GENOMICS AND ENGINEERING OF STRESS-TOLERANT MICROBES FOR LOWER COST PRODUCTION OF BIOFUELS AND BIOPRODUCTS *(USDA Agricultural Research Service Project 3620-41000-123-00D)*

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Objectives: The objective of the project is to 1) determine the metabolic, physiologic, and genetic fundamentals underlying stress tolerance of ethanologenic yeast strains and other microbes, and 2) to use this fundamental knowledge to engineer improved strains and/or process conditions that foster stress tolerance and functionality of microbes for production of ethanol and bioproducts from corn fiber and other lignocellulosic materials, despite exposure to harsh environments.

Accomplishments: Furfural and HMF are key toxic byproducts of the dilute acid hydrolysis of lignocellulosic biomass, the most economical method of releasing sugars for fermentation to ethanol biofuel. Our research has addressed the lack of yeasts able to tolerate these toxic byproducts and ethanol as significant factors limiting commercial-scale biomass to ethanol conversion. We have expanded our knowledge base and strategies to accomplish a lower-cost lignocelulose to ethanol process.

- Certain natural strains were better able to tolerate the presence of furfural and HMF than others and that natural strains of the yeasts *Saccharomyces cerevisiae* and *Pichia stipitis* can survive and adapt to the presence of furfural and HMF allowing gradually increasing levels of each inhibitor to lead to the development of strains able to tolerate relatively high levels of HMF and furfural (30 mM).
- Adapted strains are more efficient than their parent strains in the reduction of the aldehyde functional group of the inhibitors to the corresponding less toxic alcohol, ie. furfural to furfuryl alcohol and HMF to 2,5-bis-hydroxymethylfuran, suggesting the role of *in situ* detoxification in inhibitor-tolerant strains. Our work provided the first chemical identification of the HMF reduction product.
- In microarray studies comparing HMF-treated wild type and adapted strain cultures, tolerant strains have distinct expression profiles of selected genes compared with that of the parent strain. Genes in all categories of biological process, cellular component, and molecular function were involved; some were HMF-specific while others could be associated with a core set of stress genes, such as those belonging to the pleiotropic drug resistance gene family. Our development of a unique quality-controlled gene microarray for ethanologenic yeast study has allowed these phenotype differences to be exploited to track down not just key genes, but also gene networks and regulatory systems involved in stress tolerance mechanisms.
- Screening a *S. cerevisiae* disruption library identified 40 genes involved in tolerance of 8-10% ethanol and 65 genes involved in tolerance of 15 m*M* furfural. The identified gene mutants implicated several pathways in ethanol tolerance, including macromolecule modification and biosynthesis, organelle dynamics, plasma membrane and cell wall maintenance, fermentation, cell cycle, endocytosis, metabolite biosynthesis, and membrane transport mechanisms. Notably, our work provides the first evidence that the pentose phosphate pathway (long known for converting xylose to ethanol) plays a critical role in protecting yeast against furfural stress perhaps via generation of nicotinamide adenine dinucleotide phosphate (NADPH), a cofactor that is necessary for protein, nucleic acid, and lipid biosynthesis, and for protection from oxidative stress.
- Regarding yeast cellular physiology, furfural induced the accumulation of free radicals, which are known to cause severe DNA, protein, and membrane damage. Consequently, mitochondria and vacuole membrane damage were observed as well as nuclear chromatin

damage. This new knowledge is key to the engineering of yeast and fermentation processes to prevent and repair damage to cells, thereby allowing more efficient and cost-effective conversion of biomass substrates to ethanol.

- Optimization of culture nutritional factors found critical to inhibitor stress tolerance mechanisms and maintenance of yeast cell viability during fermentation. The striking influence of nitrogen source and mineral composition on the achievement of ethanol yields up to 70 g/L from media supplied high xylose concentrations (150 g/L) was noted for the natural pentose-fermenting yeast *Pichia stiptis*. Nitrogen source composition was further investigated and found to influence the ability of *P. stipitis* to survive in the presence of concentrations of furfural (100-150 mM/L) and ethanol (70-100 g/L) that were high enough to halt cell growth when spiked to cultures in logarithmic growth and early stationary phases. The inclusion of amino acids in addition to urea in the culture medium significantly improved ethanol yields and resistance of cells to furfural and ethanol. This finding will be utilized in the development of efficient fermentation processes that foster inhibitor tolerance and allow rapid ethanol production from both the hexose and pentose sugars released by dilute acid hydrolysis of low-cost lignocellulosic biomass.

Future Work: Key genes identified using the approaches above will be verified and characterized with respect to protective gene function in laboratory strains using techniques such as over-expression, disruption, specific mutation, microarray genome analysis coupled with computational modeling, assessments of fermentation performance and physiological damage to cells. Ultimately, a tolerance specific gene microarray will be designed and used in combination with strain engineering and fermentation optimization schemes (involving culture nutrition and environmental controls) to probe and direct the optimization to foster the expression of known stress tolerance genetic mechanisms to enhance the profitability of biomass to ethanol conversion.

Research Capacity at NCAUR: This project is carried out within the Crop BioProtection (CBP) Research Unit, National Center for Agricultural Utilization Research, Peoria, IL which is well positioned for expanded contributions to biofuels research areas of sustainable energy crop development, management, and utilization. The mission of the CBP Research Unit is to develop natural biological activities to reduce exposure of the environment and food supply to potentially harmful pollution due to overuse of chemicals for pest management. Fundamental activities of microbes, insects, and plants are identified and used by researchers of varied scientific disciplines (microbiology, chemistry, molecular biology, microbial physiology, plant pathology, entomology, biochemical engineering) to design new biological methods, processes and products for agriculture that foster crop protection and environmental health. Collaborating research units at NCAUR, include: Fermentation Biotechnology, Bioproducts and Biocatalysts, Microbial Genomics and Bioprocessing, New Crops and Processing Technology.

Collaborators Outside ARS: Dr. Mingzhou (Joe) Song, New Mexico State University and Dr. Steven Dolins, Bradley University (NRI Grant); Drs. Nate Mosier, Mike Ladisch, and Nancy Ho, Laboratory of Renewable Resources Engineering, Purdue University; Dr. Tom Jeffries, USDA Forest Products Lab, Madison, WI; Dr. Barbel Hahn-Hagerdal, Lund University, Sweden.