

Final Report

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Recipient: Iowa Corn Promotion Board

Project Title: Value Added Products from Hemicellulose Utilization in Dry Mill Ethanol Plants

CRADA Number: CRADA 218

Consortium:

Iowa Corn Promotion Board (ICPB)

Pacific Northwest National Laboratory (PNNL)

Idaho National Lab (INL)

Dyadic

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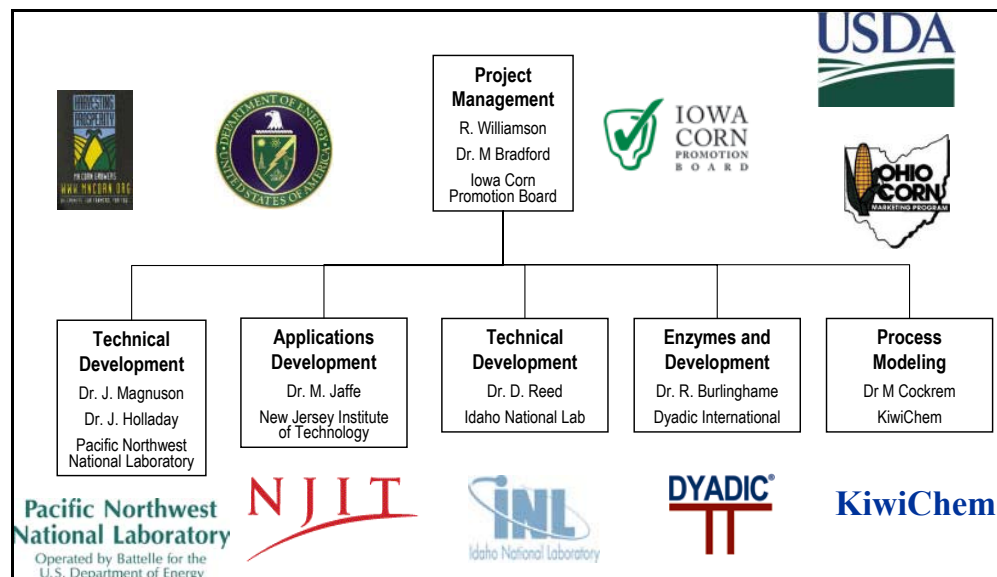
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Executive Summary

The Iowa Corn Promotion Board is the principal contracting entity for this grant funded by the US Department of Agriculture and managed by the US Department of Energy. The Iowa Corn Promotion Board subcontracted with New Jersey Institute of Technology, KiwiChem, Pacific Northwest National Lab and Idaho National Lab to conduct research for this project. KiwiChem conducted the economic engineering assessment of a dry-mill ethanol plant. New Jersey Institute of Technology conducted work on incorporating the organic acids into polymers. Pacific Northwest National Lab conducted work in hydrolysis of hemicellulose, fermentation and chemical catalysis of sugars to value-added chemicals. Idaho National Lab engineered an organism to ferment a specific organic acid. Dyadic, an enzyme company, was a collaborator which provided in-kind support for the project. The Iowa Corn Promotion Board collaborated with the Ohio Corn Marketing Board and the Minnesota Corn Merchandising Council in providing cost share for the project. The purpose of this diverse collaboration was to integrate the hydrolysis, the conversion and the polymer applications into one project and increase the likelihood of success.

Figure 1. Organization of the Hemicellulose Project



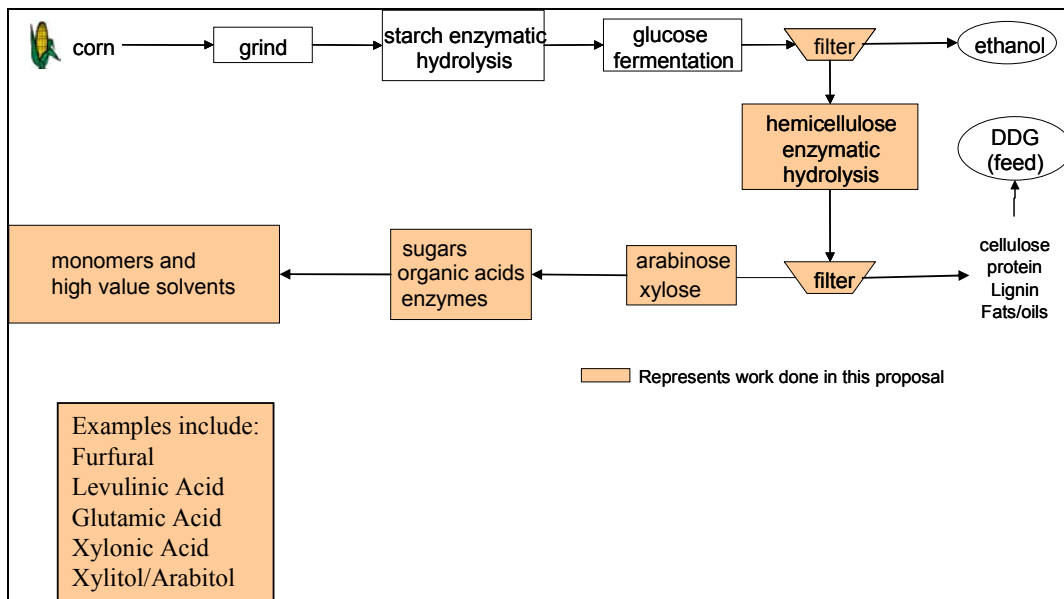
This project had two primary goals: (1) to hydrolyze the hemicellulose fraction of the distillers grain (DG) coproduct coming from the dry-mill ethanol plants and (2) convert the sugars derived from the hemicellulose into value-added co-products via fermentation and chemical catalysis.

How research adds to understanding of the area investigated. Many projects have investigated the hydrolysis of cellulose to glucose and further conversion to ethanol.

This project was designed to develop a system to hydrolyze the hemicellulose portion of the fiber. The resulting sugars would be xylose and arabinose. Then the resulting sugars would be fermented into value-added industrial products. Dyadic has a proprietary collection of enzymes that are used extensively in other market sectors. This collaboration was designed to evaluate those enzymes for this new application into the hydrolysis of hemicellulose in the DG. The results were a better understanding of the complex molecule and the enzymes necessary to hydrolyze the hemicellulose.

The other elements of this project were to convert the C5 sugars (arabinose and xylose) into value-added products. Pacific Northwest National Lab worked on discovering an organism for fermentation for a specific organic acid. The successful element was the discovery of an organism to convert glucose to the targeted organic acid. Idaho National Lab worked to genetically engineer an organism to withstand a lower pH while producing an organic acid. This work was moving toward a successful organism to produce organic acids in a low pH environment when the project ended due to funding constraints.

Figure 2. Dry Grind Ethanol with Hemicellulose Extraction and Conversion



Technical effectiveness and economic feasibility. This project is intended to find a new use for the co-product of the dry-mill ethanol plants known as distillers grain (DG). With the expansion of the ethanol industry, DG has increased in supply. Developing new uses for this co-product will be necessary as supplies increase. Currently the United States produces about 27 billion pounds of DG annually. Corn based ethanol will easily reach the 15 billion gallons within a few years. When this goal is achieved, the United States will produce 42 billion pounds of DG.

The enzymes were analyzed compared to the hydrolysis of starch to glucose. The chart below shows the targets that we were trying to achieve in this project. The

estimated cost of glucose hydrolyzed from starch is \$0.08/lb. We have a target scenario for producing xylose and arabinose from DG is equivalent to glucose (\$0.08/lb).

Table 1. Enzyme Economics to Hydrolyze DG

	DG target scenario	DG low cost scenario	DG high cost scenario	Starch to Glucose
raw material	2 cents/lb	2 cents/lb	4 cents/lb	6 cents/lb
enzyme	2.2 cents/lb	1.25 cents/lb	3.15 cents/lb	0.1 cents/lb
purification	3.8 cents/lb	2.85 cents/lb	4.75 cents/lb	1.9 cents/lb
Cost target	8 cents/lb	6.1 cents/lb	11.9 cents/lb	8 cents/lb

KiwiChem developed an engineering model of a corn dry-mill ethanol facility. This model was to be used as a base for future modification of a conceptual process. However, the research results were not advanced far enough to implement this modified process.

How otherwise benefits the public.

There are a number of ways the public will benefit from this work. First the displacement of petroleum derived monomers with biomaterials provides a new example of how high value and high volume products can be produced using renewable resources in a sustainable manner. These products will add value to farmers in rural parts of the United States as new uses are developed for corn and other starch-rich crops. Finally, there is significant value to the DOE and USDA, who funded this work, in empowering biorefineries with the choice of a new product that could develop into a significant market.

Accomplishments versus goals and objectives

Task 1: Project Management

Rod Williamson, Iowa Corn Promotion Board

The Iowa Corn Promotion Board was the project manager of this research. The ICPB hired technical consultants Bob Mustell and Marion Bradford to help guide the technical progress and evaluate milestones. Frequent conference calls and meetings were held to discuss the progress.

Task 2: Characterization of whole stillage

Jon Magnuson, Pacific Northwest National Lab

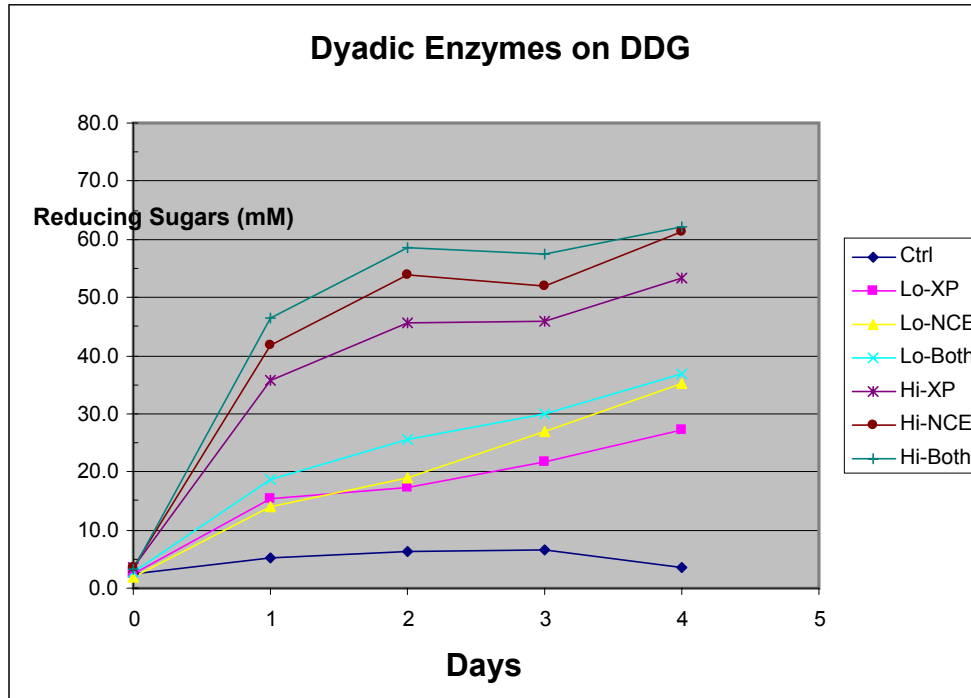
This task was aimed at determining the composition of DDGS feedstocks originating from dry mill ethanol plants. The composition was found to be as expected for the hemicellulose (arabinoxylan) portion. In other words, the hemicellulose was relatively unaffected by the cooking, saccharification, fermentation, distilling and drying steps. Depending on which dry mill ethanol plant was the source of the DDGS, its composition varied not with respect to arabinoxylan but with respect to free glucose and glycerol. Specifically, there was variation in the amount of residual glucose and glycerol from the yeast fermentation (less than 10% of total sugars). The presence of free glucose indicates incomplete carbon source utilization by *Saccharomyces cerevisiae* during the fermentation to ethanol. The presence of glycerol indicates some level of inefficiency in the fermentation, as glycerol is a sink for reducing equivalents and an undesired by-product common in yeast based ethanol fermentations. This variation is likely to exist on both a plant-to-plant and batch-to-batch basis.

Task 3: Develop hemicellulases necessary for hydrolysis of arabinoxylan

Jon Magnuson, Pacific Northwest National Lab

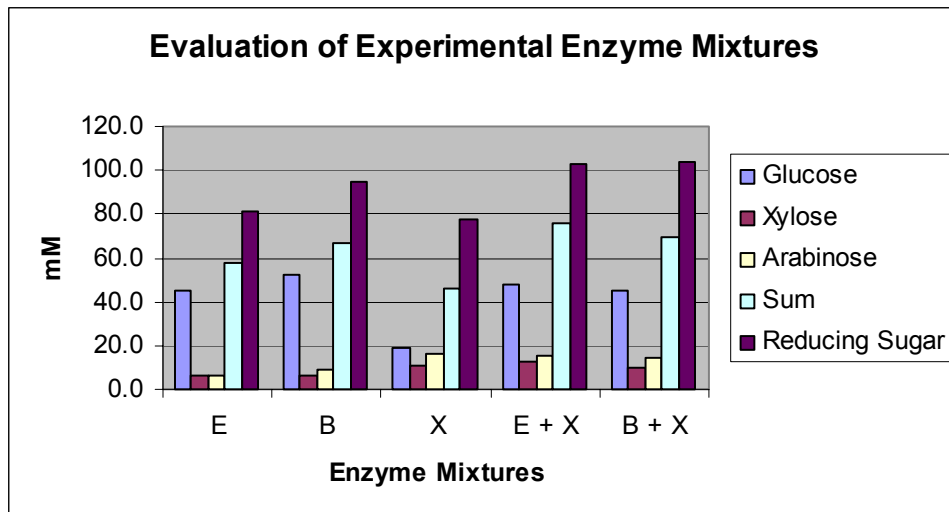
This task was aimed at identifying the enzyme activities needed for better hydrolysis of hemicellulose and identifying hemicellulases with improved enzymatic properties. The preferred enzymatic properties were increased thermal stability of the enzyme and a higher temperature optimum for the enzyme activities of interest. There are six to seven enzyme activities required for the hydrolysis of corn glucuronoarabinoxylan. These include the glycosyl hydrolase enzymes, endoxylanase, β -xylosidase, α -arabinofuranosidase and α -4-O-methyl-glucuronidase. The first two enzymes are necessary for hydrolysis of the xylan backbone and the other two are needed to cleave side chain sugar groups. In addition, the carbohydrate esterases, acetylxyLANesterase and feruloyl esterase are required to cleave acetyl groups from the xylose residues and ferulic acid groups esterified to the L-arabinose side-chains.

Our hypothesis was that current commercial hemicellulase preparations (mixtures of enzyme activities) were inadequate for complete conversion of hemicellulose to the constituent sugars. We found this to be the case (Figure #). However, hemicellulase and cellulase preparations could act synergistically to improve overall free monomeric sugar release and net solubilization of the fibrous solids, i.e., conversion of a portion of the insoluble polysaccharides to soluble oligosaccharides. Nevertheless our assays of individual activities present in these preparations indicated that they were low in β -xylosidase activity relative to endoxylanase activity. This is very likely one of the reasons that complete hydrolysis to free xylose was not observed.

Figure 3. Hydrolysis of DDG by commercial xylanase and cellulase products.

A variety of experimental hemicellulase mixtures from different fungi were evaluated in the same manner and improved preparations were identified. The best variety was evaluated in combination with commercial products to determine if there were synergistic components available in the different enzyme preparations. Figure 4 indicates that this was the case. Further evaluation is necessary to determine the best pre-treatment option and if supplementation with additional single enzyme activities, e.g., β -xylosidase, would improve the release of xylose and/or arabinose.

Figure 4. Evaluation of experimental hemicellulase and cellulase mixtures.



In parallel with the exploration of commercial and experimental hemicellulase and cellulase mixtures we evaluated different fungal strains, especially thermophilic species, for novel enzyme activities. We evaluated them for improved thermo-stability or improved temperature optima for the following enzyme activities: β -xylosidase, α -arabinofuranosidase, endoxylanase and acetyl esterase. A number of candidate enzymes with improved thermal stability and kinetic properties were identified for β -xylosidase, α -arabinofuranosidase and acetylxyLANesterase activities. The genes for a β -xylosidase and an acetylxyLANesterase were cloned from a thermophilic fungal species. Protein expression and analysis of enzyme kinetic parameters would be dependent on continuing support from a subsequent publicly funded or commercial project.

Task 4: Fermentation of Pentoses to Selected Organic Acids

Linda Lasure, Pacific Northwest National Lab

The purpose of this task was to examine fungal processes for the production of organic acids on pentoses using a selected fungal species and a target organic acid production as the model process. The ability of the fungus to utilize pentoses in a model hemicellulose hydrolyzate was tested. Finally, whole ground corn that had been subjected to starch liquefaction and enzymatic saccharification was obtained from the process stream of an operating dry mill ethanol plant. This sugar stream was used to evaluate the effectiveness of the fungal strain for organic acid production under non-carbon nutrient concentrations found in an authentic potential feedstock.

Microbial strain evaluation in shake flask experiments resulted in selection of strain 5 as the best strain (Table 2). Media optimization experiments in shake flasks examined a number of parameters and revealed the best conditions for conversion of glucose to organic acid in the laboratory. Mixtures of xylose and arabinose using commercially pure substrates as starting materials resulted in organic acid production exceeding 20 g/liter. High titers were also achieved when the enzymatic starch hydrolyzate from the dry mill ethanol plant accounted for up to 25% of the free sugars. Higher proportions of

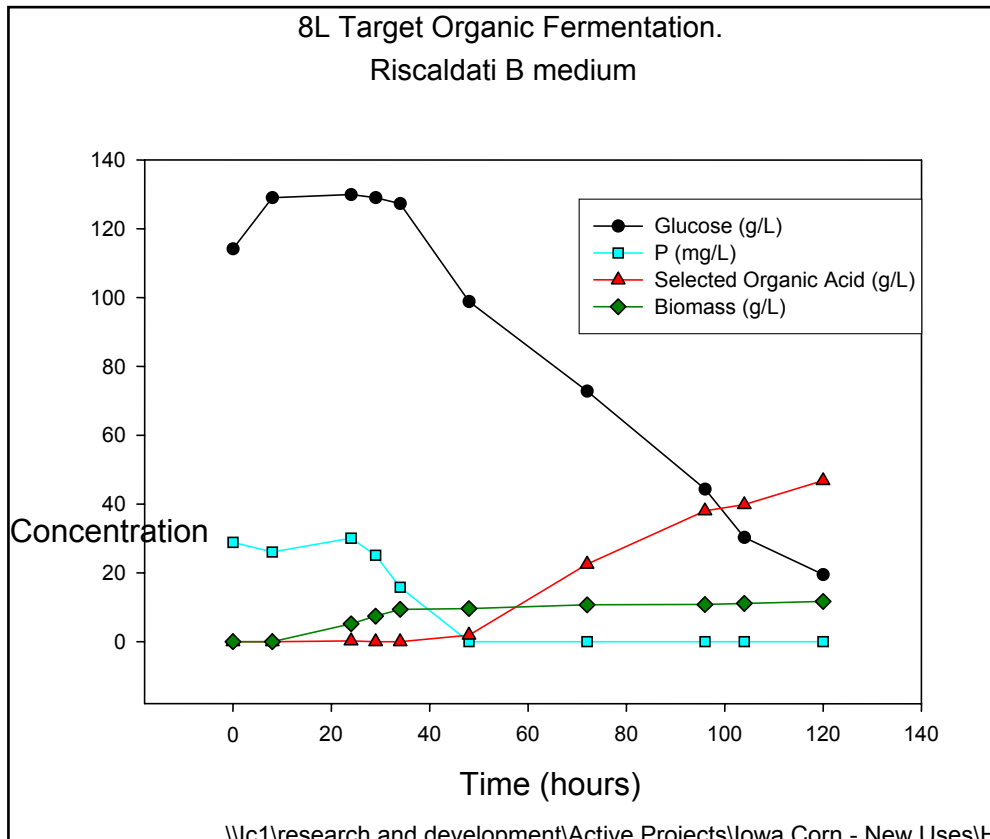
starch hydrolyzate led to decreased titers. We believe this was likely due to the measured higher free phosphate concentration in these authentic feedstocks. The phosphate concentration is known to be critical, as a target organic acid production does not commence until after free phosphate in the media is completely consumed (see Figure 4).

Table 2. Seven fungal species strains were grown on a medium containing 10% xylose, 0.3% ammonium sulfate, 0.01% potassium phosphate-monobasic, 0.05% magnesium sulfate, 0.3% calcium sulfate.

Selected Strains	Target Organic Acid (g/l)	Selected Strains	Target Organic Acid (g/l)
Strain 1	11.1	Strain 5	26.1
Strain 2	7.2	Strain 6	12.2
Strain 3	18.5	Strain 7	0.7
Strain 4	12.3		

Evaluation of a selected fungal species in an 8 L stirred tank fermenter with pure glucose media containing inorganic nutrients (N and P sources) resulted in 42 g/liter a target organic acid in five days (Figure 4). Scale-up to a 30 L stirred tank fermenter with glucose media resulted in greater than 50 g/liter a target organic acid.

Figure 5. Conversion of glucose to a target organic acid by a selected fungal species.



Task 5: Development of novel organism and process for target organic acid production.

William A. Apel and David W. Reed, Idaho National Laboratory

Biological-based organic acid production utilizes microorganisms that grow near neutral pH. Although some of these organisms can produce high levels of a target organic acid and can tolerate high concentrations at the low pH associated with a target organic acid is inhibitory to fermentation processes. **The goal of this work was to move toward the development of an acid-tolerant microbial process for the fermentation of the pentoses from arabinoxylan to a target organic acid.**

A biological a target organic acid process is desirable since low pH allows simplified sterility maintenance due to inhibition of most microbial contaminants, and simplified, lower cost a target organic acid fermentation and recovery by removing the need for (1) continuous neutralization to maintain microbial growth, (2) expensive downstream recovery processing to obtain the uncharged organic acid form, and (3) expensive waste salt disposal. Current organic acid producing processes requiring microorganisms to ferment at near neutral pH to maintain stable cellular activity are encumbered by the continuous addition of base to maintain fermentation and resultant build up of organic acid salt from the subsequent inorganic acid additions. These types of processes require expensive purification of the uncharged a target organic acid and disposal of the generated salt wastes. A low pH organic acid producing microorganisms would eliminate many of these difficulties associated, in addition to sterility maintenance from microbial contaminants. Direct extraction of the free acid into an organic phase of a two-phase (organic/aqueous) reactor could extract the organic acid continuously.

Our engineered organism produced the target organic acid; however, additional organic acids were being produced and reduced carbon flow into the target organic acid. Attempts to knock out the pathways for competing organic acids initially proved unsuccessful. Additional knockout pathways were added, but due to limited time and funding, the final evaluation of the new constructs was not complete.

We therefore did not achieve our initial goal. It was anticipated that approximately an additional 6 months with appropriate funding would be required to evaluate the new constructs.

Task 6. Conversion to organic acids by chemical catalysis

John Holladay, Pacific Northwest National Lab

This task was originally designed to explore the catalytic conversion of a target organic acid to other products using authentic fermentation mixtures as feedstocks. The purpose of this limited task was to examine issues that might arise with regard to robustness of the chemical catalysts in the presence of the complex chemical mixture resulting from an actual fermentation process. The scope of this task was changed in the last year of the project (after DOE approval) to evaluate sugar oxidation by

heterogeneous catalysis. The rationale for the change in scope was to maximize the limited investment of funding for this task to explore a relatively high risk but unexplored area of chemical catalysis, namely the oxidation of sugars by heterogeneous catalysis. The principal challenges in this area are the selective oxidation of hydroxyl groups in polyols (such as sugars), and the use of inexpensive oxidants, such as air. This task took advantage of a unique capability at PNNL, the Symyx® high throughput catalyst screening instrumentation obtained through a DOE OBP capital investment after this CRADA project was initiated.

A total of 19 plates were constructed and run in this study for a grand total of 1176 individual experiments including controls. Initial efforts focused on the stability of feedstocks and products with respect to pH, temperature, and different oxidants. Increased temperature led to decreased stability, especially for sorbitol, but also for glycerol. This required that temperatures be kept below 75°C for maximum recovery. The sugar acid products were even more sensitive than the substrates to temperature. All feedstocks and products were far more stable under air than in the presence of H₂O₂ as oxidant.

A variety of transition metal and mixed metal catalysts were generated using the Symyx® combinatorial system and tested with sorbitol or glycerol as the model compounds. Carboxylic acids of sugars were the targets of interest and these were among the products observed. Additional studies would be required to optimize the conditions and explore other catalysts for greater selectivity and stability of the substrates and products under oxidative conditions. This task identified promising areas for exploration with respect to sugar or polyol oxidation to sugar acids. However, this brief exploratory task also identified a number of challenges with respect to stability and selectivity.

Task 7: Process Economics and Applications

Mike Cockrem, KiwiChem and Mike Jaffe, NJIT

ICPB contracted with KiwiChem to develop a model process using Aspen to simulate the dry mill ethanol plant. This model was verified using an actual ethanol plant. This base case model was to be used as results were generated from the research lab to evaluate technical options. If additional funding is awarded to this project, the basic model would be used.

ICPB also contracted with Mike Jaffe at New Jersey Institute of Technology to investigate specific markets for targeted organic acids that were potential products in this project. Several applications were investigated and identified as possible avenues to develop new markets. Should the production of these organic acids continue, these markets will need to be further developed.

Summary of Project Activities

Our original plan was to hydrolyze the hemicellulose portion of the DG to produce the sugars (xylose and arabinose). These sugars were then going to be used as

feedstocks for industrial chemicals. These industrial chemicals would be utilized for specific market applications.

Dyadic supplied experimental enzymes that were tested for the hydrolysis of the sugars. Pacific Northwest National Lab did research on several elements of the project including the development and testing of enzyme hydrolysis, catalysis of sugars into value added chemicals, and fermentation of sugars into organic acid.

Products Developed

Under this program a novel enzymes were tested for commercial application. One provisional patent was submitted to the USPTO regarding these enzymes.

No presentations were made from the results of this project.