrophically and subjected to nitrogen deprivation for 24, 48 or 144

hours (in comparison with cells maintained in nitrogen replete medium). Bioinformatics analyses revealed that transcripts for enzymes in certain lipid biosynthesis pathways (such as the Kennedy pathway of triacylglycerol-TAG-biosynthesis) appear to be coordinately regulated. In these preliminary analyses, nine putative transcription factors that show up-regulation under nitrogen starvation have been identified, correlating with changes in mRNA levels of TAG genes, and may play a role in regulating the expression of enzymes involved in neutral lipid biosynthesis.

- The Weeks laboratory completed studies of transcriptome changes in the alga, *C. reinhardtii*, associated with changes in external carbon dioxide levels in the environment. A carbon dioxide-concentrating mechanism (CCM) is essential for the growth of most eukaryotic algae under ambient (390 ppm) and very low (<100 ppm) CO₂ concentrations. In this study, they used replicated deep mRNA-sequencing and regulatory network reconstruction to capture a remarkable scope of changes in gene expression that occurs when *Chlamydomonas* cells are shifted from high to very low levels of CO₂ (≤100 ppm). CCM induction 30-180 minutes post CO₂ deprivation coincides with statistically significant changes in the expression of an astonishing 38% (5,884) of the 15,501 nonoverlapping *Chlamydomonas* genes. Of these genes, 1,088 genes were induced and 3,828 genes were downregulated by 4-fold or more. The latter indicate a global reduction in photosynthesis, protein synthesis and energy-related biochemical pathways. The magnitude of transcriptional rearrangement and its major patterns are robust as analyzed by three different statistical methods. *de novo* DNA motif discovery revealed new putative binding sites for *Myb*-family transcription factors potentially involved in activating low-CO₂-induced genes. The (CA)_n repeat (9 ≤ n ≤ 25), is present in 29% of upregulated genes but almost absent from promoters of down regulated genes. These discoveries open many avenues for new research.
- Microalgae are gaining attention as a potential feedstock for the production of biodiesel, mainly derived from triacylglycerols (TAG). In many algae, TAG synthesis increases dramatically upon certain stresses but this is often accompanied by growth retardation. Rational improvements to strain productivity are limited by inadequate knowledge of algal lipid metabolism and gene regulatory mechanisms. In this context, systems-level approaches aimed at understanding and modeling metabolic and regulatory networks may enable hypothesis-driven genetic engineering strategies. The green microalga *C. reinhardtii* accumulates significant amounts of TAGs under nutrient starvation and provides a genetically tractable model for manipulating lipid biosynthetic pathways. To gain insight into *Chlamydomonas* TAG metabolism, the Cerutti laboratory examined the transcriptome of strain CC-125 grown photoautotrophically in nutrient-replete or nitrogen-depleted media. Deep sequencing using RNA-seq technology revealed, under nitrogen starvation, a global decrease in transcript abundance for genes involved in photosynthesis and protein synthesis, whereas many genes related to nitrogen uptake and assimilation were up regulated. The Cerutti laboratory has carried out a detailed analysis of the transcriptome focused on genes encoding enzymes for TAG biosynthesis and/or degradation pathways. These components were identified based on orthology relationships to known TAG metabolism enzymes from land plants and fungi. In plants, fatty acids (FA) are mainly synthesized in the plastid by the FASII complex. Free FAs are then exported to the cytosol, converted to acyl-CoA and used predominantly in the ER for TAG synthesis. TAG/FA degradation involves the peroxisome located beta-

oxidation pathway. Interestingly, in *Chlamydomonas*, few genes encoding enzymes in these metabolic pathways showed significant changes in transcript abundance under nitrogen depletion, despite a marked increase in TAG accumulation under these conditions. The expression of most genes involved in FA synthesis or beta-oxidation did not change or showed some reduction. In contrast, several genes putatively involved in the Kennedy pathway of TAG synthesis were up regulated. The greatest increases in transcript abundance corresponded to *DGTT1*, *MLDP* and an MBOAT acyltransferase. A few putative transcription factor genes (including Factor X, see below) also displayed enhanced mRNA levels under nitrogen depletion and, in clustering analyses, correlated with the increased expression of the TAG synthesis genes. The Cerutti laboratory is now using RNA interference approaches to verify the functional significance of these transcription factors as well as of the lipid biosynthesis enzymes in the TAG accumulation that takes place under nitrogen depletion. These components may represent potential targets for the biotechnological improvement of oil production in microalgae. However, our findings are also consistent with the hypothesis that TAG synthesis in nitrogen-deprived *C. reinhardtii* occurs without extensive changes in transcript abundance for most genes specifically involved in lipid metabolism. Instead, post-transcriptional regulatory mechanisms and rerouting of carbon fluxes upon nitrogen limitation may be key determinants of TAG accumulation.

- To overcome some of these problems the Cerutti laboratory has developed a tandem inverted repeat RNAi system that allows for the direct selection of effective transgenic RNAi lines and the simultaneous silencing of two unrelated genes. In *Chlamydomonas*, tryptophan synthase β -subunit (encoded by the *MAA7* gene) converts the indole analog 5-fluorindole (5-FI) into the toxic tryptophan analog 5-fluorotryptophan. RNAi-mediated suppression of *MAA7* leads to strains resistant to 5-FI. Thus, selection for this RNAi induced phenotype permits the identification of transgenic lines showing effective interference of any (non-essential) gene co-targeted with *MAA7*, as part of the same dsRNA trigger synthesized from a tandem inverted repeat. Transcriptional self-silencing and other problems associated with genomic transgene integration are avoided by this selection strategy. The possibility of off target effects when using long hairpin dsRNAs (produced by the tandem inverted repeat transgenes) is circumvented by silencing each gene of interest with at least two separate transgenes, homologous to distinct and non-overlapping sequences of the target gene. If similar phenotypes are found in several independent RNAi strains generated with different transgenes, it increases confidence that the observed phenotypic abnormalities are the consequence of down-regulation of the intended gene. This method has been used to suppress the expression of the putative transcription factor genes identified in the transcriptomics analysis of nitrogen-depleted cells.
- An example where RNAi mediated suppression of Factor X, in two independent strains (RNAi-B1 and RNAi-S10), triggers neutral lipid accumulation (detected by Nile red staining) even in cells grown in nitrogen-replete medium. By comparison, wild type (non-transgenic) cells do not form lipid bodies under these conditions. This finding by the Cerutti laboratory may allow the development of production systems where microalgae can be grown to high density in nutrient replete medium and then trigger TAG accumulation (without changing the medium) by inducible RNAi-mediated suppression of Factor X and/or related gene regulators.

- In the context of algal crop protection, the Weeks laboratory has reported the development of genes that allow transgenic algae to grow in the presence of normally lethal doses of three different types of herbicides (glyphosate, sulfonylurea, and diphenyl ether herbicides). This group is presently completing an extensive characterization of the growth of transgenic algae in different concentrations of the herbicides and under different environmental conditions. In all cases, the herbicide resistance trait performs well and the algae subjected to the herbicides are not impeded in their growth. They have also established two separate collaborations that will allow us to test the usefulness of these herbicide resistance genes in other types of algae. This work is expected to include establishing greenhouse experiments to determine if the presence of one or more of the three herbicides can protect algae carrying all three genes from ill effects of deliberate contaminations with bacteria, fungi and non-desirable algae.
- The Van Etten laboratory led efforts by the Joint Genome Institute to sequence two chlorella isolates because some chlorella isolates have high lipid content. The sequence and annotation of one of the isolates, *Chlorella* NC64A [now named *Chlorella variabilis*, Blanc *et al* (2010) *Plant Cell* 22, 2943-2955] is the host for a rapidly expanding family of large dsDNA viruses. The sequence and annotation of the other alga - originally named *Chlorella vulgaris* C-169 - revealed that it was miss-classified and has been renamed *Coccomyxa subellipsoidea* C-169. C-169 is interesting because it is the first eukaryotic microorganism from a polar environment to be sequenced (it was originally isolated in Antarctica). Although, C-169 retained the same enzyme set of the core lipid metabolic pathways as other sequenced green algae, it encodes additional enzyme isoforms in fatty acid biosynthesis pathways that may be involved in cold acclimation to maintain membrane fluidity. These enzymes and metabolic properties are of interest in developing technologies for converting lipids from microalgae into diesel fuel or valuable fatty acids.
- In collaboration with the DOE Joint Genome Institute, the Weeks laboratory performed large scale RNA sequencing of samples taken from *Chlamydomonas* cells switched from growth conditions replete in CO₂ (1% or 10,000 ppm) to CO₂ depleted conditions (100 ppm). These conditions trigger the activation of the CO₂-concentrating mechanism (CCM), a complex system that allows for energy-driven uptake of carbon dioxide and bicarbonate to supply the CO₂ fixing enzyme, Rubisco, with sufficient CO₂ supplies to allow continued cell growth and survival. In collaboration with a computational biologist, this group has conducted an in-depth analysis of the transcriptomes at four separate time points (0, 30, 60 and 180 minutes) after shifting cells to limiting CO₂ levels. In a manuscript that will soon be ready for submission, the Weeks laboratory will report the discovery of over 4,000 genes whose transcription is strongly affected (*i.e.*, > 4 fold) either positively or negatively. This observation emphasizes the fact that adaptation of algal cells to low CO₂ concentrations is a major physiological response that is vital to the competitive survival of algae in their natural environment. This research has uncovered patterns of gene response that were missed in earlier, less extensive analyses using DNA microarrays and microarrays. Importantly, this work reveals evidence for regulatory elements in CO₂-responsive genes that may help to explain how so many genes are coordinately up regulated during the first few minutes of CO₂ withdrawal. Given that photosynthesis is essential for algal biofuel production under large-scale conditions and that the CCM is essential for photosynthesis, the present studies may prove valuable as

others and we begin to manipulate algal cells to make optimal use of the limited supply of CO₂ that is available under most present commercial algal production conditions.

B.3. Improve methods for gene knockdown/gene knockout in algae

- The Weeks laboratory developed TAL Effector Nuclease (TALEN) technology for highly efficient, targeted gene knockout and gene replacement in yeast. They have conducted experiments with artificially assembled TALENs designed to target specific genes in the model alga system, *Chlamydomonas reinhardtii*. One of the targeted genes, *FKB12*, encodes the rapamycin target protein that binds rapamycin and, as a result, can then bind to the Target of Rapamycin (TOR) protein. Upon binding of rapamycin to the *FKB12* protein (and its subsequent interaction with TOR), cellular metabolism is perturbed and cells die. If our attempts to target the *FKB12* gene for knockout with specifically designed TALENs are successful and the *FKB12* gene is disrupted, TOR will no longer be inhibited when rapamycin is present and cells lacking the *FKB12* protein will survive. Preliminary experiments with a mutant lacking a functional FKB protein have shown that the selection scheme should work as planned. To allow for selection of cells carrying the TALEN gene targeting the *FKB12* gene in the presence or absence of rapamycin, they have inserted the zeocin resistance gene into this vector. The zeocin resistance gene has been shown to be a highly reliable selectable marker for genetic transformation of *Chlamydomonas* cells.
- Initial experiments with these new TALEN vectors have shown lower than expected rates of *Chlamydomonas* transformation. The Weeks laboratory is presently investigating the cause of this phenomenon in hopes of determining if our vectors are properly constructed, if the TALENs, themselves, are somewhat toxic or if other factors are at play in decreasing transformation efficiencies. As an important demonstration of the power of TALEN technology, they have designed TALENs that target a specific disease susceptibility gene in rice and have demonstrated that a high percentage of transgenic rice plants expressing these TALEN genes are, indeed, resistant to the rice bacterial blight disease – a significant impediment to rice production in Asia. Not only was this trait shown to be genetically stable, it was also possible to use classical plant breeding techniques to cross transgenic plants with wild type rice plants to remove the TALEN genes that caused the gene knockout event while leaving the non-linked inactivated disease susceptibility gene in place.

B.4. Controlled expression of transgenes in *C. reinhardtii*

- These methods continue to be established and are directly linked to those studies detailed in B.3.

B.5. Develop additional selectable markers for genetic transformation that also can be utilized for maintaining axenic or near-axenic cultures of eukaryotic algae.

- The development of methods for maintaining axenic or nearly-axenic cultures of eukaryotic algae for purposes of producing algal biofuels and other valuable bio-products is to find inexpensive chemicals that can block the growth of unwanted bacteria, fungi (including yeast) and other algae is underway and included primarily efforts in the Weeks, Van Etten and Oyler laboratories.

B.6. Exploit virus-derived assets for metabolic engineering and product development

- The Van Etten laboratory has isolated *Chlorella* mutants that have developed resistance to virus infection and are trying to discover the biochemical and physiological nature of the resistance(s). In some cases, virus resistance is due to changes in the receptor molecule(s) on the host cell walls; i.e., the virus cannot attach to the resistant cells.
- The Van Etten laboratory are identifying *Chlorella* genes that might be involved in virus resistance by conducting a transcriptome analysis of their prototype *Chlorella* virus PBCV-1 during the early stages of virus infection, i.e., they are isolating mRNAs at 0, 7, 14, 20, 40 and 60 minutes after adding the virus to its host. The mRNAs will be sequenced as cDNAs and mapped to the virus and host genomes. Even though PBCV-1 infection of *Chlorella* NC64A eventually leads to virus replication and cell death, the Van Etten group anticipates that at least some host genes may be up regulated in the first few minutes that are involved in combating virus infection. Identification of these genes will be used to engineer algae so that they are resistant to virus infection.
- Development of vectors, which will be used to try and transform some of the *Chlorella* species, continues in the Van Etten Laboratory. Several gene constructs have been made and are being tested in the transformation system.
- Research in the Van Etten laboratory focuses primarily on large DNA viruses that infect chlorella-like green algae, including PBCV-1. The PBCV-1 genome is packaged in the virion with a predicted DNA density of $\sim 0.2 \text{ bp nm}^3$. Occasionally infection of an algal cell by an individual particle fails and the viral DNA is dynamically ejected from the capsid. This shows that the release of the DNA generates a force, which can aid in the transfer of the genome into the host in a successful infection. Imaging of ejected PBCV-1 DNA indicates that it is intimately associated with proteins in a periodic fashion. Most of the protein particles detected by atomic force microscopy have a size of 60 kDa and two viral-encoded proteins (A278L and A282L) of about this size are among 6 basic putative DNA binding proteins found in a proteomic analysis of DNA binding proteins packaged in the virion. A combination of fluorescence images of ejected DNA and a bioinformatics analysis of the DNA reveal periodic patterns in the viral DNA. The periodic distribution of GC rich regions in the genome provides potential binding sites for basic proteins. This DNA/protein aggregation could be responsible for the periodic concentration of fluorescently labeled DNA observed in ejected viral DNA. Collectively the data indicate that PBCV-1 has a DNA packaging strategy that differs from bacteriophages; it involves proteins and shares similarities to that of chromatin structure in eukaryotes.

Task C. Development and testing of improved algal photobioreactors and generation and characterization of camelid antibodies for improved algal harvesting strategies

C.1. Photobioreactor design and optimization. The focus of the research efforts in the UNL Algae Greenhouse Growth facility have shifted from advanced photobioreactor design to the development of integrated anaerobic digestion (AD), hybrid photobioreactor/open growth systems (hybrid growth system), advanced harvesting (see nanobody section) and extraction of lipids. Through assessment of the needs of the Midwest and the environmental conditions as well as extensive techno-economic analysis, AD to hybrid algae growth systems to premium value algae has been determined to be the best fit for the 40 degrees latitude and needs of the

Midwest for algae biomass production. The algae biomass is being investigated for a number of applications including aquaculture, ruminant feed, nutraceuticals applications and advanced biofuels. These algae biomass applications are consistent with the goals of this proposal for algal biofuels and bioproducts production. To accomplish the investigation of the integrated AD/hybrid growth system/premium algae biomass growth system, the Oyler laboratory has led these efforts.

- One initial aim was to develop low cost, high productivity flat panel photobioreactor (PBR) systems. This research and development of PBR was initially accomplished with Coastal BioMarine (CBM), a PBR start-up company. The design of this PBR involved low cost plastic film membrane and slug flow design channels leading to efficient mixing and gas transfer in a low cost PBR. The performance of the PBR in peak density of *Chlorella* (6 g/L) and volumetric growth rate 0.2g/L/day were superior to other PBR systems and less optimized hanging bags. However the seam design of the PBR system ultimately proved to be problematic with recurrent failures due to delamination of the suspended system. The lessons learned from the CBM bioreactor development and testing were further applied by another start-up company CGC to develop a horizontal thin plastic prism design that allowed low cost, high productivity design and enhanced reliability due to the horizontal positioning and slightly thicker “coke bottle” thickness plastic walls.
- Based on our experience and difficulties with the CBM prototype PBR, the Oyler laboratory set up simple and well-established cultivation systems (glass bioreactors, aquarium tanks, hanging bags and raceway ponds), which have successfully been scaled up from the 500ml starter flask stage to ultimate 1000L aquariums. Biomass densities typically reach 0.5-1.0g dry weight/L within 2 weeks using a combination of natural and artificial light. This facility is in the GW Beadle Center Greenhouse A1 on the University of Nebraska-Lincoln campus. Through experimentation in the large-scale cultivation of *Chlorella* and *Scenedesmus* species, we have enhanced our understanding of these organisms’ growth kinetics and lipid productivity while developing genetic fingerprints and lipid profiles for all the strains in our culture collection.
- The algae growth facility currently has 24 x 500ml and 3 x 1L Bellco magnetic stirred bioreactors, 6 x 8L Bellco stirred bioreactors, and 2 x 36L Bellco stirred bioreactor, 4 x 100L hanging bag bioreactors, 8 x 200 L glass open tank bioreactors and 2 x 250L small raceway bioreactors. Continuous centrifuge small to medium size (25L per hour) and pilot scale (1000 to 2000L per hour) are in place to facilitate the harvesting of the algae. There are two light racks for agar culture plate maintenance and use of magnetic stirring for the 500ml and 3L Bellco bioreactors that are part of the hybrid algae growth train.
- The Oyler laboratory established an active collaboration with Galen Erickson (Professor, Department of Animal Sciences, University of Nebraska) who is developing anaerobic digestion (AD) technologies with ruminant wastes and assisting in providing anaerobic digestion effluent from mesophilic AD. Efforts are continuing to explore thermophilic AD for nutrient feed to the hybrid algae growth system. Of note is the successful large-scale cultivation capacity and culture diagnostic capabilities for measuring culture integrity, cell density, and lipid profiles, to test the growth of *Chlorella* spp. both autotrophically, heterotrophically, and mixotrophically in using anaerobic digester effluent.

- As part of the analytical set up for large scale algal growth, a Seal Analytical AQ1 nutrient analysis system has been purchased, which allows automated assessment of the total nitrogen, ammonia, nitrate, and nitrite nutrient levels as well as phosphate, and other nutrients in our algae growth systems.
- The Oyler laboratory has established protocols for reliably measuring algae growth curves using hemocytometer counts, Coulter Counter counts, OD, chlorophyll content, and wet/dry biomass per liter of culture. These measurements are performed on the cultures on a daily basis. We have established protocols, which lead to reliable and consistent determination of these growth parameters in culture grown both on initially sterile defined media and in cultures grown with AD nutrient inoculum, which contain a polymicrobial community.
- The Black, DiRusso, Cahoon and Oyler laboratories have developed standardized lipid extraction and analytic protocols to assay total and neutral lipid content as a percentage of dry weight, triacylglycerol (TAG) and fatty acid types in the algae cultures (specific details are in Section A).
- The Oyler laboratory has developed a colony PCR technique used for quick determination of species-specific 18S rRNA genetic fingerprints. They have also developed a number of techniques to monitor cultures throughout their growth cycle. These techniques range from initial phylogenetic evaluation of each species and automated cell counting software to rapid chromatographic lipid measurement. A truncated phylogenetic tree has been developed that represents a small fraction of the *Chlorella* species in our culture collection. T
- To better characterize the optimal applications of algae biofuels and bioproducts production in the Midwest, a characterization of *Chlorella* species for fresh water algae bioproduct production was pursued in the Oyler laboratory. The ability of various *Chlorella* to produce lipids under rapid growth conditions, nitrogen deprivation conditions, and autotrophic to heterotrophic two-stage growth conditions was pursued. The ability of *Chlorella* species to grow well on Bold's Basal Media (BBM) was tested and genetic finger printing performed coincident with determination of growth parameters and defining lipid content under nutrient provision from AD systems and heterotrophic conditions.

C.1.a. The Oyler, Van Etten and Weeks laboratories have extensively characterized 25 of the best growing *Chlorella* in the AD/hybrid growth systems with regards to genotype, growth curves, biomass production, and lipid content on both defined media and AD supplemented media.

- The Oyler laboratory has determined that the best growing *Chlorella* achieve maximal density by 2 weeks growth and a cell count of greater than 30 to 45 million cells per ml, a dry biomass of 1 to 3 grams per liter, a lipid content of 20 percent total lipid dry weight and 5 percent neutral lipid in log phase growth and 30 to 50 percent total lipid and 20 to 30 percent neutral lipid after moderate nitrogen deprivation. These algae will grow with these parameters in fresh water supplement only with 5% AD effluent liquid fraction. The lipid content is largely C18 fatty acids with appreciable unsaturated fractions.
- The four best growing candidate production organisms are UTEX 1230 *Chlorella sorokiniana*, CS-01 *Chlorella sorokiniana*, UTEX B25 *Chlorella protothecoides*, and UTEX 395

Chlorella vulgaris. UTEX 1230, CS-01, and UTEX B25 all demonstrate heterotrophic lipid increase after autotrophic growth as well.

- The UNL Algal Group has established active collaborations with a number of other research groups on the assessment of the *Chlorella* production characteristics including University of Maryland, Johns Hopkins University, University of California-San Diego, University of Texas, and researchers at Los Alamos. Member of the University of Nebraska team including Drs. Tom Clemente and Paul Blum are applying the research from these growth facilities to provide algae biomass for aquaculture feed and are working closely with the lipidomics team members for the lipid analyses. They are beginning to characterize a range of *Scenedesmus* species for further analysis of fresh water algae for AD supplemented growth. They will also be assessing both salt and fresh water *Nannochloropsis* species, *Tetraselmis* and diatoms for biofuels and bioproducts applications in the integrated AD/hybrid growth/premium algae production system being developing in the UNL Greenhouse algae growth facility.
- One of the greatest challenges to growing algae for biofuel production in open-air environments is the threat of gross contamination by bacteria, fungi, viruses and other organisms. One approach to combating such contamination is to genetically engineer algae to contain genes that provide the host algal cell with protection against toxic agents such as broad-spectrum herbicides, but that kill most, if not all, microorganisms that invade the algal production facility. Toward achieving this goal, as detailed above (Section B), the Weeks laboratory has completed design of three genes that provide resistance to three major broad-spectrum herbicides, glyphosate, sulfometuron methyl and several diphenyl-ether herbicides. Earlier work with a mutant acetolactate synthase (ALS) gene from *Chlamydomonas* demonstrated that high-level resistance to sulfometuron methyl could be obtained with this full-sized ALS gene.
- The Oyler laboratory has addressed large-scale growth of *Chlorella* and *Scenedesmus* as biofuel production organisms in several different bioreactor configurations (mini raceways, hanging bags and stirred tank reactors) with either defined media (Bold's Basal Media – BBM) or 10% clarified anaerobic digester effluent supplemented water. The growth curves and lipid contents were compared for approximately 15 top performing *Chlorella* species in smaller liquid cultures. The *Chlorella* tested included members of *Chlorella sorokiniana*, *Chlorella kessleri*, *Chlorella varabialis*, *Chlorella protothecoides*, and *Chlorella vulgaris*. While there was similarity between several of the different *Chlorella*, *Chlorella sorokiniana* UTEX1230 and CS01 emerged as consistently the best growing organisms. The lipid content under nutrient replete and nutrient deprived conditions was assessed along with the increase lipid synthesis that could be induced on heterotrophic growth. The total lipid content of UTEX 1230 and CS01 under nutrient replete conditions was consistently in the 15 to 22% range and neutral lipid content approximately 2%. With nitrogen deprivation this total lipid content increased to 29 to 38% with a neutral lipid content of 16 to 24%. Several methods of rapidly evaluating lipid were compared to valid the procedures and improve the ease of lipid characterization.

C.2. Efficient harvesting of algae using nanobodies followed by efficient extraction of lipids and/or other high-value products represents a significant challenge to the economic viability of the algal biofuel industry. As these new technologies are developed, they can be applied to

commercial operations in which large quantities of algae need be harvested and processed inexpensively in a very short period of time.

- The final of 5 algae antigen booster immunizations were administered to two alpaca's. One animal was immunized with only sonicated *Chlamydomonas* and the other animal was immunized with *Chlorella* NC64A, *Coccomyxa* C169, *Nannochloropsis* OZ2, and *Thallasiosira pseudonanna*. The immune response was assessed after each boost by ELISA and Western blot analysis of the sera, which demonstrated appropriate and strong immune responses in each animal. A cDNA library was generated of the variable domain of the camelid heavy chain only antibodies from peripheral B cell lymphocytes and cloned into a surface display phage library. The library represented high complexity sufficient to encompass the full immune response diversity. The surface display phage library was provided to UNL from the Tufts subcontractor. Additionally the surface display library was panned repeatedly against intact live *Chlamydomonas* to generate 3 independent high affinity phage encoding nanobodies. The phages were provided to the UNL research team and have been cryopreserved.

C.2.a Bioflocculation using algal cell surface-binding VHs and immobilized of specific algae for membrane (cellulose) biofilm growth systems

- For bioflocculation of microalgae, an ultimate goal is to expression a surface-binding nanobody on the surface of a production organism (e.g., the diatom *T. pseudonanna*) that recognizes the surface of a second production organism (e.g., *Chlorella*). At time of harvesting, the two production organisms. Intermediate steps toward these photosynthetic platform organisms include VHH expression in *E. coli* and on the surface of yeast.

C.2.b Generation of algae-specific nanobody libraries

- Algae-specific nanobodies that can be manipulated using phage display technology to screen for nanobodies of interest – surface-binders in this case. Under a subcontract to the Tufts University Cummings School of Veterinary Medicine, the single-chain antibody library was generated by immunizing two Alpaca research animals with total protein extracts from five different commercially and academically relevant microalgal species: *C. reinhardtii* UTEX 2244, *Chlorella variabilis* NC64A, *Coccomyxa* sp. C-169, *Nannochloropsis* sp. OZ-1, and *Thallasiosira pseudonanna* CMMP 1335. For each of the organisms used, complete genome sequences are available. One Alpaca received only *C. reinhardtii* and the second Alpaca received the other four algae. 500mg of algae were injected after freezing, thawing, and sonication and dilution in 1ml of sterile normal saline.
- The immunizations proceeded in four injection cycles at two-week intervals without adverse effect to the animals. Two weeks after the last injection serum was harvested from each animal for establishment of immune response by ELISA for serum antibody. This procedure was performed with polystyrene microtiter plates with wells coated with intact algae from *C. reinhardtii*, *Nannochloropsis* or *Chlorella* NC64A. The first Alpaca which was injected with *C. reinhardtii* only showed a strong immune response by ELISA to *C. reinhardtii* but not *Nannochloropsis*, *Chlorella*, *Coccomyxa*, and *T. pseudonanna*, as expected. Similarly, Sedona (injected with *Nannochloropsis*, *Chlorella* NC64A, *Coccomyxa* C-169, and *T. pseudonanna*) demonstrated a good response to these four organisms, but

not *Chlamydomonas*. The sera response of these animals was also analyzed by Western blot analysis with pre-immunization as well as subsequent bleeds post-immunization and shows specificity immunoreactivity to algae.

- Based on these positive results peripheral lymphocytes and cell mRNA and cDNA from blood obtained approximately one week following the final immunization of each alpaca. mRNA and cDNA libraries were prepared and M13 phage display libraries encoding the VHH domain of the heavy chain only antibodies were generated consisting of at least 1,000,000 independent transformants with alpaca VHH coding DNA prepared from the cDNA.

C.2.c Panning of algae nanobody libraries for surface-binders by phage display

- The phage library was panned against intact production algae organisms to identify a small number of species-specific and high affinity single chain antibodies to the algal surface. These single chain antibody cDNA clones were isolated and expressed as fusion proteins in *E. coli*. Other suitable hosts may be used to allow further characterization. The 'best' unique VHH clones (defined as having different *Bst*NI and *Hae*III fingerprints) clones are identified as those producing the strongest signal on phage ELISAs on plates coated with algae extracts from each of the four algae species.

C.2.d Cloning and characterization of select nanobodies recovered by panning

- The Oyler and Weeks laboratories have successfully cloned over 9 *Chlamydomonas*, *Chlorella*, and *Nannochloropsis* surface-binding nanobodies into a pET32 bacterial expression vector. *E. coli* expressed *Chlamydomonas* binding VHH nanobodies were characterized by testing as Western blot primary antibody recognition against whole *Chlamydomonas* lysate. Very little clear immunoreactivity could be detected with any of the 9 *E. coli* expressed nanobodies by this screening method. The failure of VHH nanobody reagents to recognize denatured antigens on Western blots is not uncommon when the screening procedure with phage panning is against native protein targets in the intact cell as occurs in the present case. The next screening procedure involves immobilization of total algae extract in 96 well ELISA plates and then screening the *E. coli* expressed nanobodies along with positive and negative ELISA controls. By this procedure all 9 of the *E. coli* expressed VHH nanobodies identified by phage panning resulted in a robust ELISA signal. The ED₅₀ of the ELISA binding curves ranged from 200nM to 500pM for the 9 nanobodies. Focus was placed on the two best binding nanobodies B11 and H7, which exhibited ED₅₀ of 500pM and 2nM respectively. The immunoaffinity curves with these nanobodies for binding to intact *Chlamydomonas* cells in suspension was determined to be essentially the same as the solid substrate ELISA data with lysed algal cells for B11 and H7. This result strongly suggests that the nanobodies B11 and H7 recognize epitopes on the cell surface of intact algae cells. This binding quality is necessary for the immobilization and biofloculation applications. B11 and H7 were also expressed in *E. coli* as mCherry and GFP fusion proteins. These fluorescently tagged VHH nanobody fusion proteins were used in confocal microscopy to demonstrate surface binding to the intact algae. B11 demonstrates an even immunofluorescence over the complete cell surface while H7 demonstrates binding over the entire cell surface but distinct enrichment at the two flagellar bases. Fusions of the B11 VHH nanobody with cellulose binding domain (CBD) and mCherry were constructed and mCherry-B11-CBD demonstrate high affinity binding to both cellulose

membrane and to *Chlamydomonas* algae cells. The mCherry-B112-CBD also demonstrated capacity to immobilize growing *Chlamydomonas* cells preferentially to cellulose membranes. H7-B11 heterodimers, B11 homodimers, and H7 homodimers *E. coli* expression vectors have been constructed and the proteins expressed. These dimer constructs are predicted to have 1 to 2 orders of magnitude higher affinity for the algae cells than the monomers based on previous experience in VHH nanobody dimerization experiments with toxin directed dimers. These dimers will be tested for bioflocculation. Additionally mCherry-B11, mCherry-H7, mCherry-B11-CBD are being expressed on the surface of yeast cells to determine if such surface expression can be a viable means of making a biological flocculent.

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2011

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- Allen, J, DiRusso, CC and Black, PN. Quantification and regiochemical determination of intact triacylglycerides using ESI-LC-MS/MS [In Preparation]
- Allen, J, DiRusso, CC and Black, PN. Nitrogen dependency of analyte profiles and stable isotope labeling of galactolipids in the green microalgae *Coccomyxa* C-169 [In Preparation]
- Brueggeman A, Kuehler D, Falkowski P, Hildebrand M, and Weeks D. Genes conferring glyphosate and oxydiazon/oxyfluorfen resistance as dominant selectable markers and crop protection agents for diatoms [In Preparation]
- Brueggeman A, Kuehler D, and Weeks, D. Genes conferring glyphosate and oxydiazon/oxyfluorfen resistance as dominant selectable markers and crop protection agents for microalgae [In Preparation]
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- Kobayashi N, DiRusso C, Black P, and Oyler GA. Detection and quantification of triacylglycerol by HPLC-ELSD in *Chlamydomonas reinhardtii*. *J Chromatography B* [Submitted]

- Kobayashi N, Noel EA, Barnes A, Rosenberg JN, DiRusso C, Black P, and Oyler GA. A rapid method for monitoring triacylglycerol accumulation in *Chlorella* by HPLC-ELSD. *Bioresource Technology* [Submitted]
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- Van Etten JL (2010). Early events associated with infection by chlorella virus PBCV-1, Keynote talk at the First International Conference on Viruses of Microbes, Paris, France

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- Black, PN (2011). The Oily Side of Algae, School of Natural Resources, University of Nebraska-Lincoln, October 2011
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- Cerutti, H (2011). First International Conference on Algal Biomass, Biofuels & Bioproducts, July 2011, St. Louis
- Weeks, D (2011). The next wave of genetically engineered crops for worldwide agriculture, LEAD Program, University of Nebraska-Lincoln

- Weeks, D (2011). Algae as a source of renewable and sustainable biofuels, NSF Congressional Briefing, Washington DC
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- Van Etten, JL (2012). Giant viruses change the perception of viruses, American Society of Microbiology Annual Meeting, June, 2012
- Van Etten, J.L. Invited talk at the Second International Conference on Viruses of Microbes held in Brussels, Belgium. July 2012. Title: Chlorella viruses: possible infectious agents of humans and experimental animals.
- Van Etten, JL (2012). Chloroviruses: giant algal viruses, Annual meeting of the Mediterranean Infestation, Gordes, France

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Grants Funded:

2010

- (*This proposal*) Department of Energy, DE-FG36-08GO88055, Research for developing renewable biofuels from algae, PN Black, PhD, PI, 02/2010-07/2012, \$2,378,750
- National Science Foundation, MRI, Development of Coherent anti-stokes Raman scattering spectroscopy (CARS) microscopy, YF Lu, PhD, PI, PN Black, PhD Co-PI with 6 others, 07/2011-07/2012, \$266,460
- National Science Foundation, EPSCoR, Nebraska Center for algal biology and biotechnology, E Cahoon and H Cerutti, PhD Lead PIs, with 5 Co-PIs, 09/2010-09/2015, \$5,928,235
- Department of Energy (Sub-award from the University of California Dan Diego), Consortium for commercialization of algae biofuels and biotechnology, G Oyler, PhD, PI, with 2 Co-PIs, 09/2010-09/2013, \$1,808,704
- Nebraska Center for Energy Sciences Research, Integration of Anaerobic Digestion and Algae production, G Erickson, PhD, PI and GA Oyler, PhD, CoI, \$160,000
- Nebraska Center for Energy Science Research, Integration of Anaerobic Digestion Effluent and Flue Gas CO₂ capture with Algae Growth Systems, GA Oyler, PhD PI, \$12,000

2011

- Nebraska Environmental Trust - Partnership Grant (South Sioux City Nebraska) Integrated anaerobic digestion with algae bioenergy and aquaculture, G Oyler, PhD, PI, \$250,000
- University of Nebraska Office of Research and Economic Development Interdisciplinary Grant Program, Real-time monitoring of biochemistry in production of biofuels from biomass using coherent anti-stokes Raman spectroscopy and microscopy, YF Lu, PhD, PI/PD and PN Black, PhD, PI/PD, 07/2011-07/2013, \$200,000

2012

- Nebraska Energy Center, High throughput screening for compounds that induce lipid accumulation in algae for biofuel production, CC DiRusso, PhD, PI, 01/2013-12/2015, \$140,000

Grants Submitted:

2011

Pre-proposals submitted:

- National Science Foundation, Science and Technology Center: Center for Integrated Synthetic Bioprocess Control Systems (CISBiC), GA Oyler, PhD, PI \$25,000,000

2012

- National Science Foundation, Mechanisms to maximize algal oil production, 04/2013-03/2016, CC DiRusso, PhD, PI, \$599,506
- National Science Foundation, Partnership for Innovation; Building Innovation Capacity, Development of a single-chain antibody toolkit to manipulate the microalgal cell, GA Oyler, PhD, PI, \$600,000

- Department of Energy, Biosystem discovery and design for next-generation biofuels in oleaginous species of *Nannochloropsis*, D Weeks, PhD, PI/PD and I Ladunga, PhD, PI/PD, \$1,793,116
- National Science Foundation, Processes, devices, energy sources, and tools for sustainable energy research and education, Y Demirel, PhD, PI, and GA Oyler, PhD, CoI \$1,624,385
- Department of Energy, A systems biology approach to algae biomass and biofuels production. DOE BER. Microbial systems design for biofuels, from computer modeling to experimental validation, GA Oyler, PhD, PI, \$5,800,000

Pre-proposals submitted:

- Department of Energy, An integrated agro bioenergy nexus in South Sioux City Nebraska, DOE/USDA BRDI, UNL lead institution, G Oyler, PhD, PI, \$6,900,000.
- National Science Foundation, Emerging frontiers in research innovation- photosynthetic biorefineries. Development biofuels secreting synthetic leaf biorefinery module, G Oyler, PhD, PI, UNL lead institution, \$2,000,000