Biochemical Platform

Introduction

The Biochemical platform is focused on reducing the cost of converting lignocellulosic biomass to mixed dilute sugars and their further conversion to liquid transportation fuels. While ethanol is the Program's current fuel of focus, the Program is expanding its interest into other biofuel technologies which have the potential for development into successful integrated biorefineries. Biochemical conversion uses biocatalysts, such as enzymes and microorganisms, in addition to heat and chemical catalysts, to convert the carbohydrate portion of the biomass (hemicellulose and cellulose) into intermediate sugar streams which are then fermented to ethanol and other products.

Biochemical platform R&D will further improve the feedstock interface, pretreatment and conditioning, and enzymes and fermentation processes, in addition to process integration, in order to reduce ethanol costs. These economically viable technologies will act as a springboard for launching the next generation technology to produce ethanol and other products from a wide range of cellulosic feedstocks.

Platform Performance Goal

Reduce the estimated mature technology processing cost for converting cellulosic feedstocks to ethanol.

Objectives

- Short-term objectives are to reduce the modeled production cost of cellulosic ethanol to $1.33/gallon (2007$ estimated) by 2012.
  - By 2007, achieve a modeled cost target of $0.125 (2007$ estimated) per pound of sugars (equivalent to $2.43 per gallon of cellulosic ethanol) through the formulation of improved enzyme mixtures and pretreatments.
  - By 2012, develop improved saccharifying enzymes to meet the target of reducing the cost of enzyme systems to $0.10 per gallon of ethanol produced.
  - By 2012, validate integrated production of ethanol from corn stover at integrated scale at the NREL process demonstration unit facility.
  - Develop cross-cutting technologies useful for producing fuels (i.e., improved catalysts and biocatalysts)
- By 2017, validate integrated production of ethanol from switchgrass at pilot scale.

FY 2007 Accomplishments

- Creation of successful collaboration between the Feedstock and Biochemical Platforms with INL, NREL and ORNL as leading organizations. This group identified the most likely pioneer feedstocks to a biorefinery, analyzed how pre-processing methods could affect ethanol price, and developed an experimental plan for ensiled feedstocks.
- Continued operation of the Biomass Surface Characterization Laboratory (BSCL) at NREL. The relationships between pretreatment and the chemical/structural changes in corn stover stems that result in biphasic xylan hydrolysis have been better defined. Also, within this task, NREL has developed the first generation computational model of cellobiohydrolase I (CBH I) capable of describing structure and function, and verifying CBH I structure.
- The Consortium for Applied Fundamentals and Innovation (CAFI) is a university consortium, formed in 2001, of biomass pretreatment technology experts to investigate a wide variety of pretreatment processes (AFEX, Dilute Acid, Hot Water, Lime, etc.) for corn stover and hybrid poplar. Funding will continue through 2008 to expand pretreatment activities for switchgrass (CAFI 3).
- Increased xylose yield to 75% from 63%, in laboratory-scale high solids pretreatment reactor on corn stover.

- Issued a Funding Opportunity Announcement for improved ethanologens and selected five applicants to undertake R&D to develop microbial strains suitable for commercialization.
- Issued a Funding Opportunity Announcement for developing improved saccharifying enzymes that will allow for selection of potential awardees in FY 2008.
- Increased the platform range of technology options for meeting technical targets through advances:
  - Improved yields of ethanol from distillers dried grains; and
  - Production of value-added products from sugars.

Budget

The President's FY 2008 budget allows for the acceleration of research into cellulosic ethanol conversion from a wide range of feedstocks in order to meet the near and longer-term goals of the Biofuels Initiative. The FY 2008 budget is approximately $40MM.

2008 Plans

Ethanol cost reductions will reflect the results of work in the areas of pretreatment, conversion of cellulosic components of biomass to mixed, dilute sugar streams; and process integration. Specific objectives include determining which feedstock types will be used in pioneer plants, and reducing the severity (harshness) of thermochemical pretreatment while optimizing the digestibility of the pretreated material. Selecting optimal pretreatment chemistries along with improving the overall effectiveness of pretreatment processes; further reducing enzyme costs; and increasing the solids loading for the process to reduce equipment size, energy requirements, and reagent requirements will further reduce overall process costs.

In FY 2008, pilot-scale evaluation of one or more additional chemistries or configurations for thermochemical pretreatment will continue from 2007. Pretreated biomass will be reduced to simple sugars and residue by the action of hydrolytic enzymes. Further improvements are needed to: (a) increase the specific activity of cellulases; (b) exploit the synergy between cellulase and non-cellulase hydrolases that attack the hemicellulose, protein, waxes, perhaps lignin, and other compounds that contribute to recalcitrance; and (c) optimize the cellulase preparations to specific thermochemical pretreatments.

Specific planned activities include:

- Release a Funding Opportunity Announcement (FOA) soliciting applied R&D from the university system to address a wide range of topics related to improving biochemical processing.
- Select potential awardees from the FY 2007 issued FOA to undertake R&D to improve enzyme functionality leading to lower-cost enzymatic processing.
- Undertake a revised state of technology for the biochemical platform at NREL suitable for publication in FY 2009.
- Undertake an initial analysis of pretreatment options for switchgrass through the CAFI3 project with preliminary results available by the end of the fiscal year.
- Conduct multiple runs in the biochemical process demonstration facility at NREL to obtain operational data suitable for designing a fully integrated system available to process biomass to meet the 2012 cost targets.
- Participate in the Biomass R&D Board Biomass Conversion Science and Technology Working Group.

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Biochemical Platform Support

Biochemical Platform Analysis

Andy Aden, National Renewable Energy Laboratory

Principal Investigator:	Andy Aden	Funding Partners:	N/A
HQ Technology Manager:	Amy Miranda	Sub-contractors:	N/A
PMC Project Officer:	Gene Petersen		

Goals and Objectives: The objective of this task is to perform analysis for the Biochemical Platform in order to support the on-going research in biochemical conversion of biomass. This task supports the following research areas: pretreatment and enzymatic hydrolysis, processing integration, feedstock/process interface and targeted conversion research. Specifically, the biochemical platform analysis goals are to:

1) Identify task level targets that will contribute to the overall critical project targets.
2) Track and report on overall progress to the goal of $1.31 per gallon ethanol (using $2007) via Annual State of Technology assessments
3) Perform quantified risk/uncertainty assessments to evaluate risk of project components and uncertainty in process parameters and costs.
4) Evaluate alternative processes that might improve the process economics/feasibility
5) Integration with other groups' activities including
   a) Strategic analysis of biorefineries and the bioindustry
   b) Systems Integration activities including protocol development
   c) OBP program activities including planning and reviews
   d) Thermochemical platform analysis

Relevance to 30x30 goals: Success in technology deployment hinges on technically viable, cost effective processes. This task translates R&D targets, achievements and milestones into quantitative performance and cost measures that help identify the cost sensitive parts of the technology and subsequently the

priority R&D work to increase the technical and economic feasibility of the process under development. For the 2012 target, the analysis task provides annual evaluations of the state of the technology that play a role in determining whether the program is on schedule and if the plan should be altered based on findings during the year. For the 2030 goals, the analysis task develops advanced process designs that integrate current understanding of biomass conversion, possible learning curve improvements with advanced scientific concepts to get a picture of the ultimate potential of biomass.

Project Description: Platform analysis has historically either been part of the R&D projects or a separate project. Regardless of the program structure, analysis is closely tied to the R&D and program staff to provide information and guidance both to R&D projects and to program management. This analysis work is aligned with the planning documents for the program including multi-year program, technical and analysis plans. The goal of the techno-economic analyst is to have a closed loop communication with the researchers and OBP managers. The process-engineering analysts:

1) work with the researchers at the beginning of a project to determine what the targets should be and how they will be measured/reported;
2) provide process design, mass/energy balance modeling and economic analysis input to the project to direct and show progress; and
3) report analysis results to research staff and OBP management and work with both to keep the project focused.

This project is part of the NREL Biochemical Platform which conducts R&D across the breadth of fundamental, applied and integrated science to develop robust bioconversion technologies. Directed by rigorous process engineering and cost analysis, the R&D projects work together to address the key R&D barriers to technically feasible, cost effective ethanol production from biomass. Data and results from fundamental studies on biomass recalcitrance and enzyme action in the Targeted Conversion Research project are used to design improved pretreatment and hydrolysis technologies in the Pretreatment and Enzymatic Hydrolysis project. Those core technologies are tested in an integrated process to reduce the risk to commercial developers in the Biochemical Processing Integration project. The Feedstock/Process interface project in the platform ensures that the process technologies developed are complementary to feedstock collection and storage methods and vice versa. Additionally, the biochemical platform at NREL includes validation activities for the DOE ethanologen development projects to monitor progress to the platform's fermentation goals.

Summary of Work to Date - Accomplishments (FY05-current): The techno-economic analysis work to support the Biochemical Platform has been primarily focused on showing how research can affect the process to convert lignocellulosic biomass to sugars and ethanol. As such, many of the work products are incorporated into the researchers' milestones. Two major stand-alone reports are the design reports produced in 1999 and 2002, which detailed processes to produce ethanol from wood and corn stover, respectively. The annual State of Technology cases are other important work products, documenting the research progress and providing the basis for budget projections needed by OBP staff. State of Technology cases have been developed since 2000 to reflect technologies demonstrated in the lab and pilot plant. These cases address integration targets and document how well research is overcoming techno-economic barriers. Incremental developments required to achieve market target production costs have also been developed to aid OBP in developing their budget targets.

Other analysis of note in recent years includes:

- A Joule target completed in FY05 validated the cost of sugars from stover at $0.12/lb. This intermediate cost provides a more complete picture of the process economics for a biorefinery scenario (multiple products).
- Support to Pilot Plant's Pneumapress Operations. The Pneumapress experiments (in particular the hot wash experiments) were analyzed for component material balances and the resulting effect on the process economics determined. Hot washing the corn stover hydrolyzate did not have a significant effect on improving digestibility.
- Feedstock Study. Analysis was performed to determine the effect of corn stover variability on the minimum ethanol selling price in the market target case. This analysis was done using Monte Carlo

(uncertainty analysis) techniques developed in FY03. Variations in stover composition have a significant effect on the process economics of ethanol production.

- Liaisons to Enzyme Subcontracts. In FY05, a 4-year support effort concluded for the Novozymes and Genencor subcontracts to determine the impacts of the cellulase enzyme developments. Final enzyme preps were evaluated and the cost metric applied to determine the final almost 30-fold improvement from the contracts.
- High Solids Saccharification. Initial scenarios were run to determine if there are technical and economic benefits of performing saccharification at high solids and with high temperature cellulases. The effect of "process intensification" (running at higher solids) was found to be significant and further R&D work was planned in this area.

Schedule

Project Initiation Date: Friday, October 01, 1999
Planned Completion Date: Sunday, September 30, 2012

Preprocessing and Storage Systems Development/Qualification

Corey Radtke, Idaho National Laboratory

No project summary provided.

Biochemical Processing Core R&D

Pretreatment and Enzymatic Hydrolysis

Rick Elander, National Renewable Energy Laboratory

Principal Investigator:	Rick Elander	Funding Partners:	N/A
HQ Technology Manager:	Amy Miranda	Sub-contractors:	Hauser, Neoterics International, CICSCO, University of Georgia—Complex Carbohydrate Research Center, Oklahoma State University, Baylor University, Virginia Tech University, University of Maine
PMC Project Officer:	Gene Petersen		

Goals and Objectives: The objectives of Pretreatment and Enzymatic Hydrolysis Task include the investigation and evaluation of pretreatment approaches that are aimed at reducing the cost of pretreatment and increasing the enzymatic digestibility of residual cellulose and hemicellulose in pretreated biomass in a process relevant manner. Activities in this task are aimed at overcoming barriers associated with high capital and operating cost and sub-optimal sugar yields resulting from pretreatment and subsequent enzymatic hydrolysis of biomass. The effect of different pretreatment processes and chemistries on the enzymatic digestibility of pretreated biomass residues are being evaluated in terms of lowering the overall costs of saccharifying biomass that are associated with pretreatment and enzyme usage. Basic insights into the mechanisms of pretreatment catalyst transport and compositional and ultrastructural effects of pretreatment and enzymatic saccharification on biomass are continuing to be developed. Efforts are now expanding to include the investigation of how various pretreatment approaches and conditions affect hydrolyze toxicity properties and resulting hydrolyzate conditioning requirements and the associated response of ethanologens to various raw and conditioned hydrolyzates.

Project Description: This project is part of the NREL Biochemical Platform which conducts R&D across the breadth of fundamental, applied and integrated science to develop robust bioconversion technologies. Directed by rigorous process engineering and cost analysis, the R&D projects work together to address the key barriers to technically feasible, cost effective ethanol production from biomass. Data and results from fundamental studies on biomass recalcitrance and enzyme action in the Targeted Conversion Research project are used to design improved pretreatment and hydrolysis technologies in the Pretreatment and Enzymatic Hydrolysis project. Those core technologies are tested in an integrated process to reduce the risk to commercial developers in the Biochemical Processing Integration project. The Feedstock/Process interface project in the platform ensures that the process technologies developed are complementary to feedstock collection and storage methods and vice versa. Additionally, the biochemical platform at NREL includes validation activities for the DOE ethanologen development projects to monitor progress to the platform's fermentation goals.

More specifically, efforts in the Pretreatment and Enzymatic Hydrolysis Task are focused on understanding, developing and improving process concepts to achieve higher yields of monomeric sugars from important feedstock categories in pretreatment and enzymatic saccharification processes. The effects of pretreatment on enzyme requirements to achieve high sugar yields and on the properties of liquid hydrolyzates and their requirements for conditioning to allow for effective hydrolyzate fermentation are being investigated.

The proposed major activities in the Pretreatment and Enzymatic Hydrolysis Task for FY08 are listed below:

1. Understand pretreatment factors and implement equipment systems needed to achieve 2012 intermediate xylose yield and sugar degradation targets in continuous, pilot scale pretreatment systems.

2. Develop information on inhibitory compounds generated under different pretreatment processes and conditions and relate findings to hydrolyzate fermentability and conditioning requirements.
3. Further develop the concept of “fundamental reactivity” of feedstocks in order to achieve high sugar yields from pretreatment and enzymatic saccharification processes.
4. Develop improved knowledge of pretreated slurry rheological changes during enzymatic hydrolysis and relate findings to leading high solids saccharification process and equipment designs.
5. Apply findings from enzymatic oligomer saccharification systems to process concepts to reduce pretreatment capital and operating costs, lessen inhibitor product formation, and improve overall yields of monomeric sugars from less severe pretreatment conditions.

Summary of Work to Date - Accomplishments (FY05-current): In the period of FY05-FY07, the Pretreatment and Enzymatic Hydrolysis Task has been organized into 5 main subtasks:

- CAFI 2 Support Subtask
- Feedstock Qualification Subtask
- Enzymatic Hydrolysis Subtask
- Oligomer Saccharification (formerly Exploratory Saccharification) Subtask
- 30 x 30 Milestone Support Subtask

Activities of each of these subtasks are directly aligned with important intermediate milestones (as identified in the attached Project Milestone Summary Table) required to achieve the Biochemical Platform 2012 ethanol cost and process performance targets.

Key accomplishments in each subtask are discussed below:
CAFI 2 Support Subtask.
The Pretreatment and Enzymatic Hydrolysis Task has provided technical and logistical support to the Biomass Refining Consortium on Applied Fundamentals and Innovation “CAFI 2” project. We obtained and pre-processed corn stover and hybrid poplar feedstocks for all CAFI research teams and properly stored and distributed feedstocks to the CAFI research teams as requested. Compositional analysis of the raw feedstocks was performed and these results were provided to the CAFI research teams. The compositional analysis work revealed important lignin content differences in 2 different hybrid poplar batches, which translated into significant process performance differences and fundamental insights into pretreatment mechanisms for several of the CAFI pretreatment processes. Additionally, NREL provided dilute acid pretreated corn stover and hybrid poplar performance data and samples to various CAFI research groups for comparative enzymatic hydrolysis and hydrolyzate fermentability studies. The NREL dilute acid pretreated samples were generated in high solids bench-scale and pilot-scale pretreatment reactors and are therefore more process-relevant that pretreated samples available from the other CAFI research teams.

Most significantly, NREL, via a subcontract with Neoterics International, has been providing rigorous and comparative process economic models and analysis for each of the CAFI pretreatment processes on both corn stover and hybrid poplar. While the final process economic modeling finding are not yet complete, a number of general trends have become apparent across the various CAFI pretreatment approaches and the feedstocks being investigated in the CAFI 2 project. Acidic pretreatment processes are relatively insensitive to feedstock type (corn stover or hybrid poplar) and appear to perform reasonably well, although with some losses of sugars to degradation products. A greater sensitivity to feedstock type is seen with the hot water and alkaline pretreatment processes, with these processes generally achieving lower sugar and ethanol yields, and therefore higher predicted Minimum Ethanol Selling Prices (MESP) on the hybrid poplar feedstock. Differences in performance on some of the pretreatment process are also seen with the two different batches of hybrid poplar (low lignin and high lignin), which translate to different predicted MESP results. Some CAFI researchers have modified pretreatment process conditions in order to achieve better pretreatment and enzymatic hydrolysis performance on the high lignin poplar. As the final CAFI 2 pretreatment, enzymatic hydrolysis and fermentation data becomes available, the data will be used in the final updates of each respective pretreatment process economic model. These results will be presented as “current case” and “goal case” models for each pretreatment and each feedstock, including process yields and costs for any required hydrolyzate conditioning steps.

Feedstock Qualification Subtask

In the Feedstock Qualification Task, we have established a standard methodology for the systematic testing of selected important feedstocks across a range of pretreatment conditions using acidic, alkaline, or no-pretreatment catalysts. This is enabling the identification of appropriate pretreatment conditions for representative feedstocks and provides important information on the “relative reactivity” (defined as the total glucose and xylose yield achieved in both pretreatment and subsequent enzymatic hydrolysis) of the selected feedstocks. Initially, this work was performed in a multi-well MultiClave 10X pretreatment reactor and sand bath heating system, allowing for efficient screening of various feedstocks across a range of pretreatment chemistries and conditions. This work supported a FY06 Joule milestone to show that per pound minimum sugars selling prices of $0.11 to $0.135 for switchgrass and $0.105 to $0.125 for wheat straw from combined pretreatment and enzymatic hydrolysis resulting form this low-solids pretreatment screening apparatus was possible.

More recently, the findings from the low-solids pretreatment screening of switchgrass were applied to a more process intensive high solids ZipperClave pretreatment reactor. Combined pretreatment-enzymatic hydrolysis yields of greater than 75% xylose yield and greater than 85% cellulose to glucose yield resulting from pretreatment in the ZipperClave reactor under several process conditions was achieved. Performance between the low-solids MultiClave 10X pretreatment reactor system and the high-solids and the high solids ZipperClave reactor was compared. The pretreatment conditions were identical, except the ZipperClave was loaded at 25% solids versus 5% solids loading in the MultiClave 10X. Comparing the two reactor systems, both reactors showed similar trends of increased feedstock reactivity with increasing pretreatment severity. At lower pretreatment severity conditions, the MultiClave 10X achieved higher sugar yields than the ZipperClave. However, as pretreatment severity increased, the differences in overall reactivity decreased to less than 10% between the two pretreatment reactor systems.

These findings are now being applied to conduct more fundamental feedstock reactivity studies with the goal of further ascertaining factors that control the fundamental reactivity of various feedstocks. This work will highly leverage the chemical and biological processing fundamentals work being conducted within the Targeted Conversion Research Task. Additionally, work has been initiated to better understand how various combinations of feedstock and pretreatment approach affect the generation of potentially inhibitory compounds in hydrolyzate fermentation liquors. This work is primarily being conducted by university subcontractors to identify and quantify inhibitory compounds in hydrolyzates and to develop and implement genomic enrichment methods for use in revealing toxicity mechanisms of selected hydrolyzate compounds.

Finally, activities to support the capability enhancements and operational maintenance of the Biochemical Process Develop Unit (BCPDU) at NREL to service various programmatic activities associated with Biochemical Platform and Integrated Biorefineries Platform have been managed and supported by the Feedstock Qualification Subtask.

Enzymatic Hydrolysis Subtask

One key activity in the Enzymatic Hydrolysis Subtask has been to conduct experimental studies to quantify the effect of solids concentration and background sugars on enzymatic cellulose saccharification on dilute acid pretreated corn stover. Process economic analysis has indicated that operating the enzymatic hydrolysis under high solids conditions without separation and washing steps to remove soluble compounds in pretreated slurries are economically advantageous, but the effects of such a process intensive reaction environment for enzymatic hydrolysis is not fully understood. The results were compared to control conditions where the pretreated corn stover was washed to remove monomeric and oligomeric sugars and other soluble compounds there were released during pretreatment that could inhibit enzymatic saccharification performance. Experiments were carried out at different enzyme loadings, solids concentrations, and temperatures. It was found that the enzymatic saccharification performance was affected by both solids concentration and enzyme loading, as high solids loadings resulted in lower glucose yields, presumably to sugar product inhibition and/or mass transfer limitations under high solids conditions. Also, it was found that the unwashed pretreated slurries, where sugars and

other compounds solubilized during pretreatment remain, achieved significantly lower enzymatic saccharification yields than the control experiments under identical conditions using thoroughly washed pretreated slurries. These findings indicate that there are significant challenges in applying commercially available enzyme systems to such process intensive reaction conditions that will require further understanding of enzymatic hydrolysis process and reactor design, along with improved knowledge of the inhibitory nature of compounds solubilized during pretreatment upon saccharification enzyme systems.

As motivated by process economic analysis findings, another key element of the Enzymatic Hydrolysis Subtask is to develop improved knowledge of pretreated slurry rheological changes during enzymatic hydrolysis and relate these findings to leading high solids saccharification process and equipment designs. Work has been performed to characterize how changes in rheological properties of slurries undergoing enzymatic saccharification relate to enzyme digestibility performance. Using pretreated slurries described in the high-solids pretreated corn stover enzymatic saccharification study described above, results from rheological measurements also showed that viscosity changes were more pronounced during digestions with the lower initial solid concentrations of 25% and 30%, when compared to digestions at higher initial concentrations of 35% and 40%. These results indicate that lower conversions at high solid concentrations are likely due to sugar inhibition of enzymes. At higher solid concentrations, it is likely that enzymes are inhibited either due to lack of sufficient free water or due to build up of sugars in the liquid phase. Further kinetic and rheological studies are required to better elucidate this decrease in digestibility. These advanced studies are being conducted using a new advanced rheometer system at NREL, along with two-university led subcontracts to more fundamentally characterize rheological properties of pretreated slurries and how those properties change during enzymatic hydrolysis. These findings are being applied to develop leading process and reactor design systems for high solids enzymatic saccharification. This aspect of work is led by an engineering design firm subcontractor.

Oligomer Saccharification Subtask

As motivated by programmatic drivers to lower the cost of the pretreatment operation and to achieve higher overall sugar yields with lower losses to inhibitory sugar degradation products, milder pretreatment approaches are being investigated in many aspects of the Biochemical Platform. In many cases, such pretreatment approaches may decrease the extent of hemicellulose hydrolysis that occurs during pretreatment and may result in greater proportions of oligomeric sugars from pretreatment that will ultimately need to be converted to monomers for fermentation. Such a process configuration will likely require a variety of enzyme activities to fully liberate monomeric sugars from cellulose, any remaining hemicellulose, and soluble oligomers resulting from such milder pretreatments.

Work in the Oligomer Saccharification Subtask is focusing on identification and application of enzyme systems to effectively convert oligomers released from such pretreatment conditions. Closely related work on insoluble hemicellulosic compounds in mildly pretreated biomass is being conducted within the Targeted Conversion Research Task in a highly leveraged manner (and was conducted within the Pretreatment and Enzymatic Hydrolysis Task in FY05 and FY06). This work has indicated that the addition of hemicellulase components (particularly xylanases) and in some cases, esterases, appear to work in a synergistic manner to improve the saccharification performance of both cellulose and hemicellulose. As specifically related to soluble oligomers in mild pretreatment hydrolyzates, a significant amount of advanced analytical work (LC-MS and MS-MS) has been performed to develop improved methods of complex oligomer identification, including identification of side-chain properties. These methods have shown that both the size and the complexity of oligomers is greater from milder pretreated conditions, and that there are fewer ester-linked side chains in mild alkaline pretreatments than in mild acid pretreatments. It has also been shown than xylanases, esterases, and in particular, xylosidases all demonstrate a significant impact on converting xylo-oligomers to monomeric xylose. To support these efforts, development of high-throughput enzyme-linked assays for glucose, xylose, and cellobiose determination is being conducted to improve the efficiency of the enzyme screening studies. Finally, university-led subcontracts have been awarded to generate enzyme genes for hydrolysis of recalcitrant biomass and oligomers, as well as to further identify structures for pretreatment hydrolyzate oligomers.

30 x 30 Milestone Support Subtask

The 30 x 30 Milestone Support Subtask was formed in FY07 to directly support FY07 and FY08 intermediate xylose yield milestones leading to the 2012 Biochemical Platform ethanol cost and process performance targets. Recently, the FY07 xylose yield milestone “Achieve 75% xylose yield in laboratory scale high solids pretreatment reactor on corn stem internode” was completed. In order to report results with greater process relevance, whole corn stover was used instead of a more narrow corn stem internode feedstock. These experiments were conducted using a variety of dilute acid conditions in two reactor systems (ZipperClave reactor and steam explosion reactor) at feedstock total solids loadings of greater than 45% using dilute acid pre-impregnated whole corn stover using two different feedstock particles sizes (¼ inch and ¾ inch milled corn stover). Eleven of the 14 experimental conditions exceeded 75% total yield of both monomeric and oligomeric xylose, with seven of the experimental conditions exceeding an 80% total xylose yield. These results were achieved in both reactors for both particle sizes. Four of the 14 experimental conditions exceeded a 75% yield of monomeric xylose, with two of the experimental conditions meeting or exceeding an 80% yield. The highest yield of xylan to total xylose was 87% at 180°C for 90 seconds using the steam explosion reactor. The 87% total yield value included a 5% oligomeric xylose yield and an 82% monomeric xylose yield.

There are significant challenges in achieving the higher xylose yield targets in continuous pilot-scale pretreatment reactor systems in 2008 and beyond. These include temperature control, feedstock heterogeneity and anatomical fraction segregation, less-than-ideal pretreatment catalyst impregnation, potentially detrimental feedstock compression in the plug-screw feeder, sensor calibration, process control, and residence time variability, any of which could contribute to lower xylose yields in such reactor system. To overcome the current limitations at larger scales, we must better understand both the fundamental reaction mechanisms and kinetics during pretreatment under process relevant operating conditions and the impacts that specific reactor equipment have on pretreatment performance. In future work, we propose to test hypotheses related to upstream processing operations on pretreatment performance. Some examples are the methods for pretreatment chemical impregnation and dewatering, feedstock milling, how feedstock is fed into the high pressure pretreatment reactor, reactor residence time and internal mixing, and pretreatment reactor discharge and decompression, among others. These factors will be studied in designed experiments at the bench scale and will provide guidance to future operations in continuous pretreatment systems.

Pilot-scale pretreatment equipment capabilities enhancements are currently being pursued that will increase the range of high solids operating conditions and upstream processing options that will be available in our efforts to achieve better pretreatment performance in the existing continuous pilot-scale pretreatment reactor systems at NREL. These enhancements include the procurement of a steam jacketed reactor shell for the 200 kg/day horizontal pretreatment reactor system, along with the design and installation of a non-compression feeder option (in addition to the existing compression screw feeder system) that will be integrated into the horizontal pretreatment reactor system. These equipment capabilities will be heavily utilized to achieve the FY08 (and beyond) pretreatment R&D area xylose yield milestone in continuous reactor systems.

Finally, while the xylose yield targets achieved in this milestone report were produced directly as a result of xylan hydrolysis in the pretreatment step, additional efforts both within and outside of the Biochemical Platform are investigating pretreatment approaches that may not achieve extensive xylan conversion to monomeric xylose in pretreatment. But such approaches, especially when combined with advanced hemicellulase and accessory enzyme systems, may provide for a cost effective means of achieving the higher overall xylose yields (ultimately, 90% monomeric xylose yields) resulting from both pretreatment and enzymatic hydrolysis steps for some feedstock types, including corn stover. These efforts are on-going in the Pretreatment and Enzymatic Hydrolysis Task (Feedstock Qualification and Oligomer Saccharification Subtasks), the Targeted Conversion Task, and the CAFI 2 project. Findings from these efforts will be evaluated and considered in strategies to achieve the higher xylose yield targets in intermediate milestones leading to the 2012 targets.

Schedule

Project Initiation Date: Friday, October 01, 2004
Planned Completion Date: Sunday, September 30, 2012

Novel Enzyme Products for the Conversion of Defatted Soybean Meal to Ethanol

Larry Allen, Lucigen Corporation

Principal Investigator:	Dr. Phillip Brumm	Funding Partners:	N/A
HQ Technology Manager:	Amy Miranda	Sub-contractors:	U.W. Dept of Bacteriology
PMC Project Officer:	Kevin Craig		

Goals and Objectives: The purpose of this work is to overcome the recalcitrance of cellulosic biomass by developing enzyme products capable of substantially degrading the carbohydrates in defatted soybean meal (DSM) into a fermentable substrate suitable for alcohol production.

Project Description: The purpose of this work is to overcome the recalcitrance of cellulosic biomass by developing enzyme products capable of substantially degrading the carbohydrates in defatted soybean meal (DSM) into a fermentable substrate suitable for alcohol production. This project will clone, express and characterize thermostable bacterial enzymes capable of degrading >70% of the carbohydrates in DSM into monosaccharides and disaccharides fermentable by yeast. The fermentability of the monosaccharides and disaccharides will be confirmed in alcohol fermentations using *Saccharomyces cerevisiae*. To achieve this goal, the following work will be performed.

Two thermostable cellulases will be cloned and over expressed in the *B. subtilis* system. Four thermostable *beta*-glucosidases will be identified either by screening of small insert libraries of thermophilic organisms on plates containing 4-methylumbelliferyl-β-D-glucopyranoside, or by *in silico* analysis of genome sequence information generated by JGI. The *beta*-glucosidases identified by *in silico* analysis will be recovered by PCR amplification from genomic DNA. At least two of these *beta*-glucosidases will be subcloned and expressed in the *B. subtilis* system for characterization and evaluation. Four thermostable *alpha*-galactosidases will be identified either by screening of small insert libraries of thermophilic organisms on plates containing 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-Gal), or by *in silico* analysis of genome sequence information generated by JGI. The *alpha*-galactosidases identified by *in silico* analysis will be recovered by PCR amplification from genomic DNA. A minimum of two of these will be subcloned and expressed in the *B. subtilis* system for characterization and evaluation. Four to six thermostable hemicellulases will be identified by *in silico* analysis of genome sequence information generated by JGI. The hemicellulases identified by *in silico* analysis will be recovered by PCR amplification from genomic DNA. A minimum of four of these will be subcloned and expressed in the *B. subtilis* system.

The cloned and secreted enzymes will be purified and characterized for temperature and pH ranges, the range of substrates utilized, and the products produced. The enzymes will be evaluated for their ability to improve the fermentability of soy meal by *Thermoanaerobacterium saccharolyticum* B6A, both as individuals and as combinations of enzymes. The best enzyme combinations will then be evaluated in alcohol fermentations using *Saccharomyces cerevisiae*. A large fermentation will be performed using the best blend of enzymes, and the protein quality of the material remaining after fermentation will be determined.

Summary of Work to Date - Accomplishments (FY05-current):
Task number: A Host and vector construction
Subtask A1 Develop sporulation and protease negative strain of *B. subtilis*
The production strain was constructed via DNA mediated transformation of the low protease strain of *Bacillus subtilis* 1A751.

Subtask A2 Develop shuttle vector for *B. subtilis* and *E. coli*
Two *E. coli/B. subtilis* shuttle vectors capable of secreting cloned enzymes have been constructed. The first secretion plasmid was derived from pNW33N (obtained from the Bacillus Genetic Stock Center). This plasmid replicates in *E. coli, B. subtilis* and *B. stearothermophilus*. Chloramphenicol is the selectable antibiotic in all three bacteria. The second vector, pB7, was derived from the *E. coli* plasmid pSMART

LCKan (Lucigen Corp.) and pHCMC02. pHCMC02 replicates in *E. coli* and *B. subtilis*. An *E. coli* clone containing a 2 kb insert with a thermostable xylanase gene, its promoter and signal peptide was amplified using vector sequencing primers and blunt cloned into the SmaI site of the pB7 bacillus shuttle vector. The ligation waste transformed into *E.coli* and plated on agar plates containing 0.1mg/ml 4-methylumbelliferyl-β-D-cellobioside (MUC). Colonies that showed activity were confirmed by restriction analysis before transformation into *Bacillus subtilis*. Heat-treated supernatant from cultures was tested for secretion of active thermostable xylanase protein using AZCL-Xylan (Megazyme). Supernatants containing active thermostable xylanase enzyme were run on an SDS gel with purified xylanase; the SDS PAGE confirmed the expression and secretion of thermostable xylanase by *Bacillus subtilis*.

Task number: B Cellulase and *beta*-Glucosidase Cloning

A genomic library from the thermostable cellulase producing organism *Clostridium thermocellum* ATCC 27405 was prepared (*Cl. thermocellum* culture a gift of Dr. Paul Weimer) and used to transform electrocompetent *E. coli* cells. A novel soluble cellulase from *Clostridium thermocellum*, designated Cth10H6, was identified. Library clone Cth10H6 containing the cellulase enzyme was sequenced. The sequence aligns to a cellulase from *Clostridium thermocellum* (genbank accession AM62817). Primers were designed for the cellulase ORF without the predicted signal peptide and used to amplify the 1.6kb gene from library the clone Cth10H6. The cellulase ORF was ligated to pET vector. The ligation was transformed into BL21 (DE3) *E. coli* and activity was selected for on plates using 0.1mg/ml 4-methylumbelliferyl-β-D-cellobioside (Research Products International Corp.). Five expressing clones were analyzed for protein production using SDS gel and reducing sugar assays from lysate. The final clone was sequenced confirmed. Cth10H6 cellulase, was also transformed Lucigen OverExpress™ BL21(C43) cells; these cells have a history of improved production of lethal proteins. The gene was also cloned into BL21 Rosetta Stone (Novagen) to overcome any problems with rare codons. Cells were grown to log phase in a tryptone-free medium to prevent premature expression, induced with 1 mM IPTG for overnight, harvested by centrifugation, and lysed by sonication. Protein production levels were examined by enzymatic assay and SDS PAGE; no improvement in cellulase production was observed with either strain of *E. coli*.

In an effort to identify more cellulases the sequence of Cth10H6 cellulase (described previously) was blasted (tBlastx) against the completed genome sequence assembly of *Clostridium thermocellum*. Three high homology hits were identified: Cthe2872, Cthe0536, and Cthe2147. Cth10H6 and all three candidate cellulases are predicted to have signal peptide sequences in the protein. Primers were designed to amplify each of the four genes plus 400bp of flanking sequence on either side of the ORF. The cellulases plus flanking sequence were PCR amplified directly from *Clostridium thermocellum* genomic DNA. The PCR fragments were blunt cloned into the SmaI site of the pB7 *bacillus* shuttle vector. The ligations were transformed into *E.coli* and plated on agar plates containing 0.1mg/ml 4-methylumbelliferyl-β-D-cellobioside. Colonies of Cth10H6 were weakly active on the plates, Cthe2872 colonies were moderately active and colonies of Cthe0536 and Cthe2147 showed no activity. Restriction analysis confirmed cloning of fragments for Cth10H6, Cthe2872 and Cthe2147. Cthe0536 did not clone, only empty vectors were found. The three cellulase clones were transformed into *Bacillus subtilis* and tested for secretion of active cellulase protein on AZCL-HE Cellulose reagent (Megazyme). The Cthe2872 clone did produce active cellulase protein secreted into the growth media; Cth10H6 and Cthe2147 clones did not. A 2L culture of the Cthe2872 clone in *Bacillus subtilis* was grown, the supernatant was clarified by centrifugation, and proteins were ammonium sulfate precipitated. The crude cellulase is awaiting further purification/characterization.

Thermophilic organisms producing *beta*-glucosidase were identified by plating planktonic and biofilm samples from the hot springs at Yellowstone National Park on plates containing 4-methylumbelliferyl-β-D-glucopyranoside. Over 12 isolates were recovered using this method. Positive colonies were restreaked, grown in liquid culture, and preserved at –80°C. Four of these isolates were grown in 2 liters of clear medium, harvested and used for genomic DNA preparation. The DNA preparations were used to construct small insert libraries for whole genome sequencing by the Joint Genome Institute; sequencing of these genomes is currently underway. The results from genome sequencing will be used to generate primers for PCR amplification of the *beta*-glucosidase genes from these organisms. Genomic DNA was prepared from 12 additional hot springs isolates; 8 of the isolates were positive on plates containing 0.1%

4-methylumbelliferyl-β-D-glucopyranoside (MUG). The genomic DNA was transferred to the Joint Genome Institute for whole genome sequencing of these organisms as part of an ongoing project with the JGI. When sequencing is completed, the genomic sequences will be used to design PCR primers for amplification of the *beta*-glucosidase genes from these organisms.

An acidophilic, thermophilic organism was isolated from an environmental site in Wisconsin. Genomic DNA was used to prepare a small insert library in *E. coli*, and the library was screened for cellulases using plates containing 4-methylumbelliferyl-β-D-cellobioside. A positive colony was picked, grown, and the activity of the clone determined. Based on activity assays, the enzyme was shown to be a *beta*-glucosidase rather than a cellulase, and designated AAC-1. Library clone AAC-1 containing the *beta*-glucosidase enzyme was sequenced. The sequence aligns to a putative gene from *Alicyclobacillus acidocaldarius* described as having *beta*-glucosidase and *beta*-galactosidase activity (genbank accession DQ092439). Primers were designed for the *beta*-glucosidase ORF and used to amplify the 1.4kb gene from the library clone AAC-1. The *beta*-glucosidase ORF was ligated to the prepared pET). The ligation was transformed into BL21 (DE3) *E. coli* and activity was selected for on plates using 0.1mg/ml 4-methylumbelliferyl-β-D-glucopyranoside. Five expressing clones were picked and analyzed for protein production.

Task number: C *alpha*-Galactosidase

Over 20 thermophilic organisms producing *alpha*-galactosidase were identified by plating of either local environmental samples or planktonic and biofilm samples from the hot springs at Yellowstone National Park on plates containing 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-Gal). Positive (blue) colonies were restreaked, grown in liquid culture, and preserved at –80°C. Two of these isolates were grown in 2 liters of clear medium, harvested, and used for genomic DNA preparation. The DNA preparations were used to construct small insert libraries for both whole genome sequencing by the Joint Genome Institute and for screening in a melA deficient strain of *E.coli*. Eight additional thermophilic organisms producing *alpha*-galactosidase were identified from the hot springs at Yellowstone National Park All eight of these isolates were grown in 2-4 liters of clear medium, harvested, and used for genomic DNA preparation. The DNA preparations were used to construct small and large insert libraries for whole genome sequencing by the Joint Genome Institute. Currently we are awaiting the results of sequencing by JGI to identify the *alpha*-galactosidase genes of these organisms; we will then amplify the genes directly from genomic DNA by PCR and clone the enzymes into pET vector .

Transposon mutagenesis and screening was used to identify a number of *E. coli* strains deficient in *alpha*-galactosidase activity (melA gene product); these strains give white colonies when grown on plates containing X-α-Gal. One of these clones was used to prepare high efficiency electrocompetent cells for library screening. Libraries from two of the isolates were transformed into the *E. coli* strain deficient in *alpha*-galactosidase activity. The transformants were plated onto plates containing X-α-Gal. Blue colonies were picked and restreaked three times to obtain colonies with stable inserts Sequencing of one of the two *alpha*-galactosidase positive clones was completed; the gene is highly homologous to the reported *Geobacillus kaustophilus alpha*-galactosidase gene. The gene has been cloned into a pET expression vector and produced at high levels. Characterization of this enzyme is ongoing.

The second gene could not be sequenced. Attempts to prepare plasmid containing the entire gene were unsuccessful; most plasmid recovered contained large deletions in the insert. The apparent cause of these deletions was the use of a high copy vector for the library construction. The library construction will be repeated using a low copy number vector, if necessary.

Task number: D Soy Hemicellulase Cloning
Work scheduled to begin July 1, 2007. We are awaiting whole genome sequencing results from JGI.

Task number: E Evaluate individual enzyme and enzyme combinations

Work scheduled to begin July 1, 2007. CRADA agreement with Paul Weimer of USDA Forage Research Center was written and submitted to the USDA for approval. Preliminary evaluation of enzymes on defined substrates and soy meal are underway.

Cost and Schedule

Project Initiation Date: October 1, 2006
Planned Completion Date: December 31, 2009

Energy Corn Consortium

Michael Blaylock, Edenspace Systems Corporation

Principal Investigator:	Michael Blaylock	Funding Partners:	NREL, Oklahoma State University
HQ Technology Manager:	Amy Miranda		
PMC Project Officer:	Fred Gerdeman	Sub-contractors:	

Goals and Objectives: As the principal member of the Energy Corn Consortium, Edenspace is working with NREL, Oklahoma State University, and several other partners on a recently funded USDA/DOE proposal "Energy Corn for Ethanol Production" (Funding Opportunity Announcement Number DE-PS36-06GO96002F). Edenspace is the primary partner in this consortium and is the focal point for work done by other consortium members. The primary research role of Edenspace is to implement technology developed in collaboration with NREL and Oklahoma State University into a commercially viable product, i.e. "Energy Corn^{tm}". As part of this effort, OSU is mining multiple bacterial genomes and cloning the genes of potentially useful enzymes into expression vectors. NREL will characterize and evaluate enzymes cloned by Oklahoma State University for their efficacy in biomass conversion. NREL, in collaboration with OSU, is developing a high throughput screening protocol for enzyme synergy testing on relevant substrates such as corn stover, switchgrass, and other potential biofuels feedstocks. Promising candidate genes will then be expressed in plants by Edenspace and evaluated by NREL. Edenspace will select the genes most effectively expressed in plants for incorporation into Energy Corn.

Project Description: The overall goal is the development and demonstration of a commercially viable line of Energy Corn^{tm} amenable to auto-digestion in a biomass-to-ethanol plant. Factors impacting this highly desirable function include selection/development of a suitable corn variety, *in planta* expression of the enzymes necessary to digest the biomass, development of a mild pretreatment or "enzyme activation" step to induce the autohydrolysis, and a pilot-scale demonstration of the technology. The project is broken down into discreet areas of research and integration according to the strengths of the partners.

Oklahoma State University will identify, clone, and express biomass hydrolyzing genes from a variety of sources in several expression vectors. These vectors, after transformation into suitable expression hosts, will be provided to NREL for expression, purification and evaluation of the enzymes. After expression of the desired activity, the active enzyme will be purified, assayed for activity on a suitable substrate or analogue, and then evaluated for efficacy in conjunction with other activities on biomass. Successful or promising enzymes and enzyme combinations will be reported to Edenspace for evaluation *in planta*. Edenspace will evaluate the genes with promising candidates transformed into corn for further development and evaluation.

Summary of Work to Date - Accomplishments (FY05-current):
Identification of candidate enzyme gene sequences. Oklahoma State University has initiated the genomic studies to isolate promising candidates. Several promising candidates have been identified and confirmed through initial screening procedures.

Develop automated screening assay for enzyme efficacy testing (NREL). NREL has been doing enzyme evaluation methods development using a 96 well microtiter plate format integrated into a Beckman FX robotics platform. Product (sugar) detection is currently being carried out by enzyme-linked sugar-specific assays.

Schedule
Project Initiation Date: October 6, 2006
Planned Completion Date: September 30, 2009

Lab Validation for Organism Development Solicitation Recipients

Kent Evans, National Renewable Energy Laboratory

Principal Investigator:	Kent Evans	Funding Partners:	N/A
HQ Technology Manager:	Amy Miranda	Sub-contractors:	N/A
PMC Project Officer:	Fred Gerdeman		

Goals and Objectives: DOE/GO announced an FOA for the development of robust, highly efficient fermentative organism. NREL's role and responsibility is to validate the organism performance, and to provide technical assistance in reviewing and evaluating various reports generated by the contractors. NREL will assist, as needed by DOE/GO, in evaluating the proposals. Since part of the proposal is for the applicants to perform fermentation experiments under specified conditions, we will perform on-site visits to the awardees to validate the data from those fermentation tests. We will prepare a detailed plan that will be used by the staff doing such validation. The plan prepared by NREL will be evaluated, modified and approved by DOE/GO. The audit will include the performance data on the microorganism and the overall ethanol production process that is being used by the contractors.

Project Description: This project is part of the NREL Biochemical Platform which conducts R&D across the breadth of fundamental, applied and integrated science to develop robust bioconversion technologies. Directed by rigorous process engineering and cost analysis, the R&D projects work together to address the key barriers to technically feasible, cost effective ethanol production from biomass. Data and results from fundamental studies on biomass recalcitrance and enzyme action in the Targeted Conversion Research project are used to design improved pretreatment and hydrolysis technologies in the Pretreatment and Enzymatic Hydrolysis project. Those core technologies are tested in an integrated process to reduce the risk to commercial developers in the Biochemical Processing Integration project. The Feedstock/Process interface project in the platform ensures that the process technologies developed are complementary to feedstock collection and storage methods and vice versa. Additionally, the biochemical platform at NREL includes validation activities for the DOE ethanologen development projects to monitor progress to the platform’s fermentation goals.

Summary of Work to Date Prior to FY07: In response to DOE/OBP's determination that improved microbial strains need to be available for an economic process for conversion of biomass sugars to ethanol, effort to date has focused in helping GFO to develop a Request for Information on developing robust ,highly efficient fermentative organism, and subsequently, to prepare an FOA for the same purpose. In order to identify the issues for the RFI, a thorough review and analysis of relevant public information on fermentative organisms was done and the suitable metrics for addressing the technical barriers of yield, titer, and rate for production of ethanol from biomass sugars were developed. The RFI responses from various companies, institutions and individuals were analyzed and summarized. This information and further analysis of the status of technology with respect to fermentative organisms for the conversion of biomass sugars to ethanol in process-relevant conditions were used to provide input to GFO for preparing an FOA on development of robust, highly efficient fermentative organisms for the conversion of lignocellulosic biomass to ethanol.

Accomplishments (FY07): NREL assisted DOE in developing the technical qualifications for applicants to follow in submitting proposals for review. The performance criteria were developed to provide guidelines for the organism performance and requirements that the applicant needed to supply to allow reviewers the opportunity to compare performance data from each proposal. The criteria were developed so that applicants could submit proposals to either research topics 1 or 2. Applicants needed to perform a series of qualification fermentations to demonstrate organism characteristics followed by a benchmark fermentation to show the best process conditions to date. Results were reported in tables with proposed performance targets through the period of performance. A third table was developed for the applicant to supply information on the proposed process including economics.

NREL staff was selected to contribute in selection of Topic and Topic 2 proposals during a group merit review discussion with other selected reviewers. A consensus review and score was placed on each proposal and submitted to DOE/GO staff.

NREL developed a draft plan outlining the requirements for validating the progress of the investigators research. The plan describes the procedure for conducting the initial verification, pre-Stage Gate, and final on-site audit required by the investigator. The plan will also describe the required documentation including procedures, experimental data, and final analysis that needs to be submitted during the on-site visit that shows performance and economic improvements. The researcher will be requested to perform a series of experiments to demonstrate the performance of the organism while the auditors are present. The experiments confirm the results submitted in the Fermentation Strain Performance Table A and B that was submitted in the original proposal. Intermediate and final validation performance will repeat the same series of experiments with confirmation of results based on the performance targets outlined in the original table. The NREL validation team will also confirm the Process Details and Costs Table C in the original proposal. Investigators will need to provide supporting information to show cost benefits to improved strains.

Currently, NREL and DOE/GO has completed the negotiation of confidentiality agreements with 2 of 5 total awards which will allow the initiation of the contract.

Schedule
Project Initiation Date: Sunday, October 01, 2006
Planned Completion Date: Thursday, September 30, 2010*
* Completion date is based on the 36 month period of performance once the final award is officially started.

Development of Applied Membrane Technology for Processing Ethanol from Biomass

Don Stookey, Compact Membrane Systems

Principal Investigator:	Stuart Nemser	Funding Partners:	N/A
HQ Technology Manager:	Melissa Klembara	Sub-contractors:	N/A
PMC Project Officer:	Gene Petersen		

Goals and Objectives: The program will focus on designing and developing a composite system (flat sheet or hollow fiber) that meets the chemical, thermal, and energy requirements for FGE. CMS will focus on testing at various temperatures and pressures consistent with operating conditions in ethanol plants. Initial testing related to screening and demonstration of feasibility will be done at one set of operating conditions (e.g. 95 oC,). Subsequent ruggedness tests and optimization tests in support of economic analysis will be done over a range of conditions (e.g. 110 oC, 45 psig). The excellent properties of the CMS membranes should provide the needed ruggedness to operate over this large range of operating conditions.

CMS will use above CMS membrane test results and do an economic evaluation in comparison to these controls. Milestones will show needed flux, selectivity and overall economics in combination with overall ruggedness.

The following tasks will be performed to meet the project objectives.
Task 1. Prepare Flat Sheet Porous Supports
Task 2. Prepare Hollow Fiber Porous Supports
Task 3. Coat Flat Sheets
Task 4. Coat Hollow Fibers
Task 5. Make Small Modules
Task 6. Membrane System Modeling
Task 7. Membrane Characterization
Task 8. High Temperature Gas Testing (Single Component)
Task 9. High Temperature Vapor
Task 10. Defect Repair
Task 11. Test Fermentation Ethanol System over Various Operating Ranges
Task 12. Economic Evaluation
Task 13. Design for Optimal Support and Module
Task 14. Multiple Component Evaluation

Project Description: CMS will adapt its membranes for making fuel grade ethanol (FGE) from fermentation based ethanol. The program will focus on designing and developing a composite system (flat sheet or hollow fiber) that meets the chemical, thermal, and energy requirements for FGE. This feasibility work will entail evaluating commercial porous supports, both flat sheet and hollow fiber (HF) materials. CMS will also do key modeling studies to identify the preferred design (e.g. flat sheet or (HF)). Overall design will address enhanced mass transfer and pressure drop and multi-stage features to make high purity (99.5%) fuel grade ethanol. Significant work will be done with commercial flat sheet materials. This will be followed by initial customization of process, product, or raw materials. Overall economics and basic data gathered from this research will lead to the eventual building of a prototype system (construction and testing of this prototype system is NOT included in the effort proposed here in).

Summary of Work to Date - Accomplishments (FY05-current): CMS is in the business of forming membrane devices from a family of chemically-resistant, perfluoro polymers. CMS polymers have very high membrane transport rates due to their high internal free volume, the spaces between the polymer molecules, which allow tiny molecules such as water to readily pass across the membrane while the larger molecules are blocked or hindered to membrane transport. CMS membranes are successfully used to separate air from volatile organic compounds, to dewater transformer fluids and hydraulic oils and to

drive conversion by water removal from an esterification reaction involving an organic acid and alcohol mixture.

Such applications experiences suggest that drying of the ethanol water azeotrope to produce FGE would be a logical extension to CMS' platform technology. The perfluoro polymers themselves are known to be excellent for handling strong solvents, such as ethanol, giving further assurance as to their utility in FGE production. Thus the development of an ethanol-water separation membrane, the purpose of this project, involves the tasks of identifying membrane supports, sealing materials, and system components, membrane fabrication and application techniques that result in an efficient, long-life, membrane device that is compatible with ethanol and its mixtures in the intended operating environment.

The CMS polymers must be supported on a microporous sheet or on porous hollow fibers to form the membrane structure. This load-bearing structure must withstand the fluid pressure forces and separate the feed, for example the ethanol-water azeotrope, from the water-rich permeate stream that is removed in order to produce the dry FGE. Ethanol's incompatibility with the usual CMS membrane supports, such as polysulfone, led to an exhaustive search and screening of available microporous materials. CMS tapped eight sources and screened six different polymeric materials for their potential use. Key to this screening was the formation of trial membranes and characterizing the membranes for flux and selectivity. CMS has identified two prime candidates of differing materials, one a microporous sheet, the other a hollow fiber, that are now undergoing more extensive testing. CMS intends to carry both candidates into the FGE application to insure against unforeseen obstacles to either in downstream manufacturing and application related tasks.

During the period, CMS also formalized a supply agreement with a hollow fiber manufacturer who offers a variety of hollow fibers in varying dimensions, including the prime candidate identified in our screening. While we cannot disclose the supplier's identity, this major volume hollow fiber producer also has entered a licensing agreement, secured rights to producing and distributing other CMS products, and is making substantial investment in hollow fiber supply and the related CMS products. By insuring a product supply with an interested partner, CMS is assured the potential for carrying the FGE product under development into rapid, full commercialization.

Screening studies for the flat sheet materials lead to two other important developments, namely a tool for characterizing the membranes and a simple bench scale technique for screening five different membrane forming methods and the associated polymer solvents. Typically membranes have been characterized by their gas flux, the permeance, and their ability to separate gas species, their selectivity. Both parameters involve intrinsic polymer membrane properties and the gas species being separated. Thus, the usual membrane permeance and selectivity measures are simply a reflection of the membrane's thickness and any non-separating defects or leaks. The newly developed CMS tool utilizes permeation flux measurements at multiple pressures with multiple gases to quantify the membrane's thickness and its viscous and Knudsen pore defects, parameters that are more closely related to and influenced by coating and processing techniques. By quantifying the defects we have been able to more readily direct our research to formation of defect-free membranes, thereby avoiding need for a defect repair step common in gas separation membrane manufacture. This will also enhance the membrane service life since defects and repair techniques are common detractors and initiators for membrane failure.

CMS designed and installed a new hollow fiber coating line for forming membranes on the outside surface of the hollow fibers. Approximately 25 different conditions have been tested using three different hollow fiber types. The resulting fibers are being characterized and qualified candidates will shortly become available for testing in the ethanol-water mixture and for screening in the FGE application.

Traditionally, membranes are formed and applied to the microporous flat sheet or hollow fibers before their assembly into a cartridge configuration. We have recently shown that some of the materials can be successfully coated in the final processing step after the membrane cartridge has been formed. We are very encouraged by this accomplishment. By *in situ* coating any damage to the coating layer arising during membrane handling and cartridge processing are necessarily avoided, further insuring a defect-

free product. Our partner is most interested in this potential, low cost method for forming the membrane and is now supplying small cartridges in support of our *in situ* coating trials.

CMS licensees form pleated sheet devices from CMS coated microporous flat sheet for non-FGE applications. One CMS partner has expressed interest in forming pleated sheet devices of the type material identified as a prime candidate for our FGE product. Thus, as noted previously for hollow fibers, we have accomplished another viable commercialization path to the FGE market.

Bench testing of candidate flat sheet and hollow fiber membranes has commenced with membranes operating at atmospheric feed pressures in pervaporation and vapor separation modes. Results are confirming preliminary tests that surprisingly showed CMS membranes, unlike polyvinyl alcohol (PVA) membranes, did not loose permeability at the low moisture levels necessary for the FGE application. Our investigation and research have now explained the fundamentals supporting these differences and validated our early permeation findings. Permeability is a combination of diffusivity and solubility of the permeating specie. PVA and other membrane dryers depend upon high moisture solubility for their permeation flux. Unfortunately, solubility is also related to the concentration of moisture in the mixture being separated for those materials leading to their marginal moisture removal near the dry FGE product conditions. To the contrary, CMS perfluoro polymers are hydrophobic and not influenced by the moisture. In contrast, the CMS permeability is high due to the extremely high diffusivity of moisture in the high free volume polymer and therefore remains unaffected by the low moisture levels required for FGE.

CMS has investigated several process schemes for incorporating the membrane in the FGE process. Replacement or augmentation of the cycling molecular sieve beds with a continuously operating membrane integrated into the FGE refinery show promise for substantial energy savings.

Schedule
Project Initiation Date: Friday, June 30, 2006
Planned Completion Date: Friday, June 29, 2008

Biochemical Processing Integration Core R&D

Biochemical Process Integration Task

Dan Schell, National Renewable Energy Laboratory

Principal Investigator:	Daniel Schell	Funding Partners:	N/A
HQ Technology Manager:	Amy Miranda	Sub-contractors:	Baylor University, Colorado State University, Membrane Science and Technology Center, Hauser Laboratories, Glenn Murray, Cheminformatics consultant
PMC Project Officer:	Gene Petersen		

Goals and Objectives: The goal of this task is to test integrated enzymatic cellulose hydrolysis-based biomass conversion technology using on a large-scale domestic feedstock. Corn stover is a model feedstock for much of the effort; however, we will also work with switchgrass and other lignocellulosic feedstocks. One overarching objective is to identify problems and showstopper issues relevant to the emerging biorefinery industry from an integrated process perspective. Another near-term objective is to demonstrate that the improved, lower-cost cellulase enzymes developed under cost-shared subcontracts by Genencor International and Novozyme Biotechnology reduces the cost and risk of enzyme-based process technology. Additionally, the improvement and development of both wet chemical and rapid analysis techniques also remains a major focus of this task to both improve the efficiency of in-house research activities and to support the emerging industry needs for accurate rapid analysis methods for quality monitoring and process control.

Project Description: This project is part of the NREL Biochemical Platform which conducts R&D across the breadth of fundamental, applied and integrated science to develop robust bioconversion technologies. Directed by rigorous process engineering and cost analysis, the R&D projects work together to address the key barriers to technically feasible, cost effective ethanol production from biomass. Data and results from fundamental studies on biomass recalcitrance and enzyme action in the Targeted Conversion Research project are used to design improved pretreatment and hydrolysis technologies in the Pretreatment and Enzymatic Hydrolysis project. Those core technologies are tested in an integrated process to reduce the risk to commercial developers in the Biochemical Processing Integration project. The Feedstock/Process interface project in the platform ensures that the process technologies developed are complementary to feedstock collection and storage methods and vice versa. Additionally, the biochemical platform at NREL includes validation activities for the DOE ethanologen development projects to monitor progress to the platform's fermentation goals.

Biochemical integration work will continue efforts to advance core process knowledge with emphasis on understanding factors affecting integrated process performance and producing process-relevant residues and waste streams for testing. Ultimately, this research reduces risk as well as capital and operating cost by overcoming technical barriers associated with high-solids processing, understanding the impact of feedstock variability, and developing a better understanding of the key interactions controlling process efficiency and performance (process integration).

In support of the 2012 goals, this task has been working and will continue work at the bench scale to demonstrate integrated process technology using the best available enzymes and microorganisms using an applied experimental approach. The results of this research will provide guidance to enzyme producers and microorganism developers on key technical performance parameters. It will also provide information that will be required to design pilot scale systems necessary to demonstrate good integrated process performance at the pilot scale and validate the 2012 technical target. As in any sophisticated conversion process, combining the individual unit operations into an integrated systematic process is a significant challenge. Individual pilot-scale operations to demonstrate the required performance of the unit operations as well as complete integrated pilot development runs will be required to demonstrate the $1.07 technology target. A specific challenge will be to demonstrate high solids processing at pilot scale

to reduce capital costs throughout the entire process (at least 30% in pretreatment, 20% in hydrolysis).

Additional work is also needed to improve and further develop analytical methods to facilitate research efforts requiring accurate compositional information. Realizing the goals of the 30 x 30 initiative will require the development, standardization, and validation of hundreds of new analytical methods specifically for biomass feedstocks and intermediate process streams. The data generated by these analytical methods will be used to obtain accurate performance information to accurately evaluate process economics. This will improve the efficiency of R&D activities in the biomass community and support the emerging industry needs for accurate and rapid analysis methods for quality monitoring and process control.

Summary of Work to Date - Accomplishments (FY05-current): This project formally started in FY01 as a placeholder for commercial-track projects, completed Stage 2 activities and began Stage 3 work in FY02. The Gate 3 review held in January 2002 was extremely well attended by representatives from industry, academia, and government. The distinguished panel of external reviewers unanimously agreed that the project was ready to begin Stage 3 work. In FY03, the DOE awarded grants to a number of industrial/academic/national laboratory collaborations with the purpose of developing commercial bioenergy/bioproducts processes. Therefore, in the May 2003 interim review meeting, the review panel suggested that this task should become a research track project (in Stage B) that supports these and other commercially focused projects. In a September 2004 Stage B interim review, the panel recommended that the project continue its focus on identifying process integration problems and work to generate process relevant residues and waste streams for characterization.

In FY03, we improved our ability to perform high solids pretreatment and were able to produce initial performance data for dilute sulfuric acid pretreatments carried out at solids loadings as high as 35%. Other accomplishments included extending our understanding of corn stover compositional variability, performing a preliminary assessment of process economic uncertainties using Monte Carlo analysis, and improving rapid analysis methods for compositional analysis of corn stover. In FY04, we developed an empirical model of high solid pretreatment performance (i.e., hemicellulosic sugar yields and cellulose digestibility), demonstrated high solids enzymatic cellulose saccharification (80% cellulose conversion at 25% insoluble solids loading), gathered preliminary evidence indicating the potentially overriding importance of agronomic influences on corn stover composition, and further improved the accuracy of rapid corn stover compositional analysis. More recently, we performed first-of-a-kind integrated performance testing of a corn stover to ethanol process and demonstrated deficiencies with some of the currently available ethanologens. We also demonstrated that recycling a significant portion of the stillage for makeup water will be challenging and appropriate strategies will be needed to overcome these problems. We also completed the survey of corn stover composition began in FY02, and believe we have capture the extent of compositional variability inherit in this material. We also made progress in deploying rapid analysis methods as demonstrated by the successful installation of a NIR sensor in the pilot plant for on-line stover compositional measurement. We also improved the accuracy of methods for measuring biomass-derived sugars and developed an improved technique for measuring total solids of biomass slurries.

In FY06, we advanced efforts to transfer rapid biomass compositional analysis methods to industry. Specifically, a NIR/PLS method for rapid analysis of corn stover was successfully transferred to an instrument at INL as a prelude to transfer of the method to industry-based instruments. Additionally, we demonstrated that online analysis of corn stover in an industrially relevant setting is possible and ready for deployment to industry. A mass pyrolysis technique was used to demonstrate that conventional lignin analysis (i.e., lignin defined as the acid insoluble residue of a concentrated acid treatment) doesn't correctly measured lignin in corn stover. This problem is likely present, and perhaps to a greater degree, in herbaceous materials. Also in the analytical methods area, a subcontract with Baylor University was completed that developed methods for identifying and quantifying biomass extractives in corn stover. These methods will be extended to other feedstocks in the future. This year, we began work to test Genencor's advanced enzyme preparation by basing performance of a commercial enzyme. Our effort to complete work on the advanced enzymes was delayed due to unanticipated problems in receiving the enzymes. However, work completed this year supports an FY07 D milestone. We also installed a semi-

automated centrifuge that will enhance future efforts to produce separated pretreated material to support core R&D activities.

In FY07, we tested an advance enzyme preparation from Genencor and have given the results to them for further analysis. We will begin shortly to test other enzyme preparations. We have also advanced knowledge of the conditioning process and have found that ammonia hydroxide-based conditioning has the potential to significantly reduce sugars losses that occur during this process, ultimately producing higher conversion yields based on initial sugars present in the hydrolysate liquor. We have also evaluated several ethanologens in preparation for selecting the best strains to demonstrate our September 2007 milestone target listed in the milestone table above. Higher conversion yields can be achieved by tailoring the conversion process to take advantage of the best characteristic of the enzyme and microorganisms. We will continue to build upon this work to fully understand the process and associated requirement that will be necessary to achieve the out year milestones to generate pilot scale data on an integrated process. Therefore, this task will take an active role in specify equipment for the new biochemical pilot facility and will ultimately heavily use this facility.

In the analytical area in FY07, we are continuing work to transfer rapid compositional analysis methods based on NIR spectroscopy (i.e., the calibrations equations) to stakeholders and to other manufacturer's instruments to facilitate widespread deployment of this technology. We are also improving current analytical methods for both speed and accuracy and have recently develop a HPLC method for analyzing ethanol and organics acids that reduced instrument run time to 25% of the normal time.

Finally, work is in progress to understand the impact of corn stover variability on process performance as measured by hemicellulosic conversion yields and enzymatic cellulose digestibility. Several varieties of corn we have received from various locations through out the U.S. are being pretreated in the pilot scale reactor and ANOVA will be used to determine the influence of various factors. This work is anticipated to be completed in early FY08.

Schedule
Project Initiation Date: Monday, October 01, 2001
Planned Completion Date: Sunday, September 30, 2012

Fundamentals and New Concepts

Targeted Conversion Research

Mike Himmel, National Renewable Energy Laboratory

Principal Investigator:	Mike Himmel	Funding Partners:	Genencor International
HQ Technology Manager:	Amy Miranda	Sub-contractors:	University of South Dakota, Colorado School of Mines, Vanderbilt University, Brookhaven National Lab, Cornell University, Colorado State University, Weizmann Research Institute, Oregon State University, University of Georgia, UC San Diego
PMC Project Officer:	Gene Petersen		

Goals and Objectives: The objective of the FY07 research proposed for the Targeted Conversion Research Task (TCR) is to conduct activities that ensure the success of the 2012 OBP goals of $1.31 ethanol technology. The TCR task also proposes to conduct OBP Translational Science designed to acquire new understanding of chemical and biological fundamentals underlying biomass recalcitrance. Specifically, this work will ensure the availability of new science knowledge needed by industry to develop future biorefinery processes. To achieve the DOE goals of enabling new technologies to provide 60 billion gallons of bioethanol by 2030, considerable improvement in enzyme saccharification of plant cell walls must be achieved. The correlative development of more active cellulases and improved energy plants will ensure attainment of DOE's post-2012 goals. This research utilizes the Biomass Surface Characterization Laboratory (BSCL) at NREL.

Project Description: This project is part of the NREL Biochemical Platform which conducts R&D across the breadth of fundamental, applied and integrated science to develop robust bioconversion technologies. Directed by rigorous process engineering and cost analysis, these R&D projects work together to address the key barriers to technically feasible, cost effective ethanol production from biomass. Data and results from fundamental studies on biomass recalcitrance and enzyme action in the Targeted Conversion Research project are used to design improved pretreatment and hydrolysis technologies in the Pretreatment and Enzymatic Hydrolysis project. Those core technologies are tested in an integrated process to reduce the risk to commercial developers in the Biochemical Processing Integration project. The Feedstock/Process Interface project in the platform ensures that the process technologies developed are complementary to feedstock collection and storage methods and vice versa. Additionally, the Biochemical Platform at NREL includes validation activities for the DOE ethanologen development projects to monitor progress to the platform's fermentation goals.

The recalcitrance of biomass to conversion processes has been related to the natural barriers that lignocellulosic materials present to saccharifying chemical and biological reagents. These barriers can be reduced via an adequate pretreatment. Pretreatment conditions are selected for their ability to modify the structure of biomass to increase the accessibility of the cellulose to enzymes, or the susceptibility to their action. The characteristics of pretreated biomass are also being measured to determine which have the greatest affect on the susceptibility of cellulose to enzymatic hydrolysis. The Targeted Conversion Research Task is subdivided into specific objectives that address key biorefinery technology barriers, listed as subtasks below.

The broad objective of the Chemical Processing Fundamentals subtask is to increase our understanding of the chemical and structural changes that occur in biomass during prehydrolysis over a range of treatment chemistries and severities through theoretical, modeling, and experimental studies. For example, under acidic conditions hemicellulose solubilization increases the accessibility of the remaining cellulose to enzymatic hydrolysis. A better understanding of the interaction of enzymes with biomass solids modified by dilute acid and other treatments is needed so that the rate and yields of sugars can be

increased. In addition, as the hemicellulose is solubilized, mono- and oligosaccharides are released into the hydrolyzate. Under acidic conditions the solubilized sugars can be degraded into non-fermentable products and fermentation inhibitors resulting in loss of yield. Therefore, we wish to understand the relationship between pretreatment conditions and the chemical and structural changes that occur in pretreated biomass. The goal of the Biological Processing Fundamentals subtask is to advance the fundamental principles that underlie the complex biochemical mechanisms of the key enzymes used for biomass hydrolysis. Our approach is to use the tools of modern biochemistry and protein engineering supplemented with advanced imaging and computational models to solve how the cellulases work to degrade biomass. The action of processivity in the enzymatic degradation of recalcitrant polysaccharides is influenced not only by enzyme characteristics but by the nature of the substrate itself. Because of this we have focused not only on the properties of the enzymes but also on identifying key factors that improve substrate accessibility. Using this knowledge, the cellulose hydrolysis, pretreatment cost, and cellulase cost goals for 2012 technology will be more readily achieved. Specially, we are working towards understanding how CBH I, a principal part of cellulases, work to decrystallize and hydrolyze cellulose. The objective of Advanced Cell Wall Characterization research is to develop and apply cutting edge technologies for cell wall imaging. In FY07, our results from the characterization of native corn stover ultrastructure, as well as changes resulting from the bioconversion process, have revealed that plant cell wall biomass is highly complex at all length scales and especially chemically heterogeneous at the molecular level. However, the instrumental tools needed to analyze biomass at sufficiently small scale are not available. Thus, we will focus on developing and applying advanced imaging tools and associated theory to demonstrate efficacy with regard to the plant cell wall and its chemistry.

Summary of Work to Date - Accomplishments (FY05-current): The following milestones contribute to the 2012 platform target of cost competitive ethanol. D milestones discussed are listed in the table below. E milestones contribute to D milestones.

Chemical Processing Fundamentals: Progress to completion of D Milestone: *Define the relationships between pretreatment conditions and the chemical/ultrastructural changes in corn stover stems that result in biphasic xylan hydrolysis (Joint milestone with Plant Cell Wall Deconstruction).* This knowledge will allow us to improve pretreatment and/or enzyme processing to more easily convert the recalcitrant xylan fraction of biomass. This work is a continuation of research begun in FY06 in which we attempted to identify if there was a structural component, in plant cell walls, that was responsible for the biphasic kinetics of xylan hydrolysis. We found, as had other researchers, that hydrolysis of the xylan could be described as two first-order reactions, an initial fast reaction followed by a much slower reaction. That biphasic xylan hydrolysis was found even in this very select tissue type means that the reason for the biphasic kinetics must be at the cellular level or smaller and cannot attributed to a difference in hydrolysis rates between different tissue types, such as between leaves and stalk. Based on our experiments, it appeared that there was an increase in the fraction of fast hydrolyzing xylan as the temperature and acid concentration were increased, which implies that the biphasic kinetics was not due to the presence of xylans with differing chemical structures. The first of these is the use of linear and surface plots across cell wall profiles to characterize xylan localization in the cell wall, i.e., center versus edges, and how these change in response to various pretreatments. This year's work continues to test the validity of our hypothesis that the biphasic kinetics is linked to the migration of xylan, in close association with lignin, to the exposed surfaces of the cell walls. Fast xylan hydrolysis, then occurs when the xylan comes into contact with the liquid hydrolyzate. Slow xylan hydrolysis occurs when either xylan must be hydrolyzed directly from the secondary cell walls or when xylan migration becomes rate limiting. Methods for quantitating images and for tracking changes in xylan concentration and subcellular localization are developed by the PCWC subtask.

E MS: *Determine chemical pathways for oligosaccharide hydrolysis and reversion reactions using Carr-Parinello MD modeling.* It is important to maximize the hydrolysis of xylan into xylose and minimize the formation of intermediates (xylooligomers) and degradation products (furfural and reversion products). Our prior work has used quantum mechanical calculations and experiments to determine the relative rates of hydrolysis and dehydration of xylose, xylooligomers, and xylan. Calculations and experiments showed that the barriers for hydrolysis reactions were much lower than the barriers for dehydration processes. Experimental measurements also showed that xylobiose and xylan could be hydrolyzed at

much lower temperatures than is required for dehydration. *These results indicate that dehydration reactions of xylooligomers should be of no concern during acid pretreatment.* In FY07, our research in this area is focused on the effects of mass transport on xylooligomer hydrolysis, and the mechanisms for the formation of unwanted reversion products from xylose. It has been observed that the temperatures needed for optimal yields of xylose from xylan and corn stover are much lower in bench scale experiments (110 to 130^{o}C) with low solids loading (1 to 10%) compared to the temperatures needed in larger scale pretreatment studies (140 to 200^{o}C) with higher solids loadings (10 to 30%) where mass transport is more restricted. There is evidence that the reason for the lower xylose yields is that there is a higher yield of xylooligomers at the higher solids loadings. Subcontract with Colorado State University to support this work placed 5/1/07.

E MS: *Define the critical substrate properties of corn stover stem tissue necessary for efficient cellulase enzymatic digestibility (Joint with Biological Processing Fundamentals).* Current pretreatment technologies utilize thermochemical processing to improve the accessibility of the cellulose fraction of biomass to cellulase enzymes. To improve current pretreatment regimes with more benign and thus less costly procedures, an in-depth understanding of barriers to cellulase enzymes becomes critical. Defining the complex interactions between enzymes and lignocellulose is critical to the deployment of both advanced pretreatments and improved enzymes. Prior work conducted by the Biological Processing Fundamentals and Chemical Processing Fundamentals subtasks, examined select biomass structural features that impact enzyme digestibility. Xylan content, sample drying, lignin and cellulose morphology were evaluated using a single highly purified cellulase component, the cellobiohydrolase (CBH I), obtained from a commercial *Trichoderma reesei* enzyme preparation. Our findings indicated that improving cellulose accessibility to enzyme attack is critical to efficient conversion and that factors which directly impact accessibility following pretreatment include xylan content, cellulose fiber interactions and hydration, and finally lignin content and morphology.

E MS: *Understand how pretreatment conditions impact lignin extrusion and deposition in corn stover stems cell walls.* This work is focused on identifying the fate of lignin under pretreatment conditions and the impact of changes in lignin distribution on the digestibility of pretreated substrates. Our previous work (FY06) was focused on the lignin fraction that made it into the aqueous phase of the pretreatment hydrolyzate and subsequently formed into droplets. This work demonstrated that these droplets were lignin-derived and that the free droplets may have a minimal affect on cellulose depolymerization (>12%) The major contribution from lignin to biomass recalcitrance is more likely with the lignin trapped in the residual cell wall structure. In FY07 we are defining and characterizing lignin movement in the corn stover stem as a result of thermal pretreatment in various process chemistries.

Biological Processing Fundamentals: Progress toward completion of the D MS *"Discover how cellulase enzymes move along the cellulose chain and the respective roles of the different enzyme substructures*." The following E milestones are underway in support of this D milestone.

E MS: *Model CBH I and the Family 1 cellulose binding modules using molecular simulations.* Enzymes acting on cellulose have the challenge of first associating with and then disrupting the crystal packing of an insoluble substrate followed by directing a single-polymer chain into the active site tunnel. Almost all biomass degrading enzymes have multiple domains and contain at least one carbohydrate binding module (CBM) that is thought to enable binding of the enzyme to the cellulose surface. CBMs are also thought to be important in the initiation and processivity of exoglucanases. Because of the importance of CBM's in biomass conversion we have begun using Computational Molecular Dynamics (MD) modeling to understand the contribution of CBMs on cellulose hydrolysis. This work is being done in collaboration with the San Diego Supercomputer Center. Recently a noteworthy discovery was made by NREL scientists showed that not only are Family I CBM's highly conserved in fungal enzymes but they may undergo an induced fit with a broken-chain cellulose surface. Part of this work was published in the journal *Protein Engineering Design & Selection (PEDS).* A visualization of the molecular model can be seen at: cover art movie. To experimentally confirm the induced fit model we are producing the CBM and several mutants by solid-state synthesis. These mutations have also been introduced into the gene by site-directed mutagenesis and are being produced in *Aspergillus awamori*. (Subcontract with UC San Diego, Cornell, and Vanderbilt are planned).

E MS: *Build Data Base of Cel7A Catalytic Domain Structure and Activity Using Crystallography And Kinetics*. We are using genomic sequences that have been generated in part by the DOE Joint Genome Institute to draw inferences on structural features of cellobiohydrolase enzymes that impart functional differences. Specifically we are interested in the conformation and size of the active site tunnels of these enzymes. Protein sequences are being compared using the algorithm PSI-BLAST which searches protein databases to determine close homologs. Sequence and structure conservation scores are then calculated using the web based program ConSeq. This approach allows for the identification of functionally and structurally important residues in protein sequences using the assumption that slowly evolving sites are biologically important. These highly conserved residues within the protein core are likely to have an important structural role in maintaining the protein's fold and will provide insight to the function of the enzyme. We plan to generate structural data on this enzyme using the new crystallography tools at NREL. As part of this study we have identified and successfully engineered an addition peptide loop into the *T. reesei* catalytic core and have removed the analogous loop from the *P. funiculosum* enzyme. Subcontract with Oregon State to support this work is planned.

E MS: *Assemble A Library Of Enzymes Necessary To Determine The Functional Relationship Between Application Of Defined Hemicellulases, Accessory Enzymes, Cellulosomes, And Cbms To Untreated Corn Stover Stems And The Required Relative Severity Of Dilute Acid Pretreatment.* Diferulate esters strengthen and cross-link primary plant cell walls and help to defend the plant from invading microbes. Phenolics also limit the degradation of plant cell walls by saprophytic microbes. We have shown that incubation of corn stover with a ferulic acid esterase and a xylanase improves its overall conversion. In order to evaluate the role of this class of enzymes two fungal ferulic acid esterases (faeA and faeB) were cloned and purified in *Aspergillus awamori*. We have determined many of the biochemical properties of the recombinant enzymes such as substrate specificity, thermal stability, and glycosylation. Subcontract with University of Georgia to support this work placed 6/1/07.

Advanced Plant Cell Wall Characterization:
This subtask has developed specific molecular probes, primarily CBMs, tagged with various fluorescent proteins for mapping pretreated corn stover samples. This project is also developing mutations of CBMs specifically recognizing polysaccharides for corn stover cell walls. Image pretreated corn stover cell walls using AFM, TIRF and Confocal Laser Scanning Microscopy. We will apply fluorescent labeling techniques to understand how pretreatment chemistry modifies the specific cell wall components such as cellulose and xylan. This project will particularly focus on developing and applying approaches for high (molecular) resolution imaging and quantitative analysis. Initiate a new imaging concept that combines spectroscopy and microscopy. This subtask has begun to evaluate Coherent Anti-stokes Raman Scattering (CARS) microscopy for biomass characterization. This project will be in collaboration with Prof. Sunney Xie of Harvard University (uncompensated collaborator). We also continue to develop the imaging capability of the BSCL. We recently completed the installation of two significant pieces of visualization equipment in the DISC (Data Interpretation and Simulation Center). The first system provides the capability for passive stereo 3D visualization by a working group using a single projector 3D projection system and shuttered 3D glasses. Also, we've implemented a high resolution collaborative visualization environment by tiling 6 high definition, LCD displays into a single 25 megapixel display. We are currently working on the software solutions that will allow us to fully utilize these image visualization tools with the full range of 2D, 3D, and video file types from multiple platforms in a collaborative work environment.

E MS: Develop And Apply Biochemical Probe Technologies To The Characterization Of Chemically And Enzymatically Treated Corn Stem Cell Walls Using AFM And Total Internal Reflection Fluorescence Microscopy. In support of the goals of the Biomass Program, NREL intends to explore the application of CBMs residing in the bacterial cellulosome as well as the cellulosome itself for labeling plant cell wall specimens for enhanced imaging. Subcontract with the Weizmann Research Institute is planned.

E MS: Image Plant Cell Wall Degrading Enzymes Produced By Natural Biomass Degrading Communities Colonizing Corn Stem Cells To Identify Novel Conversion Paradigms. Using bioinformatics and genomics tools provide new GH enzymes from mycorhizal and endophytic fungi associated with corn and poplar roots. The primary objective of this project is to gain an understanding of the composition and the

metabolic potential of the lignocellulosic biomass degrading communities that decay poplar wood chips, corn stover and switch grass biomass under anaerobic conditions. Subcontract with BNL to support this work placed 6/1/07.

E MS: Develop Initial Concepts For New Biomolecular Imaging Methodologies Aimed At Generating Chemical Maps Of Corn Stem Cell Wall Structure At The Nanometer Scale. The primary objective of this effort is to develop new approach for achieving visualization of single cellulase enzyme acting on cellulose by single molecule spectroscopy. Development of a single-molecule photo-activated fluorescence imaging system: This task will involve development of a custom Total Internal Refelction Fluorescence Microscopy (TIRF-M) system for excitation and high-sensitivity detection of fluorescence. Subcontracts with South Dakota State University and Colorado School of Mines to support this work placed 7/1/07.

Schedule

Project Initiation Date: Sunday, October 01, 2000
Planned Completion Date: Sunday, September 30, 2012

Engineering Thermotolerant Biocatalysts for Biomass Conversion to Products

K.T. Shanmugan, University of Florida, IFAS

Principal Investigator:	K.T. Shanmugam	Funding Partners:	University of Florida
HQ Technology Manager:	Amy Miranda	Sub-contractors:	N/A
PMC Project Officer:	Fred Gerdeman		

Goals and Objectives: The objective of this study is to construct novel thermotolerant biocatalysts (second generation) that function optimally under environmental conditions that are also optimal for the activity of fungal cellulases (50ºC and pH 5.0). (I) Physiological and genetic characterization of selected second generation biocatalysts for optimum product production using the native lactic acid fermentation as a model system for future studies on ethanol production. (II) Metabolic engineering of selected biocatalysts to replace the native lactic acid pathway with a homo-ethanol pathway. (III) Metabolic and physiological characterization of engineered strains.

Project Description: The target is to develop technology that can support production of ethanol from biomass resources at a cost of $1.10 by 2010. The cost of cellulase enzymes for saccharification of feedstock has been identified as one of the critical cost factors in the production of fuel ethanol, from lignocellulosic biomass and this must be improved to reach this goal. With DOE support, two companies, Genencor and Novozymes, have reduced the cost of this enzyme to about 10-20 cents per gallon ethanol produced by optimizing cellulase production and activity. A parallel and synergistic approach to reduce enzyme cost is by developing improved biocatalysts that can operate at conditions that are optimal for these fungal cellulases. Present industrial biocatalysts function at temperatures that are 10-20 degrees below the optimal temperature for fungal cellulase activity (50ºC and pH 5.0). By developing second generation biocatalysts that are effective producers of ethanol under these conditions, the amount of enzyme needed can be reduced by more than half with corresponding reduction in cost. The production of these second generation biocatalysts for simultaneous saccharification and co-fermentation of both cellulose and hemicellulose hydrolysate is the focus of this study.

Summary of Work to Date - Accomplishments (FY05-current): The main objective of this study is to isolate bacterial biocatalysts that function optimally at the conditions that are also optimal for fungal cellulase activity towards reducing the amount of cellulase needed for SSF of cellulose and engineer these bacterial biocatalysts to produce ethanol as the main fermentation product using hexose and pentose sugars as the feedstock. Towards this goal we have accomplished the following.

1. **Isolation of thermotolerant microbial biocatalysts**. We have isolated over 400 bacterial biocatalysts that grow at 50-55 ºC and pH 5.0, conditions that are optimal for fungal cellulase activity. Based on 16S rRNA (DNA) sequence, many of the tested isolates were identified as *Bacillus coagulans,* which comprises a very diverse group of Gram-positive bacteria that has not been well studied. Based on various physiological characteristics, two isolates from this group, strains 36D1 and P4-102B, were selected for further analysis.

2. **Fermentation characteristics**. Strains 36D1 and P4-102B produced L(+)-lactic acid as the main fermentation product from both glucose and xylose at an optical purity of 97-100%. The lactic acid yield was about 90% from glucose and about 80-85% from xylose. The volumetric and specific productivities of lactate for strain 36D1 were, 0.86 (g/L.h) and 3.5 (g/h.g dry cell mass), respectively, during glucose fermentation. For comparison, the corresponding reported specific productivity value for *Saccharomyces cerevisiae* producing ethanol is about 2.0 g/h. g dry cell mass. On a molar basis, the specific productivity of respective products for *B. coagulans* and yeast were comparable. These bacteria also fermented sucrose and cellobiose in addition to other sugars normally found in various hemicellulose acid hydrolysates, such as galactose, mannose and arabinose. Ability of *B. coagulans* to ferment cellobiose would eliminate the need for β–glucosidase in the cellulase preparations during SSF of cellulose. Xylose was fermented by the *B. coagulans* strains using the enzymes of the pentose-phosphate pathway that convert all the xylose to pyruvate for further conversion to lactate or ethanol.

3. **SSF of cellulose**. Lignocellulosic biomass conversion to products depends on simultaneous saccharification of cellulose to glucose by fungal cellulases and fermentation of glucose to product by microbial biocatalysts (SSF). The cost of cellulase enzymes represents a significant challenge for the commercial conversion of ligncellulosic biomass into ethanol or lactate. In our experiments, the optimum temperature for the fungal cellulase from Genencor was found to be about 60ºC and the optimum growth temperature for *B. coagulans* strain 36D1 was 55ºC. The optimum cellulase concentration for SSF of crystalline cellulose with Genencor cellulases and *B. coagulans* strain 36D1 was determined to be about 7.5 FPU/ g cellulose at 50ºC. For highest volumetric productivity of the specific product in SSF of cellulose, *B. coagulans* required only 5 FPU/g cellulose at 55ºC while yeast required at least 20 FPU/g cellulose to reach the same level of volumetric productivity of strain 36D1. SSF with *B. coagulans* at 55ºC provides a 3 to 4 -fold reduction in the amount of fungal cellulase needed for optimum fermentation of cellulose to product with an associated savings in enzyme cost.

4. **Fermentation of hemicellulose acid hydrolysate**. Sugar cane bagasse acid hydrolysate (HCH) was overlimed and the pH of the overlimed hydrolysate was lowered to 5.0, the optimal pH for fungal cellulase activity. At this pH, *B. coagulans* fermented the HCH in mineral salts medium with only corn steep liquor (0.25% w/v). Lactate concentration peaked at 75% HCH in the fermentation to about 55 g/L and the yield was about 90% of the total sugars in the HCH. In addition, in an SSCF, simultaneous saccharification of cellulose (20g/L) with cellulases and cofermentation of the released sugars with 40% HCH, both cellulose and HCH were simultaneously fermented to lactic acid by *B. coagulans* at 50ºC and pH 5.0. These studies show that *B. coagulans* strains can ferment cellulose with less cellulases than yeast and also can coferment cellulose and HCH to products with savings in enzyme cost and equipment cost.

5. **Shuttle plasmid vector for gene transfer into *B. coagulans***. Although *B. coagulans* was first described in 1915, this bacterium is not a focus of intense study. Due to this lack of interest, gene transfer systems for this organism are not available. We found that only one *E. coli/ Bacillus* shuttle plasmids, pNW33N, function in *B. coagulans* strain P4-102B irrespective of the growth temperature. In addition, many of the antibiotic resistance genes from *B. subtilis* plasmids failed to provide resistance in *B. coagulans*. We constructed a shuttle plasmid using the replicon from a native plasmid found in strain P4-102B and chloramphenicol resistance gene (plasmid pMSR10). Using our electroporation protocol, plasmid pMSR10 was transferred into *B. coagulans* at about the same frequency as observed during *B. subtilis* transformation.

6. **Ethanol pathway for *B. coagulans***. Using plasmid pMSR10 as the backbone, we constructed plasmid pMSR40 that carries *Sarcina ventriculi pdc* (pyruvate decarboxylase) and *Geobacillus stearothermophilus adh* (alcohol dehydrogenase). Plasmid pMSR40 was stable in *B. coagulans* and the two genes of the ethanol pathway were transcribed. However, any increase in ethanol in fermentation broth or PDC activity was below the detection limit in the recombinant. Optimization of the expression of the ethanol pathway is in progress. Among the other *pdc* genes we tested (from *Zymomonas mobilis, Zymobacter palmae, Acetobacter pasteurianus* and Yeast), only the *pdc* from *Z. mobilis* produced detectable level of activity in a model Gram-positive host, *B. megaterium*. Using suicide plasmid vectors we are in the process of constructing mutants with deletions in the gene encoding L-LDH to shift the fermentation from lactate to ethanol while also optimizing the expression of active PDC in *B. coagulans*.

7. **An alternate homoethanol pathway**. Since the PDC activity was not detected in the *B. coagulans* recombinant, we developed an alternate pathway for production of ethanol as the main fermentation product. This development was in *E. coli* and utilized mutations in the pyruvate dehydrogenase complex (PDH). Although PDH is produced by wild type *E. coli* during fermentation, the enzyme activity is close to undetectable level. We introduced mutations in the gene encoding PDH that supported the enzyme activity in an anaerobic cell. In such a mutant, ethanol yield from glucose was close to 90%. Since the PDH of *B. coagulans* functions in a manner that is similar to that of *E. coli*, we are in the process of introducing analogous mutation in the *B. coagulans* gene encoding PDH. Introduction of this mutation into the chromosome is expected to yield a PDH complex that can support fermentation of sugars to ethanol

by this novel pathway. To increase the rate of ethanol production by this PDH-ADH pathway, we have cloned a native gene encoding ADH activity.

Summary. *Bacillus coagulans* grows and ferments all the sugars normally found in biomass to optically pure L(+)-lactic acid. Pentose is fermented by *B. coagulans* through the pentose-phosphate pathway to complete conversion to pyruvate and then to products. SSF of cellulose at 55ºC with *B. coagulans* requires only about 25-33 % of the cellulase needed for comparable SSF with yeast at 35ºC with a significant cost-saving on cellulase. An alternate homoethanol pathway has been developed for engineering *B. coagulans* for ethanol production. Towards this goal, gene transfer system and plasmid vectors for *B. coagulans* were also developed.

Schedule
Project Initiation Date: April 1, 2004
Planned Completion Date: March 31, 2008 (with one year extension)

Fungal Genomics

Scott Baker, Pacific Northwest National Laboratory

Principal Investigator:	Scott Baker	Funding Partners:	Poet, Novozymes, Verenium,
HQ Technology Manager:	Amy Miranda		Dyadic International,
PMC Project Officer:	Fred Gerdeman		Mycosynthetix
		Sub-contractors:	N/A

Goal and Objectives:

- Goal
  - Generate innovative fungal-based biotechnology to enable a robust biorefinery industry
- Objectives
  - Reduce the cost of biofuels and bioproducts
- Directly utilize complex biomass
- Enable processes with high concentration of end-product (for example, organic acids and enzymes)
  - Improve the overall tractability of filamentous fungi
  - Leverage industry needs and expertise to help guide research program

Project Description:

Our research is focused on generating basic understanding of fungal biotechnology. This basic understanding of the fungal systems will provide industry with enabling tools to rapidly and effectively develop new processes for the biorefinery industry.

Fungal biotechnology offers a promising set of technologies that are very important to the establishment of integrated biorefineries. These organisms have the potential for rapid, highly productive conversion of biomass resources to bio-based intermediates that bring economic benefit to industry. While fungal fermentations are currently used for a limited number of specialized products, the tools for a broader and more rapid implementation of these organisms are not yet available to industry. Current processes have been developed over many years without a clear understanding of the underlying ability to control and utilize fungal organisms.

To address the barriers to fungal fermentation, enabling R&D is needed in the following primary areas:

- **Morphology control**: the ability to control the morphology of the organism to increase both its productivity and the ability to functionally grow it in a fermentor
- **Genomics:** Application of genomics and proteomics tools to fungal species for selective modification
- **Hyper-productivity:** the ability to produce isolates with very high productivities of selected products, similar to those currently used for citrate production
- **Biomass to Product, Single Stage Processes:** enzyme production, biomass hydrolysis, biomass substrate utilization, cell growth, and generation of selected products
- **Filamentous fungal ethanol:** develop a model filamentous fungal ethanologen, targeted screening of a partner fungal collection for ethanol producers, metabolic flux modeling

Summary of Work to Date - Accomplishments (FY05-current):

The work has been divided into several subtasks:

1. Morphology control
2. Genomics
3. Hyperproductivity
4. Biomass to product
5. Partner Review Board
6. Filamentous fungal ethanol

Morphology control

Morphology of fermentation organisms is crucial for productivity. The most productive morphology for filamentous fungi is the pellet. Our model for a pelleted morphology in a fermentation process is *Aspergillus niger* in citric acid fermentation. In the citric process, *A. niger* forms hollow spheres wherein

the whole surface area of the cell is made up of hyphal tips. Hyphal tips are “where the action is” with regard to fungal metabolism – they are the sites of secretion and growth. Pellets are also ideal for fermentation due to their low viscosity.

We have spent the first three years of this project dissecting the genetic control of pellet morphology in *A. niger* citric fermentation. Our goal has been to determine which cell biological processes are responsible for *A. niger* pellet morphology so that we can manipulate these pathways in other fungi that produce other products and ethanol. Our research has implicated the G-protein signaling pathway, the ubiquitin mediated proteolysis pathway at the genetic level. Reactive oxygen species (ROS) are produced by metabolism, and induced by the high glucose needed for citrate production, and have been implicated at the cell biological level. We have proposed a morphology control model linking these three pathways. This model forms a framework on which to base future experiments in *A. niger* and other fungi that will have future roles in the biorefinery.

Genomics
Since 2005, the Fungal Biotechnology Team at PNNL has led the *A. niger* Genome Project in collaboration with the DOE Joint Genome Institute (JGI). The availability of the genome has accelerated our research considerably. The genome sequence is now complete and has been mapped onto the eight known *A. niger* chromosomes. We have used the genome sequence information to rapidly target a number of gene families for genetic and transcriptome analysis. In addition, the genome has enabled high-throughput proteomic studies of the pelleted morphological state. Currently, we are comparing and analyzing the differences between the JGI *A. niger* genome (a citric acid producer) and comparing it to another recently published sequence from an *A. niger* strain that is a protein producer.

Hyperproductivity
We have chosen Aspergillus terreus and itaconic acid as our baseline organism and product. Our examination of the A. terreus genome sequence led to the identification of three genes of interest with regard to hyperproductivity on complex plant biomass. PCR primers for one of these genes, phyA encoding the phytase enzyme, have been designed and ordered. The primers will be used to construct a gene deletion cassette. Phytase is an enzyme that hydrolyzes phytic acid from corn and other plants to inositol and inorganic phosphate. Higher levels of phosphate appear to be correlated with low itaconic acid productivity in A. terreus. Our hypothesis is that deletion of the phyA gene may keep inorganic phosphate levels low and itaconic acid productivity high when A. terreus is grown on complex plant biomass sources.

The deletion cassette for phyA will be constructed and a deletion mutant of A. terreus isolated. This deletion mutant will be compared to the wild type with regard to itaconic acid production in shake flask studies using glucose media with or without phytic acid amendment, and enzyme treated ground corn. These studies will continue into FY08. Three 30L fermentation runs of A. terreus grown on glucose will be prepared and samples will be isolated at three time points. The times are before the onset of itaconic acid production, just after onset, and in the middle of the high productivity phase. These samples will be used for preparation of RNA for EST (expressed sequence tag) sequencing to be performed by JGI and for proteomics analysis to be performed at PNNL in FY08.

Biomass to product
Dry weight to image correlations work was continued to evaluate the potential for using this approach for analyzing process kinetics in consolidated biomass processing. Our baseline organism, A. terreus, was introduced successfully to the 30L fermentors. Repeated fermentations using glucose as the carbon source resulted in final titers of itaconic acid ranging from 50-60 g/L. This is an important accomplishment for supporting all of the other subtasks since A. terreus is our consolidated bioprocess model organism, the fungus to be used for transformation with morphology control genes identified in the Morphology task, and the target of proteomic and genomic studies to support the Hyperproductivity task.

Partner Review Board
A biorefinery industry Partner Review Board was formed with Poet, Novozymes, Verenium, Dyadic International and Mycosynthetix. We have prepared Annual Reports and held Annual meetings in 2005

and 2006. A SharePoint site was established so the PNNL Fungal Biotechnology Team and the Partners Review Board can exchange ideas, reports and other information relative to the project. Final arrangements are in process to provide the Board members and the PNNL Fungal Team access to this secure site.

Filamentous fungal ethanol
Like yeast, filamentous fungi possess the ability to produce ethanol when grown in low oxygen/anaerobic culture conditions. However in contrast to yeast, filamentous fungi are able to degrade lignocellulosic biomass, including cellulose and hemicellulose, as well as efficiently utilize xylose and other pentoses. Despite their positive characteristics and perhaps due to their inherent biological complexity compared with yeast or bacteria, filamentous fungi have only been explored as ethanol production organisms in a limited way. In previous experiments using Fusarium oxysporum grown on 10% glucose + 0.2% DDG we were able to generate up to 5.8% (w/w) ethanol. We have been authorized to add a task to our existing OBP supported Fungal Biotechnology research program to support characterization and optimization of filamentous fungal ethanol production from both simple sugars and directly from lignocellulosic biomass.

Schedule
Project Initiation Date: Saturday, January 01, 2005
Planned Completion Date: Monday, December 31, 2012

Chemicals and Products R&D

Integrated Biorefinery – Separations/Separative Bioreactor – Continuous Bioconversion and Separations in Single Step

Seth Snyder, Argonne National Laboratory

Principal Investigator:	Seth W. Snyder, YuPo J. Lin	Funding Partners:	Archer Daniels Midland Company
HQ Technology Manager:	Valerie Sarisky-Reed	Sub-contractors:	N/A
PMC Project Officer:	Fred Gerdeman		

Goals and Objectives: The objective is to identify and overcome technical hurdles and to demonstrate the technoeconomic feasibility for an integrated biorefinery to produce biobased chemicals. This project includes C-milestones for a pilot-scale process demonstration and economic analysis for commercialization. The D-milestones are focused on solving technical barriers for process development of the Separative Bioreactor. It includes energy efficiency and productivity of organic acid production; product purification; durability of process components; stability of biocatalysts without neutralization agents. The E-milestones are materials development and characterization of process parameters including: ion-exchange resin wafer development; automation of the Separative Bioreactor; biocatalyst immobilization; identification of key operational parameters.

Project Description: The Separative Bioreactor holds great promise for dramatically reducing the cost of production of many charged biobased products including organic acids and amino acids. The Separative Bioreactor integrates continuous bioconversions and separations into a single step. The DOE Office of the Biomass Program is interested in reducing technical barriers to production of biobased fuels and biobased chemicals. Technical analysis indicated that several organic acids are potentially "Top Ten" candidates for biomass-derived platform chemical intermediates. Overcoming the costs associated with acid neutralization and product separation was identified as a significant technical barrier to reducing the cost of production of organic acids.

Summary of Work to Date - Accomplishments (FY05-current): In initial bench-scale performance demonstrations of this electrically-driven membrane-based technology, the Separative Bioreactor produced and separated both lactic acid and gluconic acid. Initial calculations clearly show the economic advantages of using the Separative Bioreactor. Process economic calculations reveal that the Separative Bioreactor could produce organic acids at significant cost savings in comparison to conventional fermentation technology and even compete with petrochemical processes. Therefore, we believe that the Separative Bioreactor could 1) enable production of biobased chemicals as major platform intermediates and 2) facilitate the commercial success of the emerging integrated biorefinery industry. The scope of products that could be produced as well as long term bench scale and pilot testing are required to confirm the economic impact of this new technology.

Argonne developed an economic model to assess the production cost of biobased chemicals using our preliminary laboratory experimental results. Argonne selected the enzyme glucose fructose oxidase reductase (GFOR) for conversion of glucose to gluconic acid. The enzyme also converts fructose to sorbitol in a sequential reaction. Argonne completed 200 hours of continuous operation of single-stage enzymatic (GFOR) separative bioreactor to produce 18 wt. % gluconic acid without adding neutralization chemicals. The electrical energy consumption met the targets of the economic model.

Argonne and ADM developed a process to produce GFOR for full laboratory-scale separative bioreactor runs. Laboratory-scale, long-term continuous runs are in process. Pilot-scale demonstration are planned for the second half of FY2008.

ADM provided modified conditions for the whole cell fermentation using in-house strains to produce gluconic acid and lactic acid. Argonne conducted additional resin wafer screening to optimize product recovery under the modified conditions. The composition and dimensions of the resin wafer were

optimized for the targeted fermentation pH and steady-state product titer. Argonne provided a redesigned separative bioreactor stack with the new resin wafers to ADM. The composition, fabrication and utilization of these resin wafers are protected by Argonne's background IP portfolio. The specific conditions and performance results are business sensitive patent and are protected by the Argonne-ADM CRADA. ADM performed a continuous integrated whole cell fermentation/separative bioreactor run for 240 hours (gluconic acid). Pilot-scale demonstration is planned for the first half of FY2008. Currently we are commissioning the pilot-scale equipments.

Argonne and ADM have initiated quarterly project reviews alternatively held on each location. To prepare for the pilot demonstration, the project team members have switched to monthly project meetings since March 2007.

To date, two US patents were granted and several patent applications were published and we also filed international patents. They are listed below.

Two US patents granted:
- US Patent 6,797,140 Electrodeionization Method.
- US Patent 7,141,154, Single-stage separation and esterification of cation salt carboxylates using electrodeionization

Four pending US patent applications:
- US Patent Application 20040115783, Immobilized biocatalytic enzymes in electrodeionization (EDI)
- US Patent Application, Retention of counterions in a separative bioreactor
- US Patent Application, 20040917, Electrically and ionically conductive porous material and method for manufacture of resin wafers therefrom
- US Patent Application, 20040917, 20040917,Devices using resin wafers and applications thereof
- Three international patent applications in Brazil, India & Australia, "Electronically and Ionically Conductive Porous Material and Method for Manufacture Of Resin Wafers Therefrom".

Beside the patents, the team received an R&D 100 award in recognition of the commercial promise of the Separative Bioreactor. Argonne and ADM are listed as co-developers of the technology. Chemical and Engineering News (January 09, 2006, pg. 32-33) published a story about ADM's "The ADM way of making chemicals". The story mentions the Argonne-ADM CRADA. Argonne also presented : a poster highlighting the project at BIO 2006; a seminars highlighting the work at the University of Illinois Symposium on Sustainable Bioenergy; a seminar to highlight part of the work at the North American Membrane Society (NAMS) 2006 Annual Meeting; a poster highlighting the project at 2006 AICHE Fall meeting.

Schedule
Project Initiation Date: Tuesday, June 01, 2004
Planned Completion Date: Tuesday, September 30, 2008

Advanced Catalyst Development for Polyols Production

John Holladay, Pacific Northwest National Laboratory

Principal Investigator:	John Holladay / Alan Zacher	Funding Partners:	UOP
		Sub-contractors:	Michigan State University
HQ Technology Manager:	Valerie Sarisky-Reed		
PMC Project Officer:	Fred Gerdeman		

Goals and Objectives:

The overarching goal of our work is to develop catalytic processes to displace petroleum with biomass for the production of commodity chemicals and fuels. To address this goal we have the following objectives:

- Develop broadly enabling catalyst technologies that will achieve catalyst life, specificity and activity targets while operating with actual feeds (rather than pristine feeds).
- Convert sugar alcohol waste streams to polyols. To do this better control of C-C and C-O bond scission is needed. Selective bond scission technology could be widely applicable in numerous applications, including fuels. Success will result in enhanced capability of extracting value from waste streams to improve the overall economics of biorefineries.
- The starting point for this work is to demonstrate the technology required to convert glycerol to propylene glycol.

Project Description:

The overarching objective of this work is to develop technology to control C-C and C-O bond scission reactions. Broadly enabling technology could have a wide range of applications in producing chemicals and fuels from sugar alcohol waste streams. In this stage of the work we are concentrating on an integrated process for the production of propylene glycol from sugar alcohols, specifically glycerol. PNNL has developed a number of catalysts for the production of propylene glycol from sorbitol. Sorbitol is derived from the hydrogenation of glucose. In order to make these technologies commercially viable, further improvements in the catalyst systems need to be developed. Improvements need to come primarily in the form of increased selectivity to propylene glycol. One of the major co-products from the conversion of sorbitol is glycerol. It is critical that a catalyst system be developed for converting glycerol to propylene glycol in high yields. In addition, other sources of glycerol are available and could be used for the production of propylene glycol. Glycerol is a co-product of bio-diesel production or from the transesterification of fatty acids. The utilization of glycerol from biodiesel production or fatty acid production could provide near-term commercial opportunities for implementation of the technology.

UOP has substantial interest in providing the engineering and catalyst manufacture for the conversion of glycerol into propylene glycol for use in high purity applications. The processes for biodiesel production and for the transesterification the fatty acids are very similar and it is expected that a single catalyst technology can be developed that will be applicable to both sources of glycerol to produce propylene glycol.[1]

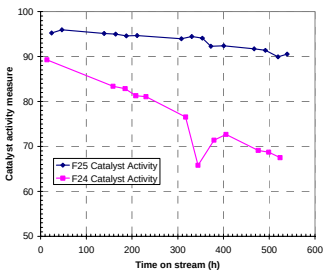
In this work PNNL and UOP have assembled a multi-disciplinary team with defined roles working closely together (see presentation slide 7). PNNL has primary responsibility in catalyst discovery and life-tests, process definition and optimization. Working with Michigan State University, PNNL is also tasked with fundamental kinetic and mechanistic studies. UOP has primary responsibility for commercialization, reaction engineering, and economic modeling; they are also responsible for catalyst characterization and commercialization. UOP maintains a web portal in which all data, notes and other information generated by either party is centrally stored and made available to the team. The team holds a joint web conference on the fourth Thursday of each month and hold informal discussions on a weekly basis. DOE funds are going to PNNL; UOP's research is being done as cost share.

[1] At the inception of the project in April of 2005 ADM was part of the cooperative research and development agreement (CRADA) along with PNNL and UOP. ADM opted out in August of 2006. UOP took over assignments held by ADM and the project was refocused for FY07 research.

Summary of Work to Date - Accomplishments (FY05-current):

Work flow: Catalyst development/process development culminating in techno-economic model

To understand the accomplishments in the last two years it is helpful to break down the work flow into ***catalyst development*** research and ***process development*** research which culminate in a detailed process flow diagram and ***process economics*** (See presentation **Slide 8**). In the presentation we have divided Catalyst development research into (1) determining what supports should be used, (2) defining optimal metals, (3) understanding interactions between support, metals and substrates, (4) defining catalyst activation its performance and (5) validating catalyst production by industry. Development of catalyst must be done in conjunction with process development and we have completed tasks to define the operational parameter space around pressure, temperature, space velocity (flow rates), concentrations, and pH. In the integrated process feed conditioning must be done as well as product recovery and purification. Since raw materials often predominant costs in biobased processes, minimizing feedstock processing is integral. Product separation and purification is essential to meet market acceptance. Accomplishments for each of these areas are summarized in the paragraphs that follow. In the Annual Operating Plan (AOP) milestones are based on process economics as related to cash cost of production (CCOP) and return on investment (ROI). This is presented in the Project Milestone Summary Table at the end of the report.



Brief summary of efforts through August 2005

At the time of the FY05 conference we had completed a significant amount of combinatorial / high throughput screening studies. These studies were aimed at identifying catalyst supports, metals, co-metals, and additives (pH modifiers). At the same time we initiated batch studies to validate findings from the continuous reactor and flow reactor studies to examine catalyst life. The initial flow study data was sobering; catalyst life was unacceptable. In both runs the drop of activity was unacceptable for a 500 h test. Further catalyst discovery work was implemented using high throughput techniques. While at the same time additional batch reactor work and flow reactor work using pristine feed was carried forth.

Where we are today: Technology demonstration for glycerol to propylene glycol

Catalyst development studies. Based on data obtained from extensive combinatorial research, carbon supports exhibited substantial promise. Catalyst activities were higher with carbon-based supports than observed for stable metal oxides such as monoclinic zirconium, rutile titania or carbon doped with metal oxides. Furthermore, a significant difference in performance was exhibited among different carbon supports that were examined. The greatest impact was on catalyst activity. Although the impact on selectivity was less severe, to meet the selectivity target (>90%) *one particular carbon was singularly effective* (see presentation slides 9 and 10).

The support plays many roles in the catalyst. For example it keeps the metal finely dispersed to prevent sintering and leaching. The support can modify catalytic behavior of the metals. In addition supports may also interact with the substrates and products. This is particularly true for activated carbon. Hence the local pore concentration of products and reactants can vary greatly from that of the bulk solution. One can consider the support as a delivery system of the substrate to active catalytic sites. We have found that propylene glycol (PG) competitively adsorbs on carbon compared to glycerol at both low and high temperature. However, the trend appears to be reversed in regards to the metal where glycerol is preferentially adsorbed on Ru sponge. One implication of this finding is that PG could have stability problems. Another is that reaching high conversion could be difficult. We are able to circumvent both problems through operating under continuous flow reactor (see presentation slide 11).

Although much work was done prior to August 2005 on identifying optimal metals, we ran a significant number of combi plates to further define metal composition. A portion of the work was designed to expand metal options and replace certain metals in bimetallic systems. We discovered new metal compositions that were effective in hydrogenolysis chemistry. Approximately 1000 individual runs were

completed in the combi system during this study. From that work about ten catalysts carried on to flow reactors (see presentation slide 12), two of which were chosen to go forward (see presentation slide 13).

Flow reactor testing turned out to be integral to the program. Hydrogen mass transfer is better controlled in a trickle bed flow reactor resulting in higher selectivity (see presentation slide 14 and 28). Approximately 125 flow reactor runs have been completed since August 2005, ranging in duration from 24 h to 2000 h. The focus of the runs has been to demonstrate catalyst life, optimize catalyst activation procedures, define process operating parameters and determine impact of feedstock impurities. One key accomplishment from this work was reducing the total metal loading on the catalyst (slide 13). A second key accomplishment was completing a demonstration run of over a quarter year without loss of catalyst performance (slide 14). A third key accomplishment was determining the optimal method to activate the catalysts. To our surprise the two catalysts required different procedures to maximize activity (slide 15). Catalysts were examined by a battery of surface science tools which included testing at the Advanced Photon Source at Argonne National Lab (by Simon Bare of UOP). The work was used to direct activation studies and determine morphology changes on the catalyst over time. Catalyst research culminated with industry replication and scale-up of the two PNNL catalyst systems, a significant milestone (slide 16).

Process research. To optimize selectivity and conversion several experimental design sets were complete which examined pressure, temperature, liquid space velocity (flow rates), hydrogen to substrate ratio, substrate concentration, and pH. The work was completed in flow reactors. Statistical tools were used to analyze results (slide 17). Several surprises were found that are the basis for new intellectual property. The work culminated with industry validating the process at UOP in 150 cc flow reactors (slide 18). Industry validating the process in a scaled-up reactor system was a significant milestone.

An important aspect of flow reactor work was to reduce raw material costs. Initial work was done on purified feed. The impact of various impurities was tested by doping pure feeds and running for 24-48 h. At the same time process options for raw material processing were evaluated. We were able to remove several costly unit operations and two process options were down selected for raw material processing. We have achieved a key accomplishment of reducing feed costs while demonstrating catalyst life (slide 19).

Coming out of the catalytic reactor is a crude propylene glycol stream with various impurities. For the final process, the propylene glycol needs to be separated and purified. This work is underway driven by modeling along with experimentation. The goal is to minimize cost to produce proypylene glycol streams that will meet market acceptance (slide 20).

Process economics. The catalyst and process work, described in the paragraphs above, culminate in a detailed process flow diagram and a full technoeconomic model. Models of this sort are invaluable in determining sensitivity analysis to direct future research efforts. The CCOP and ROI have been determined. Although we are not publishing numbers in this document, the resultant numbers are the basis for an internal UOP Go/No-Go decision point. A technology review has occurred which received high marks. The primary show stoppers at this stage are market based and are listed on slide 21. In the longer term the knowledge gained from this work can be used to widen the range of feedstocks and products.

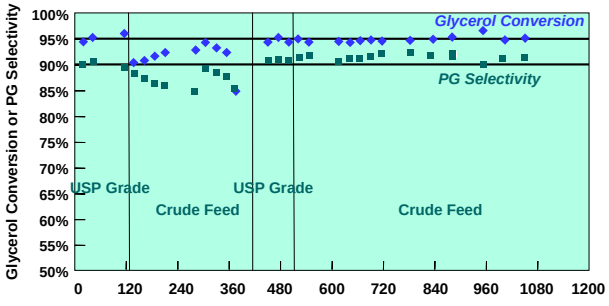


Figure 1. Accomplishments: met catalyst life and performance targets; industry validated

Schedule

Project Initiation Date: April 1, 2005
Planned Completion Date: September 30, 2008

Isosorbide, the Continuous Isosorbide Production from Sorbitol Using Solid Acid Catalysis

Rodney Williamson, Iowa Corn Promotion Board

Principal Investigator:	Norm Olson	Funding Partners:	The University of Iowa
HQ Technology Manager:	Valerie Sarisky-Reed	Sub-contractors:	N/A
PMC Project Officer:	Fred Gerdeman		

Goals and Objectives: The main objective of this project is to develop and/or enhance cost-effective methodologies for converting biomass into a wide variety of chemicals and products using supercritical fluids. Supercritical fluids will be used both to perform reactions of biomass to chemicals and products as well as to perform extractions/separations of bio-based chemicals from non-homogeneous mixtures.

Through this project we hope not only to empirically develop new paths for the conversion of biomass to chemicals but also to enhance the understanding of the science behind the nature of supercritical fluids.

Project Description: Supercritical fluids processing of biomass represents a very versatile and diverse path to the production of chemicals. Supercritical fluids can be used under a variety of conditions to quickly convert cellulose to sugar or to convert biomass into a mixture of oils, organic acids, alcohols and methane. Supercritical conditions exist when a fluid is pressurized and heated to conditions where the fluid behaves as neither a gas nor a liquid. This high pressure/moderate temperature zone is called the supercritical (SC) region. Most fluids exhibit significant changes in properties in the supercritical region, for example, water exhibits the characteristics of a high strength acid in the SC region while carbon dioxide exhibits solvent characteristics similar to hexane. Supercritical pressures and temperature are different for each fluid.

In this project numerous chemical reactions and extractions that take place when biomass is exposed to supercritical fluids will be investigated. With the exception of some well-known industrial processes the mechanisms underlying supercritical fluid interaction with solid biomass is not well understood.

This project will investigate several conversion reactions that may include 1) cellulose to sugars; 2) sequential dissolving and separation of cellulose, hemi-cellulose and lignin; 3) catalyst free biodiesel esterification; 4) other catalyst free esterifications; 5) direct biomass conversion to numerous chemicals; and 5) direct biomass conversion to hydrogen and CO.

The end result of many processes used to convert biomass to chemicals and fuels is often a somewhat complex mixture of products. This is true for many supercritical fluids reaction systems and most sterile and non-sterile fermentation systems. Supercritical fluids will be investigated with the goal of extracting key industrial chemicals such as furfural, organic acids (e.g. acetic acid and lactic acid) and alcohols (e.g. ethanol) from mixtures.

Summary of Work to Date - Accomplishments (FY05-current): Preliminary experiments and analysis on cellulose reactions to sugars in supercritical water have been performed using existing, old reaction equipment at the University of Iowa. Yields of approximately 50% sugars (glucose + fructose) have been demonstrated so far. Progress has been made in the development of reactor models for the hydrothermal conversion of cellulose to sugars. COMSOL Multiphysics finite element modeling software was used to model the temperature and flow patterns in the laboratory reactors. Accurate temperature- and pressure-dependent water properties were incorporated into the models, and the models yield results consistent with experiment. The reactor models will be used in interpreting experimental data for the determination of rate constants for cellulose hydrolysis and decomposition of sugars under hydrothermal conditions.

The work performed using the old reactor at the University of Iowa will be used to define starting conditions for the new reactor being installed at Iowa State University.

Reaction kinetic parameters were obtained for several reactions involved in the hydrothermal decomposition of cellulose. Specifically, activation energies and pre-exponential factors were obtained for the hydrothermal conversion of glucose, fructose, cellobiose, and cellotriose. Kinetic constants were calculated for the formation of 5-HMF, glyceraldehyde, levoglucosan, and erythrose during decomposition of monosaccharides, and for the isomerization reaction between glucose and fructose.

Preliminary experiments have been performed on catalyst free esterification of carboxylic acids in supercritical alcohols. The purpose of this research track is to demonstrate the use of supercritical fluids to make esters from soybean oil via a catalyst-free method.

A flow type reactor was designed and built to increase conversion of reactants. An esterification reaction was performed to test the new system. Technical grade linoleic acid purchased from Sigma was reacted with methanol. The solvent in the reaction products was removed under reduced pressure, then analyzed using NMR. The NMR spectrum of the esterification product does not indicate the presence of any unreacted linoleic acid, only methyl esters. Thus, the reaction appears to have proceeded to 100% conversion.

Work was started on setting up the reactor for organic acid esterification in supercritical alcohols and on developing protocols for chemical analysis

Schedule
Project Initiation Date: May 31, 2006
Planned Completion Date: June 30, 2008

Succinic Acid as a Byproduct in Corn-Based Ethanol Biorefineries

Susanne Kleff, Michigan Biotechnology Institute

No project summary provided.

Development of Sustainable Bio-Based Products and Bioenergy in Cooperation with the Midwest Consortium for Sustainable Bio-Based Products and Energy

Mike Ladisch, Purdue University

Principal Investigator:	Dr. Michael Ladisch	Funding Partners:	Michigan State University,
HQ Technology Manager:	Valerie Sarisky-Reed		University of Illinois, Iowa
PMC Project Officer:	Gene Petersen		State University
			N/A
		Sub-contractors:	Ames Laboratory,
			Competitive Pool-Recently
			Awarded, USDA NCAUR

Goals and Objectives: The work scope is based on the focus of this research on distillers' dry grains. Distillers' dry grains are co-products of dry-mills after starch in ground-up corn kernels has been fermented to ethanol. Distillers' grains contain cellulose (6 carbon sugars), pentosans (5 carbon sugars), residual starch (6 carbon sugars), some lignin, and protein. This composition provides opportunities to add value to the product mix from a dry-mill ethanol plant, reduce the net cost of ethanol, and support the DOE sugar platform. The proposed scope of work is defined by: 1) pretreatment of the distillers' grains; 2) hydrolysis of the resulting streams; 3) fermentation of the xylose and glucose in the hydrolysates to alcohols and chemicals and chemical intermediates; 4) analysis of composition of various streams for generating preliminary material balances and advanced separations to enhance overall process economics; 5) life cycle analyses to assess environmental impacts of new processes to allow for more rapid implementation of technology, and 6) economic assessment of the impact of process improvements resulting from this research on market potential. Participants for each area may come from any or all of the consortium members.

Our goal is to develop process technologies for (1) creating new markets for DDGS and (2) transitioning cellulose conversion technologies for use in existing corn to ethanol plants. The absence of a heavily lignified cell wall structure in DG makes DG particularly susceptible to pretreatment using either hot water or ammonia freeze explosion. The economic analysis compares added cost of conversion to the increased yields of fermentable sugars and ethanol in order to project the impact of cellulose conversion technology to increase income in dry grind plants.

Project Description: The new renewable fuel standards will push ethanol production to 7.5 billion gallons per year within two years. This project addresses processing that occurs post-fermentation and therefore minimizes impact on existing equipment in an ethanol plant, and facilitates retrofit of cellulose conversion capability into dry grind facilities. The project integrates multiple institutional capabilities to help solve a crucial problem: the proliferation of low value, fiber rich distillers grains (DG) now being produced in the corn dry milling industry.

The dry grind industry is growing rapidly, and the proliferation of the DG and DDGS has the potential to depress the market for this by-product and decrease the profitability of dry mills. This concerted effort will add value to distillers' grains by further processing them into additional fermentable sugars and ethanol, while leaving a solid that is reduced in weight and rich in protein. The project involves and integrates among members of the consortium: 1) advanced pretreatments to enhance the digestibility/reactivity of the fiber component (cellulose and hemicellulose) of DG, 2) enzymatic hydrolysis of pretreated DG to produce fermentable sugars and thereby remove part or all of the cellulose and hemicellulose, and thereby increasing the feed value of the residual unhydrolyzed solids, 3) fermentation of these sugars to ethanol and other biobased products, 4) analysis of composition and advanced separation methods applicable to ethanol and other products, 5) life cycle analysis to quantify key environmental features of these corn based biorefineries and the crop production systems that support them, and 6) comprehensive economic analysis of the processes, technologies, and markets, incorporating uncertainty in key technological and market parameters. As part of the Biomass Program within DOE's Office of Energy Efficiency and Renewable Energy, it is expected that this research will contribute directly to the multi-year

technical plan particularly as related to the "Sugar Platform" and "Products," thus helping to ensure the department meets its targets to establish biomass as a significant source of sustainable fuels for the United States.

Summary of Work to Date - Accomplishments (FY05-current): The Consortium has worked together as a research team to obtain compositional analysis of DG and DDGS, to pretreat these materials, and then to systematically study their bioprocessing into sugars, ethanol, and other bioproducts. The results have been used to construct outlines of dry grind process flowsheets for untreated and pretreated DG so that compositional data, resulting from our experiments could be mapped onto the flowsheets to develop material balances, and to quantitate inputs and outputs for purposes of constructing an economic model.

The research has been carried out in close collaboration with industrial partners. One partner provided wet cake (DG) and DDGS, and insights on practical considerations for enhanced utilization of DG or DDGS. Genencor works closely with the Consortium to provide enzymes, expertise, and guidance on the types of enzymes that might be best suited for a specific set of processing conditions. These data, combined with an economic model that utilized current baseline capital costs, and cost of goods have enabled potential economic returns to be assessed if a dry grind facility were to incorporate cellulose conversion technology to reduce the volume of unfermented co-product, enhance ethanol yields, or produce other biofuels, while marketing the residual protein. While the scope of the work focuses on sugar and ethanol production, some effort was also made to analyze the composition, as well as the potential feeding value of a product that we have termed enhanced distillers' dried grains and soluble, since the processing of DDGS to additional ethanol results in a decreased mass and increased protein content of the DDGS that remain.

DDGS and wet distiller's grains are the major co-products of the dry grind ethanol facilities. As they are mainly used as animal feed, and a typical compositional analysis of the DDGS and wet distiller's grains mainly focuses on defining the feedstock's nutritional characteristics. With an increasing demand for fuel ethanol, the DDGS and wet distiller's grains are viewed as a potential bridge feedstock for ethanol production from other cellulosic biomass. The introduction of DDGS or wet distiller's grains as an additional feedstock for existing dry grind plants for increased ethanol yield requires a different approach to the compositional analysis of the material based on determining a detailed chemical composition. This is especially the case for the polymers cellulose, starch and xylan, which release fermentable sugars upon enzymatic hydrolysis. One accomplishment of this research is the detailed and complete compositional analysis procedure for DDGS and wet distiller's grains, as well as the determination of resulting compositions carried out independently by three different research groups. The compositions were comparable, thus giving an internally consistent basis for developing material balances, assessing the impact of pretreatment and hydrolysis, and developing an economic model.

One accomplishment is development of reproducible and accurate compositional analysis of DDGS and wet distiller's grains with a close to 100% mass closure. DDGS and wet distiller's grains are rich in glucan, xylan and arabinan. Total available sugars (glucan and xylan) of DDGS and wet distiller's grains for producing ethanol were measured to be 29.4% and 36.4%, respectively, based on total dry mass. Glucan includes both starch and cellulose in a ratio of approximately 1:3. Crude protein comprises 25% of the total dry mass of DDGS. Crude oil measured as ether extractives is 11.6%.

The second major accomplishment of this research is the use of controlled pH, liquid hot water (LHW) and ammonia fiber expansion (AFEX) pretreatments of DG and DDGS to enhance enzymatic digestibility of the distillers' grains . Both pretreatment methods significantly increased rates and extents of hydrolysis of distillers' dried grains with soluble (DDGS) as compared to untreated material, resulting in over 90% cellulose conversion to glucose within 24 hours of hydrolysis, at an enzyme loading of 15 FPU cellulase and 40 IU β-glucosidase per gram of glucan. Hydrolysis of pretreated wet distillers' grains at 13-15% (wt of dry distillers' grains per wt of total mixture) solids loading with the same enzyme dose resulted in approximately 70% glucan conversion to glucose within 72 hrs, regardless of the pretreatment methods applied. In addition a new approach to pretreating the DG was found. This approach forms phosphite esters of the oligosaccharides in DG and DDGS. This has been shown to be effective in dissolving the DDGS or DG in water.

A third major accomplishment is developing an understanding of how noncellulolytic enzymes act on pretreated substrates to enhance the hydrolytic efficiency of cellulases. This work was carried out with the input and assistance of Genencor. Supplementing the cellulase enzyme mixture with xylanase and feruloyl esterase for the high-solids digestion of the pretreated wet distillers' grains at 15-20% solids (w/w) enhanced the glucose yields up to 80% and xylose yields up to 50%. Fermentability of the hydrolyzed wet distillers' grains was tested by non-recombinant *Saccharomyces cerevisiae* ATCC 4124 strain, and close to metabolic (theoretical) yields of ethanol were obtained, for both LHW treated and AFEX treated wet distillers' grains. Enhanced DDGS refers to the final product of a modified dry grind process in which the distillers' grains are recycled and processed further to extract the unutilized polymeric sugars. Compositional changes of the laboratory synthesized enhanced DDGS have also been carried out and are part of an on-going activity.

The fourth major accomplishment was proving the fermentability of both glucose and xylose, derived from hydrolysis of pretreated substrates. Since industrial yeast strains are not capable of fermenting a major part of the sugars in this hydrolysate, non-traditional microorganisms were tested. These were: *Clostridium beijerinckii* BA101 for fermentation to butanol, and *Saccharomyces* 424A(LNH-ST) and *Escherichia coli* FBR5 for fermentation to ethanol. The yeast 424A(LNH-ST), under license to Iogen is in use at their new biomass/ethanol demonstration facility. Development of optimal fermentation conditions showed that all three microorganisms are capable of converting 80%, or more, of glucose and xylose to product. In the case of *E. coli,* acetic acid was found to be inhibitory, while some aromatic compounds derived from degradation of low molecular weight, lignin-like compounds in the DG could inhibit *Clostridium*. Overall, the formation of inhibitors due to the LHW and aqueous pretreatments was found to be minimal, and to have little effect on fermentation of the sugars to ethanol or to butanol.

The formation of oligosaccharides due to pretreatment led to a fifth accomplishment: the examination of a fixed bed (plug flow reactor) of meso-phase or solid catalysts. The results showed that current catalysts are capable of rapidly hydrolyzing cellobiose to glucose, but that the conversion of xylo-oligosaccharides and cellodextrins was not as efficient. While the results show a fixed bed catalyst has potential for rapidly processing aqueous streams containing soluble oligosaccharides, catalyst characteristics, including pore size, acid strength, and thermal stability need to be significantly improved for this approach to be feasible.

A sixth accomplishment was the development of an economic model, based on a process flowsheet in which pretreatment is added onto, or retrofitted to a dry grind facility. The concerted efforts of the Midwest Consortium Team enabled an integrated flowsheet to be developed, and the data on pretreatment, hydrolysis, fermentation, and co-product value to be mapped onto it in order to generate material balances around the process. The resulting flowsheets are being used to generate economic analysis, and to quantitate the value that is added by cellulose pretreatment and enzyme hydrolysis of DG and DDGS from a dry grind facility.

The seventh major accomplishment has been development of a peer reviewed, 10-paper compendium of the research results from this work. This work is being submitted to the journal, *Bioresource Technology*, to give an integrated assessment of the research results, obtained thus far, as well as a quantitative assessment of practical approaches to using cellulose conversion technology to enhance utilization of DG and DDGS in dry grind facilities. Work still remains to be done. However, the researchers in the Consortium wanted to inform colleagues and the industry of the status of this work, and the exciting potential of the near term application of cellulose conversion technology to dry grind, corn to ethanol plants. Annual meetings of the Midwest team and representatives from industry have also been carried out to communicate our results and receive feedback from a broad audience.

Schedule

Project Initiation Date: Friday, October 01, 2004
Planned Completion Date: Saturday, June 30, 2007

Iowa State University Biomass Energy Conversion Project

Norman K. Olson, Iowa State University

No project summary provided.