

Fermentation Biotechnology Research Unit, National Center for Agricultural Utilization Research, Peoria, IL

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Project Number 3620-41000-118-00D. INDUSTRIALLY ROBUST ENZYMES AND MICROORGANISMS FOR PRODUCTION OF SUGARS AND ETHANOL FROM AGRICULTURAL BIOMASS

The overall goal of this research is to develop improved processes for converting herbaceous biomass to ethanol by incorporating new enzyme and biocatalyst technologies with modern pretreatment strategies. An additional goal is to evaluate the potential of biomass as a feedstock for hydrogen production via fermentation.

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Project Number 3620-41000-122-00D. COST-EFFECTIVE BIOPROCESS TECHNOLOGIES FOR PRODUCTION OF BIOFUELS FROM LIGNOCELLULOSIC BIOMASS

This research strives to develop cost-effective pretreatment, enzymatic saccharification, fermentation and downstream processing technologies, and their integration for production of biofuels (ethanol and butanol) from lignocellulosic biomass. The overall goal is to produce biofuel from waste lignocellulosic agricultural residues and processing byproducts (e.g. barley and wheat straw, rice hulls, corn fiber) at a price competitive with corn starch-based fermentation process.

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Key Accomplishments:

Developing Better Enzymes:

Discovery of the most active β -xylosidase for releasing sugars from biomass. Sources of β -xylosidase are needed for converting xylan to xylose, the second most common sugar present in biomass after glucose, for subsequent bioconversion to ethanol. We determined that the β -xylosidase from *Selenomonas ruminantium* is the most catalytically efficient enzyme known (at least 15-fold better than those reported in the literature) for catalyzing the hydrolysis of xylooligosaccharides to xylose and has good properties of temperature and pH stability. Additionally, the enzyme can be efficiently produced in *Escherichia coli* (>4 g enzyme/liter). These properties place the enzyme at the forefront for development as a saccharification catalyst. A patent on the use of this enzyme has been applied for.

Discovered and patented two novel microbial enzymes (highly glucose tolerant β -glucosidase from *Candida peltata* and thermostable α -L-arabinofuranosidase from *Aureobasidium pullulans*) that greatly aided in the saccharification of the cellulose and hemicellulose components of biomass into fermentable sugars. Isolated a fungal (*Fusarium verticilloides*) strain capable of producing hemicellulase enzymes that are highly effective in hydrolyzing hemicellulose (complex heteroarabinoxylan) from corn fiber.

Isolated plant cell wall hydrolyzing enzymes – including cellulases, hemicellulases, and esterases, with very high specific activities from a novel source, the anaerobic fungus *Orpinomyces* PC-2. Recombinant DNA technology was then applied to construct *E. coli* hosts capable of producing these enzymes in large quantities. Many of these enzymes have been patented and are in the process of being investigated for applications by other researchers within and outside of ARS.

Expressed a highly active xylanase enzyme in a fungus (*Trichoderma reesei*) used for producing industrial hydrolases. The xylanase gene originated from an anaerobic fungus and, so, was then engineered to overcome problems with codon-bias in the new host so that high yields of the heterologous enzyme could be produced. The enzyme cocktail produced by the modified *T. reesei* should be more efficient at degrading cellulosic biomass into fermentable sugars. This is the first time an anaerobic fungal enzyme has been successfully expressed in *T. reesei*.

Developed custom hydrolytic enzyme preparations – prepared by growing fungi on liquid hot water treated corn fiber – that convert oligomers of corn fiber into monosaccharides with arabinose, glucose, and xylose yields of 80%, 100%, and 80%, respectively.

Biocatalyst Development:

Engineered and patented a microorganism (*E. coli*) that has enhanced stability and produces ethanol from mixed sugar substrates efficiently with excellent yield. The novel metabolic engineering method used for constructing these strains has been patented and the strains are being evaluated by numerous laboratories here and abroad in Europe and Australia.

Developed a method for removing inhibitory chemicals from biomass-derived sugars using a bioabatement type strategy. Bioabatement uses microorganisms to remove undesirable chemicals, and is widely used for waste water and ground pollution treatments. The fungus *Coniochaeta ligniaria* NRRL30616 was isolated from an industrial-site soil sample during a screen for microorganisms suitable for bioabatement. The microorganism is able to consume numerous inhibitory compounds commonly present in biomass hydrolysates. Furthermore, treating hydrolysates with this fungus prior to fermentation improves ethanol yield and productivity. A patent has been granted covering *C. ligniaria* and its application of bioabatement for removing inhibitors from biomass hydrolysates.

Fermentation Process Development:

Demonstrated that sugar mixtures generated from a pilot plant demonstration project on the fermentation of corn fiber were fermented to ethanol. The project used an ARS-developed, recombinant *E. coli* strain (*E. coli* FBR16) which was engineered to co-ferment arabinose, glucose, and xylose sugars. Ethanol yields were 84-97% of the maximum possible based upon beginning sugar concentrations. The demonstration project is the outcome of a highly successful collaboration with an academic institution (Purdue University), U.S. D.O.E. laboratory (NREL), a local ethanol producer (Aventine), and ARS (NCAUR)

Demonstrated that butanol, which can serve not only as fuel but also as a chemical, can be produced from dilute acid pretreated enzymatically saccharified wheat straw hydrolyzate without using any detoxification step (lime treatment) typically required for dilute acid pretreated substrate. The fermentative bacterium efficiently utilized multiple sugars present in wheat straw hydrolyzate. Butanol was recovered simultaneously by gas stripping during fermentation. This solves the problem of product inhibition and will help to reduce the production cost of butanol significantly as a result of integration of fermentation with recovery.

New Feedstocks

Demonstrated that Wheat straw pretreated with alkaline peroxide will undergo complete enzymatic saccharification to fermentable sugars. No common fermentation inhibitors were produced. Both simultaneous hydrolysis and fermentation and simultaneous saccharification and fermentation approaches worked equally well for producing ethanol from peroxide-pretreated wheat straw by use of an ARS-developed ethanologenic recombinant bacterium capable of converting multiple sugars (glucose, xylose, arabinose). Similar results were demonstrated for Barley straw.

Developed processes for dry fractionation of field pea into enriched protein and starch streams and for fermenting the pea starch to ethanol. Ethanol yields from pea starch were comparable to that from corn starch, and the enriched protein was similar in protein content to high-protein soy meal, with a well

balanced amino acid profile. This is the first work to demonstrate that pea starch can be fermented to ethanol and, importantly, that the process can be accomplished using commercial enzymes and yeast.

Other Scientific Expertise or Capabilities:

The full capabilities of NCAUR including: NMR facility for solids and liquids analysis, a DNA facility for synthesis and sequencing, a high-pressure supercritical fluid processing lab, an extrusion and injection molding lab, scanning and transmission electron microscopes, environmentally-controlled materials testing lab, and a greenhouse. NCAUR houses the ARS Microbial Collection that contains over 85,000 strains of microorganisms. The Center also has a Pilot Plant, a modular facility located in the recently renovated north wing for chemical, biological, and food processing. This plant encompasses over 65,000 sq. ft. with four flexible, two-story labs for scale-up research and concept demonstration.

In addition to the resources listed above, equipment items available for the research program are specifically located within the Fermentation Biotechnology Research Unit which are well equipped to conduct microbiological, biochemical, genetic, chemical, and engineering research. These include GCs, HPLCs, GC/MS, LC/MS, spectrophotometers, and other analytical equipment. Protein isolation and purification, gel electrophoresis, thermocyclers, quantitative PCR, and other molecular biology equipment. Fermentation equipment available ranges from shake flask to bench scale (15, 1 to 10L bioreactors) to 30L (3) and 100L (2). Equipment available for pretreatment experiments include: autoclaves and a fluidized heating bath for dilute-acid pretreatments experiments, and water bathes for ambient pressure alkaline pretreatments. Higher pressure liquid experiments can be carried out using the sand bath or an available high pressure stirred reactor (Parr, Moline, IL). Custom manufactured pipe reactors capable of being used up to 200°C are available for use with the fluidized heating bath. Some of these pipe reactors have already been modified for steam explosion and it is envisioned that these could be conveniently modified for the higher-pressure ammonia experiments. Also available is a Lab scale pretreatment reactor ("LABOMAT" Type BFA-12 Version 200°C, MATHIS) with digital controlled infrared heating system that has a temperature range of 20 - 200°C. Precise temperature control and programmable operations with a variable speed rotary disk that allows up to 12 stainless steel 200ml beakers to mix simultaneously.