"Trojan Horse" Strategy for Deconstruction of Biomass for Biofuels Production

LDRD
LABORATORY DIRECTED RESEARCH & DEVELOPMENT

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Problem

Production of renewable biofuels to displace fossil fuels currently consumed in the transportation sector is a pressing multi-agency national priority (DOE/USDA/EERE). Currently, nearly all fuel ethanol is produced from corn-derived starch. Dedicated "energy crops" and agricultural waste are preferred long-term solutions for renewable, cheap, and globally available biofuels as they avoid some of the market pressures and secondary greenhouse gas emission challenges currently facing corn ethanol. These sources of lignocellulosic biomass are converted to fermentable sugars using a variety of chemical and thermochemical pretreatments, which disrupt cellulose and lignin cross-links, allowing exogenously added recombinant microbial enzymes to more efficiently hydrolyze the cellulose for "deconstruction" into glucose. This process is plagued with inefficiencies, primarily due to the recalcitrance of cellulosic biomass, mass transfer issues during deconstruction, and low activity of recombinant deconstruction enzymes. Costs are also high due to the requirement for enzymes and reagents, and energy-intensive and cumbersome pretreatment steps.

R&D Approach

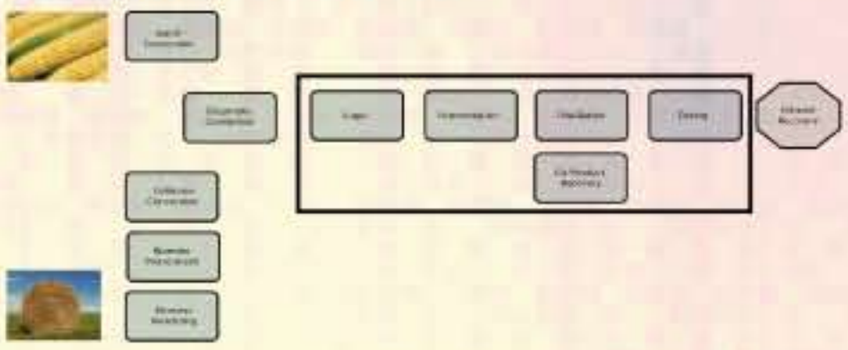
One potential solution to these problems is found in synthetic biology; we propose to engineer plants that self-produce a suite of cellulase enzymes targeted to the apoplast for cleaving the linkages between lignin and cellulosic fibers; the genes encoding the degradation enzymes, also known as cellulases, are obtained from extremophilic organisms that grow at high temperatures (60–100 °C) and acidic pH levels (<5). These enzymes will remain inactive during the life cycle of the plant but become active during hydrothermal pretreatment i.e., elevated temperatures. Deconstruction can be integrated into a one-step process, thereby increasing efficiency (cellulose-cellulase mass-transfer rates) and reducing costs. Our proposed disruptive technologies address biomass deconstruction processes by developing transgenic plants encoding a suite of enzymes used in cellulosic deconstruction. The unique aspects of this technology are the rationally engineered, highly productive extremophilic enzymes, targeted to specific cellular locations (apoplast) and their dormancy during normal plant proliferation, which become Trojan horses during pretreatment conditions. We have been leveraging established Sandia's enzyme-engineering and imaging capabilities. Our technical approach not only targets the recalcitrance and mass-transfer problem during biomass degradation but also eliminates the costs associated with industrial-scale production of microbial enzymes added during processing.

The application of enzymes and microorganisms for the sustainable production of chemicals, biopolymers, materials and fuels from renewable resources as part of efforts to move towards "green chemistry" offers great opportunities for new and novel enzymes. While the majority of the industrial enzymes known to date have been derived from bacteria and fungi, archaea, which represent the third domain of life, are increasingly finding applications in biotechnology. Most of the archaeal species identified to date have been from extreme environments (conditions that mimic those of industrial processes). These environments such as geothermal and deep-sea volcanic sites (80-121 °C), polar regions (–20 °C), acidic solfatara fields (pH < 4), alkaline springs (pH > 8) and hypersaline lakes (2-5 M NaCl) have been shown to be teeming with life forms that have unique biotechnological importance.

As a starting point we are working with two enzymes. The endoglucanase SSO 1949 and CelA encoded by S. *solfataricus* and A. *acidocaldarius* respectively. Prior to synthesizing the genes we performed extensive bioinformatics analysis on the open reading frames (ORF), removing cryptic splice sites and optimized the codon preferences for robust expression in bio-energy crops.

These enzymes therefore provide the target actuators for Trojan Horse deconstruction strategy. The substrate range, operational pH and temperatures of these archaeal enzymes make them promising candidates to optimize bioconversion of recalcitrant cellulose to fermentable sugars by running the process of saccharification at lower pH and higher temperatures as well as serve as a model systems for analysis of the process.

State of Ethanol Production

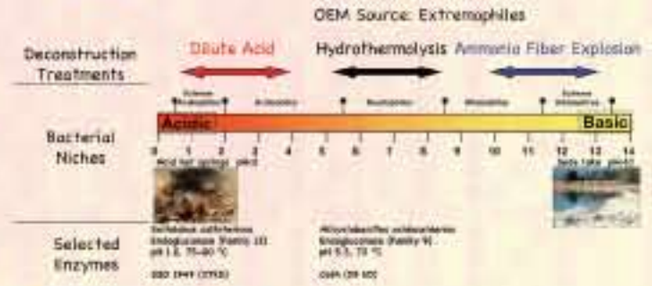


Currently, almost all of domestic ethanol production is from corn-derived starch, which in itself is a high value energy food and feed commodity. An alternative is lignocellulosic biomass (e.g., grasses, wood, agriculture waste etc.), which is renewable, cheap and readily available to the tune of 50-billion tons per year.

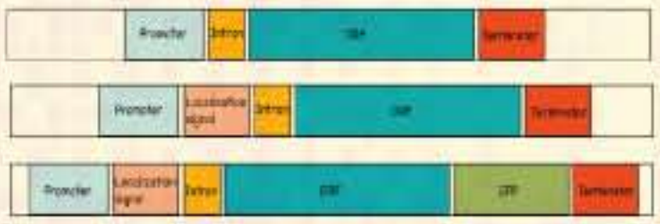
Biomass is converted to fermentable sugars for biofuels production using pretreatments, which disrupts cellulose cross-links (e.g., lignocellulose), thereby allowing exogenously added recombinant microbial enzymes to access cellulose for "deconstruction" into fermentable sugars. The low activity and high costs of recombinant deconstruction enzymes and cumbersome pretreatment steps are the major economic shortcomings of biomass refineries.

Engineered Biomass Advantages

- Consolidated bioprocessing
- Steps and cost reduction
- Elimination of enzyme costs



Actuator Construction



The actuator is driven by a strong constitutive promoter and contains an intron. Cellulase genes were cloned for free expression in the cytoplasm as well as targeted to the apoplast using a signal sequence from rice. We also constructed them as a fusion with GFP. We are using Agrobacterium to introduce the actuator into the model and bioenergy plants and use a selectable marker to enable selection of plants that have been successfully transformed. The actuator without the cellulases have been tested and GFP signal localizes as expected.

Testbeds for Actuator Testing

Our initial testbed for actuator evaluation is Arabidopsis which is a small flowering plant from the cabbage and mustard family and one of the model organisms for studying plant biology. It was the first plant genome sequenced, has a life cycle of 6 weeks allowing multiple generations to be evaluated over a short time. Not only are phenotypic changes in the plant easily observed but there are well-established molecular and growth techniques for handling the plant under laboratory conditions, thus making it a very useful model system; and information gleaned from these studies are readily applicable to Poplar — a bio-energy feedstock.

Arabidopsis thaliana

Our second testbed in Brachypodium distachyon(L) which has many qualities that make it a model for functional genomics studies in temperate grasses, cereals, and dedicated biofuel crops such as Switchgrass. These attributes include small genome diploid accessions, a short life cycle, and simple growth requirements.

Brachypodium distachyon(L)

We are also testing the actuator transiently in Nicotiana using agro-infiltration.

We were able to localize and detect GFP in the apoplast using the GRP signal sequence

Free GFP was detected in the cytoplasm

Preliminary Results

We identified and synthesized two enzymes after performing extensive bioinformatics analysis on the open reading frames (ORF). We removed cryptic splice sites and optimized the codon preferences for robust expression in bio-energy crops.

The ORFs have been cloned for free expression in the cytoplasm as well as targeted expression to the apoplast in Arabidopsis as well as Brachypodium. The genes have also been cloned as fusions with GFP which enables localization of the enzymes.

The GFP-expressing actuators were transformed into Arabidopsis using Agrobacterium and the resulting plants were evaluated for expression of GFP.

The promoter and targeting signal localizes the protein to its expected location.

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