BIOCHEMICAL CONVERSION

TECHNOLOGY AREA

CONTENTS

INTRODUCTION

The Biochemical Conversion Technology Area is one of 14 related technology areas that were reviewed during the 2019 Bioenergy Technologies Office (BETO) Project Peer Review, which took place March 4–7, 2019, at the Hilton Denver City Center in Denver, Colorado. A total of 30 projects were reviewed in the Biochemical Conversion session by five external experts from industry, academia, and other government agencies.

This review addressed a total U.S. Department of Energy (DOE) investment value of approximately $82,692,210, which represents approximately 9.6% of the BETO portfolio reviewed during the 2019 Project Peer Review. During the Project Peer Review, the principal investigator (PI) for each project was given 25–30 minutes to deliver a presentation and respond to questions from the review panel.

Projects were evaluated and scored for their project approach, technical progress and accomplishments, relevance to BETO goals, and future plans. This section of the report contains the results of the project review, including full scoring information for each project, summary comments from each reviewer, and any public response provided by the PI. Overview information on the Biochemical Conversion Technology Area, full scoring results and analysis, the Review Panel Summary Report, and the Technology Area Programmatic Response are also included in this section.

BETO designated Dr. Ian Rowe as the Biochemical Conversion Technology Area Review Lead, with contractor support from Mr. Clayton Rohman (Allegheny Science & Technology). In this capacity, Dr. Rowe was responsible for all aspects of review planning and implementation.

BIOCHEMICAL CONVERSION R&D OVERVIEW

The Biochemical Conversion Technology Area focuses on research and development (R&D) of biological processes that convert biomass to biofuels and bioproducts. Biochemical processes within the BETO portfolio can either stand alone as an entirely biological operation from feedstock to final product, or they can act as single steps within larger pathways that employ other nonbiological conversion technologies. Projects presented in the Biochemical Conversion session cover a wide variety of scientific disciplines, generally targeting one or more of four purposes: (1) to deconstruct lignocellulose into biochemical intermediates, such as cellulosic sugars (both five-carbon [C5] and six-carbon [C6]) and lignin; (2) to biologically upgrade such intermediates into fuels and bioproducts; (3) to biologically upgrade thermochemical intermediates (gaseous or aqueous); and (4) engineering bioprocesses to valorize waste streams.

Since Fiscal Year (FY) 2016, BETO's state-of-technology (SOT) model for tracking technological progress has assumed that all C5 and C6 sugars would be converted to fuels, while lignin would be valorized to bioproducts. This is the framework in which most of the core "pathway-specific" research done within the biochemical conversion R&D portfolio fits. The assumptions associated with the SOT are critical for certain national laboratory annual operating plan (AOP) projects that seek to verify BETO SOT pathways. Other projects within the biochemical conversion portfolio aim to enable the bioeconomy more broadly; most of the competitive funding opportunity announcement (FOA) projects fall within this "enabling" category.

Cost-effective and energy-efficient biochemical deconstruction is the first half of most of the efforts represented in this chapter. The low-temperature deconstruction of biomass by dilute acid or mild deacetylation treatment followed by enzymatic hydrolysis is the common process for generating intermediates within the biochemical conversion R&D portfolio. Other intermediates, such as those produced from the processing of algal biomass or from the biological deconstruction of plastics, represent additional enabling approaches to generating intermediates that can then be upgraded. The second half of the conversion processes discussed here involve a biological upgrading approach. Projects which upgrade cellulosic sugars are the focus of the portfolio; however, processes that use other intermediates such as syngas, lignin, and plastic-derived monomers are also being investigated.

A key to developing cost-competitive cellulosic biofuels is reducing the processing and capital costs and improving the efficiency of separating and converting cellulosic biomass into fermentable sugars. Current R&D focuses on high-yield feedstocks, more efficient enzymes, and more robust microorganisms to advance biochemical conversion processes. The resulting advanced biochemical conversion technologies will increase fuel yields in future biorefineries and enable a bioeconomy that leverages an abundant, renewable carbon feedstock.

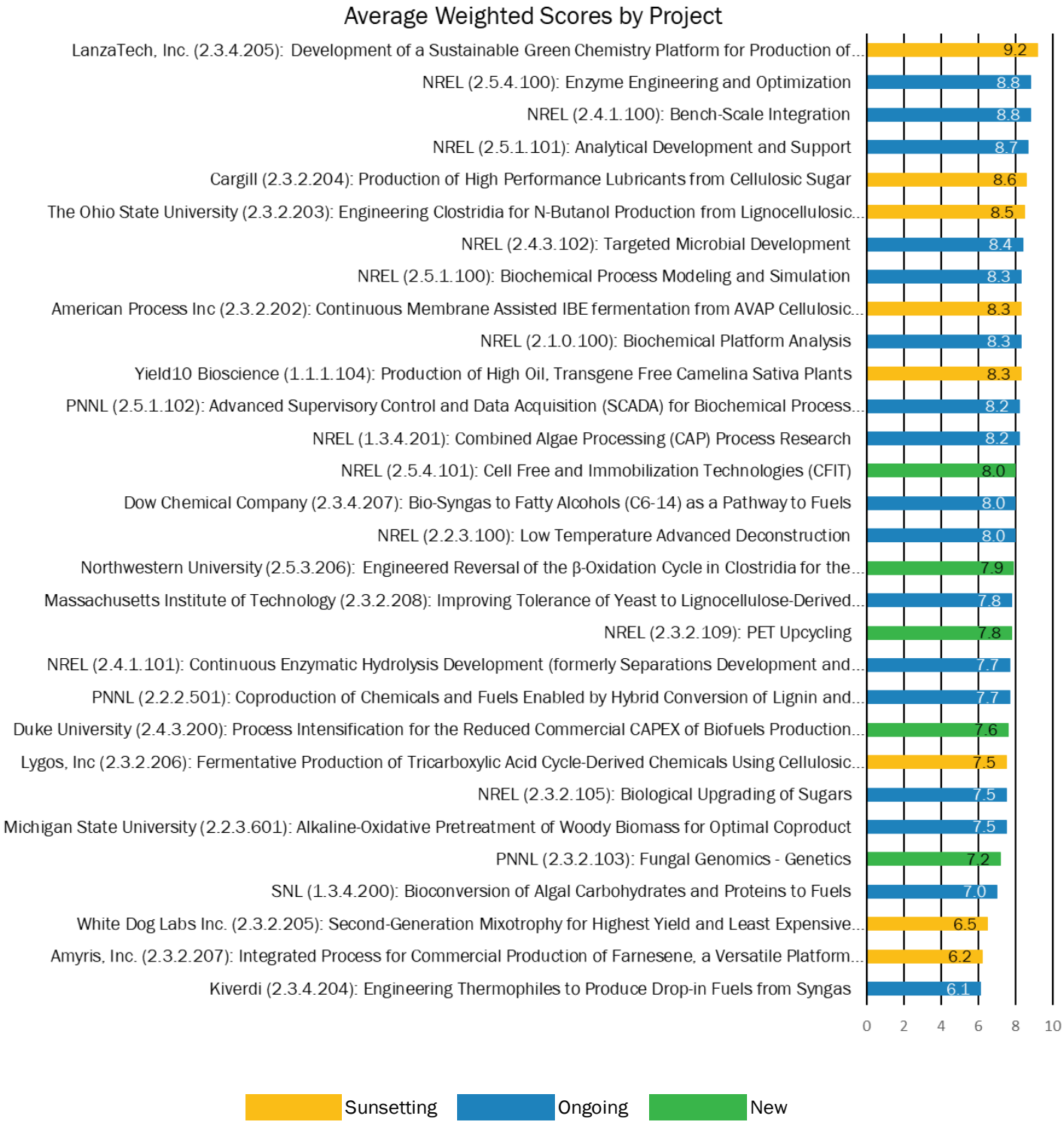
BIOCHEMICAL CONVERSION REVIEW PANEL

The following external experts served as reviewers for the Biochemical Conversion Technology Area during the 2019 Project Peer Review:

Name	Affiliation
Charles Abbas*	iBiocat
Farzaneh Rezaei	Pivot Bio
Chris Rao	University of Illinois at Urbana–Champaign
Ben Gordon	Broad Foundry
Steve Van Dien	Persephone Biome, Inc.

*Lead Reviewer

TECHNOLOGY AREA SCORE RESULTS



BIOCHEMICAL CONVERSION REVIEW PANEL SUMMARY REPORT

Prepared by the Biochemical Conversion Review Panel

The Biochemical Conversion Review Panel was tasked to provide feedback using criteria selected by BETO to assess the biochemical conversion projects based on project outlines, abstracts, and updates provided in oral and written presentations by project PIs and teams. Based on the summary provided from the different reviewers, most reviewers shared similar assessments of the projects reviewed, with five projects that the lead reviewer feels were underrated or overrated based on deliverables. Some of these discrepancies can be explained on the basis of technical success versus commercial viability or the projects being outside of a reviewer's areas of expertise. That is expected given the makeup of the review committee and the criteria used to analyze the projects.

The projects reviewed represented work done at DOE national labs, private well-established companies, small startup companies, and/or university-led research. The projects had different start and completion dates with a range of budgets, levels of difficulty, and challenges. With few exceptions, most of the PIs and project teams responded well by providing responses to the criteria in BETO guidelines, with some providing extensive details that assisted the reviewers in responding to criteria selected. The projects that did not follow the required format for reporting progress achieved an overall lower average score in spite of the fact that they had technical depth and showed good progress on achieving the targets outlined. The review process went smoothly, and presenters did a great job highlighting the progress of their work and technical merits and deliverables while allowing sufficient time for reviewers' questions and answers. The reviewers were impressed with the quality of the presentations that further provided clarity on the soundness of technical plans and deliverables.

IMPACT

The current funded projects in the Biochemical Conversion Technology Area represent several parallel approaches that deploy a range of bio-based omics technologies that have been developed over the past two decades to help address the economic challenges for the processing of biomass lignocellulosic feedstocks. The investment made by BETO is necessary in light of the knowledge gaps that increase the uncertainty of economic viability for the use of these feedstocks. These gaps are not being addressed by industry as they require mid- to long-term funding, and as a result compete with short-term industrial projects that increasingly focus on making existing biorefineries more efficient for conventional feedstocks.

The BETO-funded projects attempt to address challenges to industrial development presented by biochemical conversion in achieving cost-effective bioprocessing of biomass to biofuels, chemicals, and polymers at a biorefinery. This requires the development of cost-effective, rapid methods for compositional analysis of the starting incoming materials and the processed separated streams, as well as hydrolysis components which include sugars, phenolic compounds, and cations. It also requires cost-effective commercial enzymes that can be used to effectively hydrolyze pretreated or untreated biomass to release and separate the carbohydrates from the lignin and other organic and nonorganic constituents. Additionally, a biorefinery requires robust microbes that can withstand industrial processing conditions, can operate at pH and temperatures under anaerobic or microaerophilic conditions that are far from optimal conditions, and that can survive common process upsets. The selection of robust microbes that can be developed for biofuel and bio-based chemicals must take into account the resistance to inhibitors that are generated during pretreatment steps such as degraded sugars (furfural, hydroxymethyl furfural, levulinic acid, formic acid); lignin degradation products (phenolic compounds); and acetic acid and other cations (sodium, calcium, iron, magnesium, etc.) that are toxic, required for growth, or can result in imbalanced growth. For optimized conversion, improved online process tools need to be available, as well as biochemical process modeling and simulation capabilities. The current projects within the Biochemical Conversion portfolio address these issues directly and indirectly.

The analytical development and support platform at the National Renewable Energy Laboratory (NREL) is at the forefront of developing analytical methods for compositional analysis of biomass feedstocks. While these methods are well developed for research purposes, they are still costly and require a dedicated research lab staff with technical expertise—something that is not readily available to industry. The emerging lignocellulosic biomass industry also faces challenges in the cost and availability of effective cellulase enzymes. The enzyme engineering and optimization platform at NREL seeks to optimize a cellulase-enzyme blend that is more effective at hydrolysis of sodium hydroxide-treated corn stover deconstructed through low-temperature deacetylation and mechanical refining (DMR). This effort is necessary and worthwhile. BETO should continue to support and expand cellulase enzyme development to provide more cost-effective enzymes.

The development of new microbes for production of cellulosic ethanol, *n*-butanol, other alcohols, and lipids from sugars, as well as the use of syngas as a feedstock, is an active area of industrial research. Some of these projects are carried out in partnership with DOE national labs and university-based research. Among the projects funded within the biochemical conversion theme that look promising are:

- The engineering of acetogenic *Clostridia* to improve syngas utilization for the production of acetone and other downstream fuel and commodity products directly from biomass syngas using a novel energy-conserving route (LanzaTech, Oak Ridge National Laboratory [ORNL])
- The Ohio State University project for engineering *Clostridia* for *n*-butanol production from lignocellulosic biomass and recaptured CO_2
- The production of high-performance lubricants from lignocellulosic sugars (Cargill)
- Biosyngas to fatty alcohols (C6–C14) as a pathway to fuels (Dow Chemical Company/LanzaTech/Northwestern University)
- The Continuous Membrane-Assisted isopropanol, butanol, and ethanol (IBE) fermentation from American Value Added Pulping (AVAP™) cellulosic sugars (American Process Inc.). The production of high oil, transgene-free camelina hybrids (Yield10 Bioscience).

All of these five projects should be further evaluated for technology transfer and commercial scale-up opportunities.

INNOVATION

Most of the innovative projects reviewed received a score lower than 8.0. This reflects the higher degree of risk and technical challenges inherent in innovation that need to be addressed in funding these projects. These projects were primarily led by universities or small to midsize startup companies. Some examples of these are:

- Amyris project focused on the production of farnesene from lignocellulosic sugars (Amyris/Renmatix/Total)
- Engineering of thermophilic bacteria for production of drop-in fuels from syngas (Kiverdi/Lawrence Berkeley National Laboratory [LBNL]/NREL)
- The use of a Dynamic Metabolic Control (DMC) approach for production of biofuels and chemicals from lignocellulose with lower capital expenditures (CAPEX) (Duke)
- Engineering the reversal of β-oxidation cycle for the production of fuels and chemicals from syngas (Northwestern University/LanzaTech)
- Use of mixotrophs for highest yield and lowest cost for biochemical production (White Dog Labs)

- Fermentative production of tricarboxylic acid cycle (TCA)-derived chemicals from cellulosic sugars (Lygos)
- Improving tolerance of yeast to lignocellulose-derived sugars (Massachusetts Institute of Technology).

By funding these projects, BETO has shown a higher tolerance to risk to ensure new disruptive technologies are developed. A common weakness in some of these higher-risk projects is the use in some cases of novel microbes, poorly characterized or highly toxic feedstocks, and/or novel processes and staging. The target product selected and cost associated for recovery of finished products and detoxification of feedstocks were some of the challenges encountered, as well as low productivity, low yields, and low titers of products. These projects could have benefited greatly by providing a proof of concept or short feasibility study and a detailed techno-economic analysis (TEA) prior to multiyear funding.

Some examples of innovative projects that incorporated risk-management approaches and therefore were more successful technically are:

- The production of high-performance lubricants from lignocellulosic sugars (Cargill)
- Biosyngas to fatty alcohols (C6–C14) as a pathway to fuels (Dow Chemical Company/LanzaTech/Northwestern University)
- Production of high oil, transgene-free camelina hybrids (Yield10 Bioscience)
- Continuous Membrane-Assisted IBE fermentation from AVAP cellulosic sugars (American Process Inc.)
- Development of a sustainable green chemistry platform for production of acetone (LanzaTech).

A closer look at the more successful projects indicate that these projects were well executed. Two of these projects involved strong partners and industrial know-how. The American Process Inc. project is a good example of a highly integrated project that uses lignocellulosic sugars for the production of IBE and what looks like a promising, cost-effective recovery of end products. These projects adapted their plans to meet research needs using a stage-gate approach with deliverables and milestones, thereby incorporating appropriate risk management into the innovation process to facilitate and accelerate innovation as opposed to hindering or curtailing it.

SYNERGIES

There are several projects that were well developed with sound technical approaches and synergies that focused on a range of topics, including the development of processes for the production of biofuels and chemicals from lignocellulosic-derived sugars and syngas produced from lignocellulose, that use cellular or algal biomass as feedstocks. These synergies can be used to advance the use of these feedstocks by grouping the projects together based on the carbon source used as a fermentation feedstock. They can also be grouped based on the choice of microbial system and processing aids used. For example, some of these projects aim to develop new strains of the conventional yeast *Saccharomyces cerevisiae* for the production of ethanol from lignocellulosic hydrolysate sugars, other nonconventional oleaginous yeasts (*Lipomyces*, *Cryptococcus*, etc.) for oils, the use of bacteria (both aerobic and anaerobic; mesophilic and thermophilic), or selected strains of microalgae. These projects can also be grouped based on the target product(s). The products include—in addition to ethanol, algal oils, fatty acid alcohols, acetone, *n*-butanol, isopropanol, farnesene, mono and C15 terpenes—organic acids such as lactic and butyric, other TCA organic acids, the amino acid aspartic acid, 2,3-butanediol (BDO), the biopolymer polyhydroxyalkanoate (PHA), and monoethylene glycol (MEG).

Although the original goals of these funded projects involved multiple approaches and parallel platforms to use lignocellulosic feedstocks with different microbes for different target products, it may be possible to aid these projects to more effectively achieve their goals. This can be accomplished through a collaborative approach to

assess bottlenecks in each project. BETO can provide support by assisting with TEA, the development and implementation of analytical tools, improving process monitoring, and process design and engineering. These capabilities already exist within DOE labs, in particular at NREL and Pacific Northwest National Laboratory (PNNL), but are not currently deployed to support external projects supported by BETO. Creating a mechanism for this open collaborative exchange would be worth pursuing, and the projects can be helped by providing access to DOE in-house capabilities. Other tools that the external projects may need that are not available at DOE labs can be pursued with external partners as necessary.

An example of how this approach is used are the platform projects focused on the development of processes that use all of the constituents of lignocellulose to improve processing of these feedstocks by greater valorization of lignin and other byproducts such as fermentation-derived microbial biomass. There are four complementary projects that use a fungal genomics platform: the production of a C15 terpene hydrocarbon for use as a fuel and the organic acid maleic acid at PNNL; the Hybrid Conversion of Lignin and Bioconversion Intermediates at Idaho National Laboratory (INL), NREL, and PNNL to produce oleaginous yeast and other cellular biomass; the combined algal processing (CAP) to produce precursors to polyurethane, butyric acid, or hydrocarbons at NREL; and the bioconversion of algal carbohydrates and proteins to PHA and lactic acid at Sandia National Laboratories (SNL). These projects, while unrelated, have great synergies and can benefit from an open collaborative approach across the four DOE labs. The above projects, when taken in the collective, bring together an excellent dedicated team of scientists within the DOE national laboratory system. BETO can use a similar approach to group other external projects to assist these projects in achieving their targets through an open innovation model by facilitating the dialogue and exchange between these different projects.

FOCUS

There are several gaps that need to be addressed with greater support from BETO. The areas that come to mind are:

- **Developing new cellulases and supporting more enzyme discovery work, as the current cellulase cost needs to be reduced and effectiveness needs to be improved to cover a broad range of lignocellulosic feedstocks.** Enzymes form extremophilic organisms that are well suited to industrial applications but have not been used much within industry. Greater use of directed evolution and rational protein design and engineering tools are needed to improve enzyme stability and performance.

- **Developing more robust, cost-effective analytical tools to measure composition of lignocellulose.** The current wet chemistry approaches require a considerable investment of funds and manpower while the new spectroscopic analytical approaches are expensive and require routine instrument calibration by an experienced operator. Expediting this work with companies that are providing the equipment and funding of nanoscale measuring instruments can drive innovation and cost down.

- **Exploiting more of the fungal genomes that have been sequenced by the DOE's Joint Genome Institute (JGI) to use and mine additional microbes and genes for metabolic engineering using the fungal genome portal or MycoCosm.**[1] Since the publication in 2011 in *Mycology Journal* of the article on fueling the future with fungal genomics, there has been little progress on industrial applications using the fungal sequences analyzed.[2] The fungal genomics projects within PNNL need to be expanded to include other DOE labs and university-based and private company research. There are many nonconventional yeasts where genomics tools are now available. The Lygos project attempted to

[1] U.S. Department of Energy Joint Genome Institute. "MycoCosm: The Fungal Genomics Resource." http://jgi.doe.gov/fungi.

[2] Grigoriev, Ivor V., Daniel Cullen, Stephen B. Goodwin, David Hibbett, Thomas W. Jeffries, Christian P. Kubicek, Cheryl Kuske,, et al. 2011. "Fueling the Future with Fungal Genomics." *Mycology Journal* 2:3, 192–209. https://doi.org/10.1080/21501203.2011.584577.

engineer the yeast *Pichia kudriavzevii* for the production of aspartic acid but these efforts were abandoned prematurely in favor of *Corynebacterium glutamicum*.

- **Adopting the use of TEA and in-depth modeling for all projects.** It was not clear from the presentations that all of the projects have received rigorous in-depth analysis. Some of the PIs mentioned that a TEA was carried out but there does not seem to be a consistent approach. An independent TEA should be used to confirm some of the assumptions made by researchers on what represents technical targets for a successful project.

As a point of emphasis, BETO has endeavored to find a careful balance between new high-risk projects at universities and startup companies that are not easily funded by industry and other government granting agencies, while supporting lower-risk projects with the involvement of industrial partners from well-established companies. The preceding summary clearly represents the need to support new, potentially disruptive technologies.

TECHNOLOGY DEVELOPMENT PIPELINE

Most of the projects reviewed were at the early- to mid-stage development. Some of the BETO-funded early-stage development projects represent higher risk when compared to industrial in-house projects that are funded at existing biorefineries. They also lacked an in-depth techno-economic modeling analysis or TEA that is required for industrial projects. The more advanced mid-stage projects had already gone through a proof-of-principle or feasibility study, and therefore had a lower inherent risk. While most of these projects will be technical successes, without a much more in-depth analysis it is difficult to determine commercial viability in the biofuels and bio-based chemical markets. Based on past experience, companies seek funding for higher-risk projects when there is a lack of clarity on new technology and uncertainty about the markets.

RECOMMENDATIONS

1. **Increase investment in the development of rapid analytical tools to lower costs and create more effective cellulases and more robust industrial organisms.**

The absence of cost-effective analytical methods that can be readily deployed and used for routine analysis continues to hinder industrial development of lignocellulosic biofuels. This is illustrated in the current situation with processes outlined by the U.S. Environmental Protection Agency (EPA) for renewable identification number (RIN) credit applications from first-generation ethanol plants that aim to use the corn fiber component in corn kernels.

Beyond the analytical challenges that the emerging lignocellulosic biomass industry faces are the cost and availability of effective cellulase enzymes. The enzyme engineering and optimization platform seeks to optimize a cellulase-enzyme blend that is more effective at hydrolysis of low-temperature deconstructed corn stover. While addressing the current limitations to DMR hydrolysis has great relevance to this feedstock, it is not clear if there is also a direct benefit that will reduce the cost of cellulases for other lignocellulosic feedstocks. Expanding the testing of the new cellulase-enzyme blend to other feedstocks including corn kernel fiber would be a positive development in supporting the existing U.S. corn-based biofuels industry. Beyond the current commercial suppliers of cellulase, there has been limited recent development of cellulases outside of the efforts by some research universities, small enzyme manufacturers, startup companies, and NREL. This effort is worthwhile and needs continued BETO support to spur and expand cellulase enzyme development to provide more cost-effective enzymes.

There is also the need to ensure that the strains of microbes being engineered can meet chemical and physical stresses that are common in large-scale fermentation such as the presence of toxic inhibitors and toxic end products, nutrient deficiency, poor mixing condition, poor heat transfer, and vessel back pressure. Successful fermentations also require good temperature, sufficient aeration, and pH control, which are not readily attainable in high-solid lignocellulosic fermentations. There are well-developed industrial microbes in wide use

and tapping into genes to engineer new pathways should be used when necessary, thereby reducing the risk associated with using novel organisms with poor productivity. Gene-editing technologies that have recently gained acceptance will help pave the way for more efficient, well-targeted genes that improve metabolic flux. The BIOREFINE-2G European Union-funded project that was recently completed is a good example of the use of gene-editing technology to develop more commercially attractive processes for efficient conversion of pentose-rich side streams from biorefineries into dicarboxylic acids for use as precursors for bio-based polymers (https://cordis.europa.eu/project/rcn/110369/reporting/en).

2. **Implement a rigorous, independent TEA process for all projects.**

End-product titers need to be quantified, process yields from all sugars/carbon sources need to be determined, and productivity needs to be calculated. All yields and productivity measurements should be clearly specified and not reported on the basis of unspecified inputs or baseline numbers that are not provided.

3. **Expand the advisory team from the existing industries and work towards greater industrial involvement.**

All project teams need to have chemical engineers, preferably with industrial scale-up experience. This is necessary in view of the fact that technical successes in engineering microbes do not always lead to commercial success. Greater emphasis should be placed on the cost-effective recovery of end products. Bridging the gap between bench scale and industrial scale is needed for deployment of research projects at industrial biorefineries.

BIOCHEMICAL CONVERSION PROGRAMMATIC RESPONSE

INTRODUCTION/OVERVIEW

The Biochemical Conversion Program expresses its immense gratitude to the reviewers for their thoughtful recommendations and the significant time and effort they each put into the panel and the subsequent feedback sessions. The reviewers stated that the investments made by BETO into Biochemical Conversion R&D are important mechanisms for addressing the higher-risk gaps in capabilities and knowledge that carry a significant amount of uncertainty. The funding of such technologies that have a mid- or long-term time horizon often compete for the immediate needs of industry and are thus underexamined. The panel also identified the analytical development tasks within the portfolio as a specific strength which can enable the field of in-line compositional analysis of biochemical processes to advance.

The synergies that exist within the portfolio were also specifically pointed out by the panel. The active connection between the projects that work biomass pretreatment and deconstruction techniques and those that focus on microbial development was seen as a strong collaborative effort. Similarly, praise was given to the portfolio connection between efforts on deacetylation and mechanical refining pretreatment with the enzyme engineering project, which focuses on designing an enzyme blend that specifically works with that pretreatment strategy.

The panel pointed toward more effective cellulase enzymes as a potential area of the portfolio that may require additional resources. The reviewers stated that BETO could address the industrial gap in capabilities and the development of robust microbes that can tolerate chemical and physical stresses present in many biochemical processes.

The following section specifically address the three major recommendations from the review panel.

Recommendation 1: Increase investment in the development of rapid analytical tools and lower-cost/more effective cellulases.

The program agrees with the panel's assessment that in-line, rapid analytic tools are vitally important to advancing the bioeconomy. Such technologies are essential to allowing real-time measurements and process control within intricate biochemical conversion processes. BETO continues to fund our existing projects devoted to analytical development and support and we are specifically motivated to enable industrially relevant in-line analysis tools. This is reflected in recently established efforts on this subject at PNNL. BETO also continues to fund efforts in cellulase development and points toward the long history of successful enzyme development from BETO-funded work as substantial progress already made toward this recommendation.

Recommendation 2: Implement a rigorous, independent TEA process for all projects.

BETO acknowledges the vital importance of TEA in ensuring that the efforts undertaken by this Office are sound and justified. Every competitive project within the biochemical conversion portfolio has rigorous TEA requirements and must pass an initial verification (including a robust TEA discussion) prior to receiving the bulk of the awarded funds. Similarly, our AOP efforts at the national labs often have required TEA components associated with individual projects. Lastly, there are several AOPs within the portfolio that are specifically dedicated to performing such analyses for large portions of the portfolio and comparing them to each other. Given all of these current extensive efforts, BETO believes that our existing efforts in TEA are sufficient; however, BETO will make efforts to better communicate our existing TEA efforts to future reviewers.

Recommendation 3: Improved industrial involvement.

As often as possible, BETO targets stakeholder input in the form of workshops, listening days, requests for information (RFIs), and one-on-one informal discussions with external partners in the bioeconomy. Given the smaller scale of much of the work within this R&D portfolio, requiring dedicated chemical engineers on each project within given funds would not be feasible or suitable. Although there is no dedicated "advisory team" for the biochemical conversion portfolio, members of the conversion team regularly attend relevant industrial and academic events to stay abreast of the current SOT within the industry. We feel that this is an area in which BETO excels.

PRODUCTION OF HIGH-OIL, TRANSGENE-FREE *CAMELINA SATIVA* PLANTS

Yield10 Bioscience

PROJECT DESCRIPTION

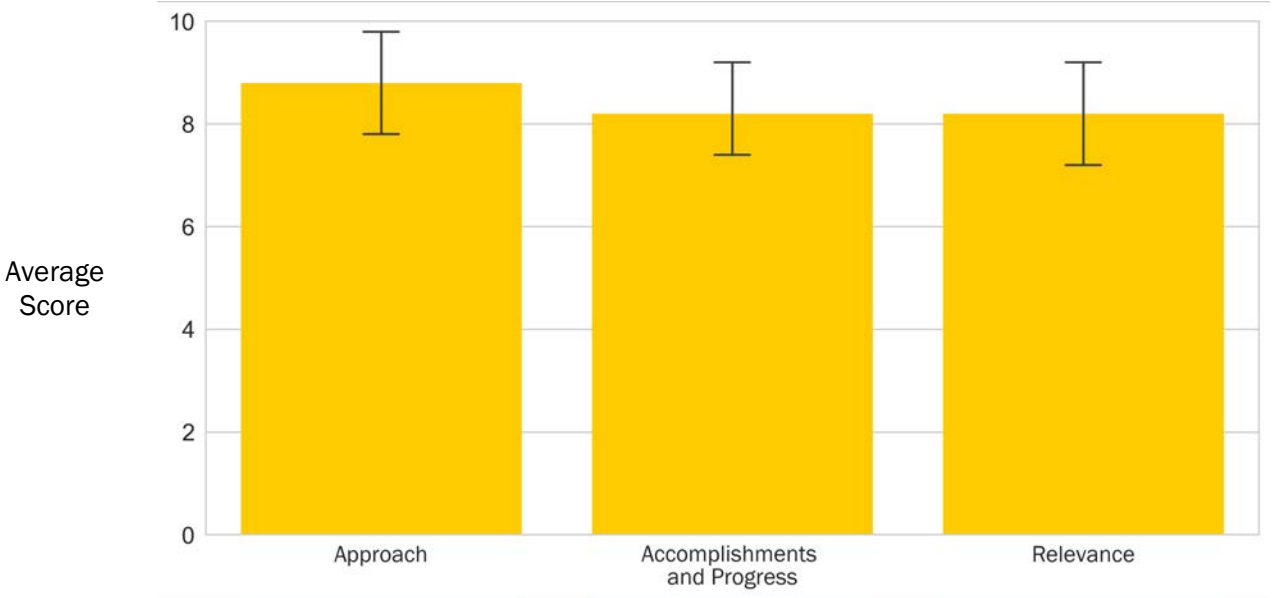
The objective of this project was to develop a *Camelina sativa* feedstock with significantly increased seed yield and oil content to maximize oil yields per acre. *Camelina* is an oilseed crop with high potential as a bioenergy feedstock due to its high oil content, low inputs for cultivation, frost tolerance, and short growth cycle (80–110 days). To accelerate market entry, a next-generation technology was used to develop genetically-modified-organism-free plants to provide an expedited path through regulatory approval. This is anticipated to significantly decrease the time and thus the costs of commercialization of these lines.

WBS:	1.1.1.104
CID:	EE0007003
Principal Investigator:	Dr. Kristi Snell
Period of Performance:	10/1/2015–9/30/2018
Total DOE Funding:	$1,996,598
Project Status:	Sunsetting

For this program, current *Camelina* lines were considered to yield, on average, 1,500 lb of seed per acre and possess a seed oil content of 40%. The program had two oil production benchmarks: (1) increase production of seed to 2,500 lb per acre with seed containing 45% oil, and (2) increase production of seed to 3,500 lb per acre with seed containing 60% oil. Hitting the first benchmark would significantly expand the potential of *Camelina* as a crop and would provide the profitability necessary to incent farmers to grow *Camelina*, resulting in the production of renewable feedstock for the bio-based energy and chemical industries. The second benchmark (3,500 lb of seed per acre with 60% oil content) would have a huge impact on the availability of renewable *Camelina* oil for the biodiesel and aviation fuel markets.

Weighted Project Score: 8.3

Weighting for Sunsetting Projects: Approach - 25%; Accomplishments and Progress - 50%; Relevance - 25%



I One standard deviation of reviewers' scores

The accomplishments of this program demonstrated that genome editing of multiple targets in *Camelina* is technically feasible, despite the complexity of the *Camelina* genome. It also demonstrated that it is possible to increase seed yield while maintaining seed oil content using genome editing. Genome-edited lines achieving up to a 40% increase in seed yield were demonstrated in greenhouse growth studies. In separate experiments, the program also achieved increases of up to 38% in the seed oil content of individual seeds. This increased seed oil content was, however, often accompanied by a decrease in seed yield. These observations demonstrate that carbon flow in *Camelina* can be significantly shifted to seed oil production but that additional research is necessary to concurrently increase or maintain seed yield. Yield10 Bioscience plans to continue to build on these results after the BETO program. During the course of this program, Yield10 Bioscience also demonstrated that lines with single edits, as well as lines with multiple edits, generated using genome-editing technology can be designated as nonregulated by the U.S. Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) through their "Am I Regulated?" process. Two separate "Am I Regulated?" letters were submitted to USDA-APHIS and edited *Camelina* lines described in the letters were deemed nonregulated by the agency, demonstrating a reduced regulatory path.

The technology developed in this program contributes to the overall understanding of seed oil biosynthesis in oilseed crops and how seed yield and seed oil content can be improved using genome-editing techniques. The technology is thus also relevant to increasing seed and oil yield in related oilseed crops, such as canola.

OVERALL IMPRESSIONS

- The goal of this project is to develop a *Camelina* strain with both increased yield and oil content using a genome-editing technique that would have facilitated regulatory approval compared to other modified crops. The team made increases in both yield and oil content but determined that with the current approach there is a tradeoff between these two metrics, so the final goals were not achieved. Plans are in place for field testing of the best strains. The team also obtained confirmation from the USDA that plants modified using *Agrobacterium* transformation of the clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (Cas9) were not regulated, as long as no foreign genes were introduced. This could have huge implications for other modified crop development. It would be good to see more projects on crop development, not only to improve yield but also to make them more amenable to downstream conversion.

- The goal of this project was to engineer *Camelina sativa* with increased seed yield and lipid content using a transgene-free approach. While the project did not meet all of its objectives, the results were very promising and merit future work. The major accomplishment was to increase seed yield by 39% while sustaining the oil content. Increasing oil content was more challenging, as this resulted in a reduction in yield. The next step will be to evaluate these lines in the field to demonstrate commercial viability.

- The Yield10 Bioscience team has applied new gene-editing technologies to define a new state-of-the-art process for *Camelina* engineering to produce non-food seed oils. Seed oil yields are not yet at desired levels, but if the team can achieve them, it could provide a new and important feedstock.

- This project attempts to promote *Camelina* as an alternative crop and to demonstrate the use of gene-editing technology, as well as the identification of target genes. Work is incomplete as it stands, and further research and development is needed. Without that, the investment made in this work may not materialize or generate interest from the current seed development companies.

- Refreshing to see a focus on the crop, which is a different aspect but as important. It is great that the PI included regulatory piece in the project as well.

RECIPIENT RESPONSE TO REVIEWER COMMENTS

- The recipients choose not to respond to the reviewers' overall impressions of their project.

BIOCONVERSION OF ALGAL CARBOHYDRATES AND PROTEINS TO FUELS

Sandia National Laboratories

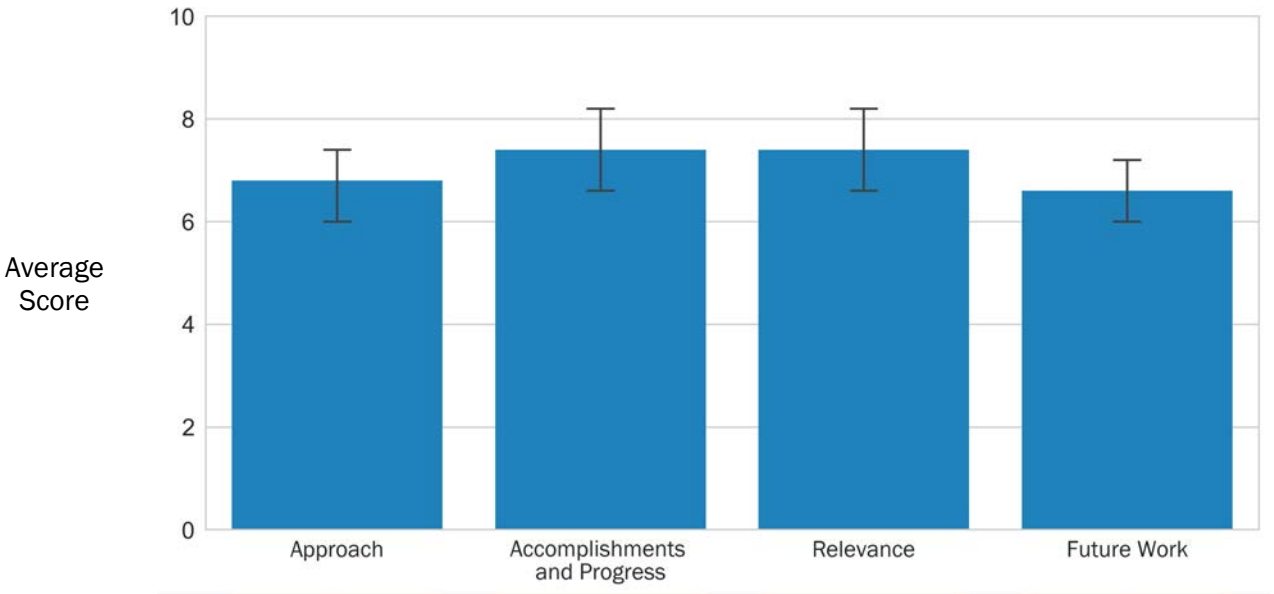
PROJECT DESCRIPTION

WBS:	1.3.4.200
CID:	NL0026336
Principal Investigator:	Dr. Ryan Davis
Period of Performance:	10/1/2015–9/30/2019
Total DOE Funding:	$1,100,000
DOE Funding FY16:	$350,000
DOE Funding FY17:	$350,000
DOE Funding FY18:	$200,000
DOE Funding FY19:	$200,000
Project Status:	Ongoing

A key challenge for scale-up of algal biomass production for renewable commodities is efficient utilization of all of the major biochemical fractions of the biomass, including carbohydrates, proteins, and lipids. Development of a combined algal processing biorefinery would facilitate coproduction of petroleum-displacing chemicals with the intermediate- to high-value products that are currently produced from algae. To overcome issues involved with highly variable feedstock composition, our group is developing means for single-pot bioconversion of amino acid and sugar oligomers from algal hydrolysates to generate a variety of petroleum-displacing end products. Through these efforts we have recently demonstrated pretreatment and bioconversion of a variety of whole-algae hydrolysates, including raceway-cultivated *Nannochloropsis sp.* and filamentous periphyton obtained from algal turf flow ways. To enable high-efficiency utilization of multiple fermentation substrates, we have developed an engineered *E. coli* biocatalyst consortium with each cohort optimized for utilization of either sugar or amino acid oligomers. A quantitative polymerase chain reaction (qPCR)-based method was developed for tracking the relative abundance of the biocatalyst cohorts during fermentation trials and was subsequently used to optimize the inoculum composition for

Weighted Project Score: 7.0

Weighting for Ongoing Projects: Approach - 25%; Accomplishments and Progress - 25%; Relevance - 25%; Future Work - 25%



I One standard deviation of reviewers' scores

maximum bioconversion yields. Using this strategy, we have demonstrated production of fusel alcohols, terpenes, and hydroxyalkanoates from diverse algal biomass, and found that retention of algal lipids as an extractive overlay in the bioprocessing provides process-intensification benefits by alleviating product inhibition and allowing co-extraction of the algal-derived products. Downstream testing of these products for fuel applications indicate that in several cases the algal-derived bioconversion products have attractive properties in both spark- and compression-ignition engines. Furthermore, TEAs suggest that the demonstrated yields of multiple bioconversion product options should offset the algal biomass production cost estimates from n^{th}-plant models.

OVERALL IMPRESSIONS

- The goal of this project is to extract additional value from algal biomass, which is currently expensive to produce. The basic idea is to use algal biomass as a substrate for a microbial fermentation process. Initial work was focused on the production of fusel alcohols. Current work is focused on the production of ethyl lactate using an engineered microbial consortium. Overall, the project is very innovative. The team is making reasonable progress. It is not clear how the metabolic engineering work fits into this project—the details are somewhat vague. The project would benefit from TEA and more focus on product recovery.

- This team has demonstrated important improvements in the production of alkanoates and fusel alcohols from microalgal biomass. So far, they have achieved near-theoretical yields in pretreatment, demonstrated the first *in vivo* production of ethyl lactate, and are well on the road toward demonstrating a two-times yield improvement over their previous target.

- On the overall, this project is technically sound and aims to add value from the refining of algal biomass from non-oil streams. Identifying the right product mix and markets for these products will be challenging.

- This project, the way it is presented, seems like a complicated one with stretch goals/targets. Better project management, clear go-no-go decision making, and a better explanation of current progress is needed to make it clear what the project status is, and how the team is planning to meet milestones.

- The goal of this project is to utilize algal biomass as a feedstock for producing chemicals. After extraction of lipid products from algal cultures, an abundance of protein and carbohydrates remains that can be used as a substrate. The team has focused on making hydroxyalkanoate esters from algal protein and carbohydrate. This is a complicated process, requiring organisms to take up a complex mixture of substrates and make two different compounds and then condense them by esterification. The team has made very commendable progress given the modest budget and is in a position to make a very high impact if the progress continues. The team has a good vision for future work, but specific tasks associated with improving product titer are not described. In the future, they should focus on some of the metabolic challenges in getting all the pathways to work in coordination. Growing multiple strains in coculture at scale is high risk and has no commercial validation.

RECIPIENT RESPONSE TO REVIEWER COMMENTS

- The recipients choose not to respond to the reviewers' overall impressions of their project.

COMBINED ALGAL PROCESSING (CAP) PROCESS RESEARCH

National Renewable Energy Laboratory

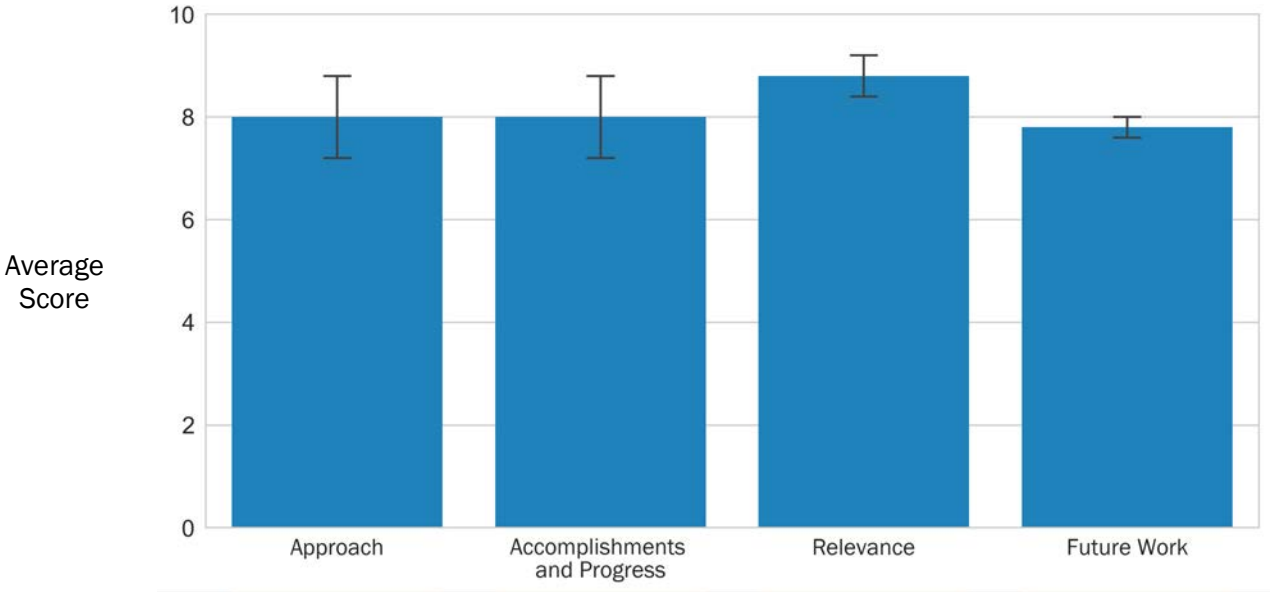
PROJECT DESCRIPTION

The CAP scheme has been developed to reduce the Minimum Fuel Selling Price (MFSP) through the development of technology for the production of a portfolio of fuels and coproducts. This work has combined a number of unit operations arising from the Biochemical Conversion Program along with novel conversion steps specific to algal biomass in order to develop an integrated process to establish a conceptual multiproduct algal biorefinery. Acid pretreatment enables wet lipid extraction with high yields and efficiently hydrolyzes algal carbohydrates with no need for enzymes, allowing for efficient fermentation of the sugars to fuels and chemicals. Additional products have also been identified by conversion of the residual biomass following fermentation and extraction, rounding out the complete valorization of the algal biomass. Recent improvements in process sustainability have been achieved through adaptation of the CAP process for use with algal biomass grown in saline media, and approaches for further cost reduction are being developed to take advantage of low-cost wet wastes to serve as blendstocks for algal biomass.

WBS:	1.3.4.201
CID:	NL0026469
Principal Investigator:	Dr. Phil Pienkos
Period of Performance:	10/1/2014–9/30/2021
Total DOE Funding:	$2,100,000
DOE Funding FY16:	$600,000
DOE Funding FY17:	$500,000
DOE Funding FY18:	$500,000
DOE Funding FY19:	$500,000
Project Status:	Ongoing

Weighted Project Score: 8.2

Weighting for Ongoing Projects: Approach - 25%; Accomplishments and Progress - 25%; Relevance - 25%; Future Work - 25%



I One standard deviation of reviewers' scores

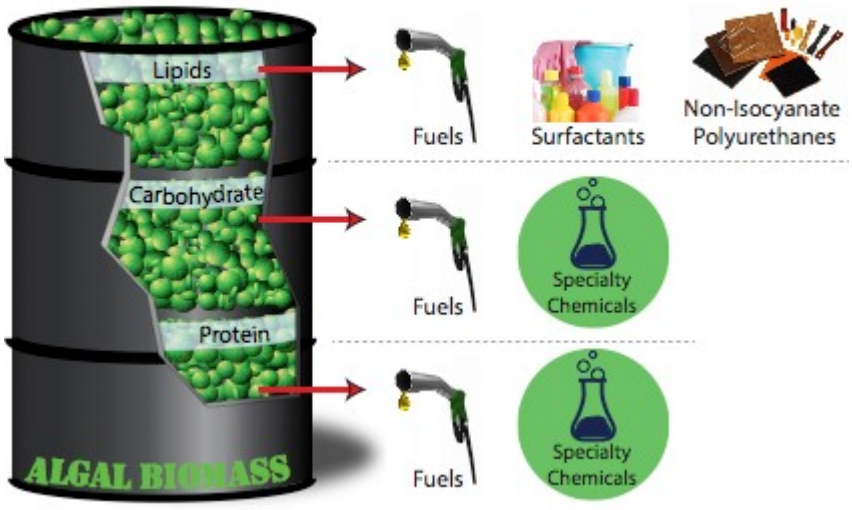


Photo courtesy of National Renewable Energy Laboratory

OVERALL IMPRESSIONS

- The goal of this project is to improve the economics of an algal-based process by generating a portfolio of products. Unlike cellulosic biomass, the composition of algal biomass is tunable, thereby enabling a range of potential products. One promising example is the development of a process enabling the conversion of lipids to polyurethane polymers, using a mixture of chemical and biological approaches. The focus for the next year will be to develop protein conversion processes to make diesel. In addition, the team proposes to develop halotolerant algae in order to reduce the need for fresh water. Overall, this is a very compelling project and the team is making excellent process. The partnerships with Patagonia and Algix are also very promising.

- This project is making impressive progress toward demonstrating an approach to significantly reduce MFSPs from algal feedstocks by integrating conversion technologies for fuel and coproducts. A major driver of the success of this project will be in matching CAP-accessible products from different algal components with industry.

- The project performers have addressed several of the challenges for the cost-effective production and use of algal biomass. They have also relied on experience gained from the processing of lignocellulose. In the long term, CAP may provide additional revenue for product and coproducts. Before that can be accomplished, a commodity supply chain with a low cost of production is needed.

- Clarification on the quad chart overview (project start date) would help understand the percentage of work completed so far (it is stated that the project started in 2013). The project needs to rely on various other projects within BETO; however, it is not clear how the project interacts with other teams, and therefore, clearer paths/approaches to work more closely with other projects for quick adaptation of their learning would be needed in this project and all other projects working in the consortia. From manufacturability, it would be great to investigate costs associated with production of various products

(fermentation and separation/purification) and if there are combinations that would make the most sense and feasibility in regard to cost of operation.

- CAP is using various approaches to extract as much value as possible from algae, because TEA has shown that algal biofuels without coproducts will never be economically viable. Having a detailed understanding of the algal biomass composition is important for this goal. This biomass can also be combined with other waste streams as a feedstock. There is a clear path to a $2.50 per gasoline gallon equivalent (GGE) biofuel cost if the goals of this project can be achieved. The team has demonstrated three different products made from algal material using biological or chemical conversion: butyric acid, polyurethanes, and hydrocarbons. The team has a good approach and has made great progress. They have a solid understanding of the economics and algal processing technology, but it is not clear that they appreciate all of the challenges associated with downstream fermentation.

RECIPIENT RESPONSE TO REVIEWER COMMENTS

- Thanks to all the reviewers for their positive feedback and constructive criticism.

- We apologize for the confusion created by the quad chart. Due to time limitations, we were unable to cover this in great detail. The project started back in 2013 but is now in the first year of a three-year annual operating plan cycle indicating a 17% completion of that cycle. Interaction with other teams is summarized in slide 28 in the supplemental section. Interactions with biochemical project leads take place formally at biweekly program meetings and with algal project leads at monthly algal program meetings. There was not enough time to provide more details on how this project interfaces between biomass production, composition, and TEA across the Advanced Algal Systems platform. Some background is missing due to the separation of this presentation from the other algal projects, including the algal TEA work. We included four supplemental slides from the algal TEA presentation (slides 29–32). Ryan Davis of NREL is responsible for TEA modeling of both biochemical processes and algal processes, and so the modeling of fermentation and product recovery costs uses the same process assumptions though with different feedstock and product metrics based on the data generated in this project.

- The comment regarding insufficient appreciation of the challenges associated with fermentation is certainly true, and in my experience every scale-up project presents a new learning curve. As noted above, the algae program uses the same TEA modeling modules for our fermentation work as are used for the biochemical program, though modified for the differences in feedstock input. It may be worth noting that our approach is to establish the applicability of various fermentation options in our integrated CAP scheme to determine the potential value based on relative contributions to MFSP, and to maximize fitness within the BETO strategic framework. As in any early-stage R&D program, the ultimate goal is to establish a conceptual process, within an appropriate TEA framework, that would warrant addressing the various challenges that occur during scale-up.

BIOCHEMICAL PLATFORM ANALYSIS

National Renewable Energy Laboratory

PROJECT DESCRIPTION

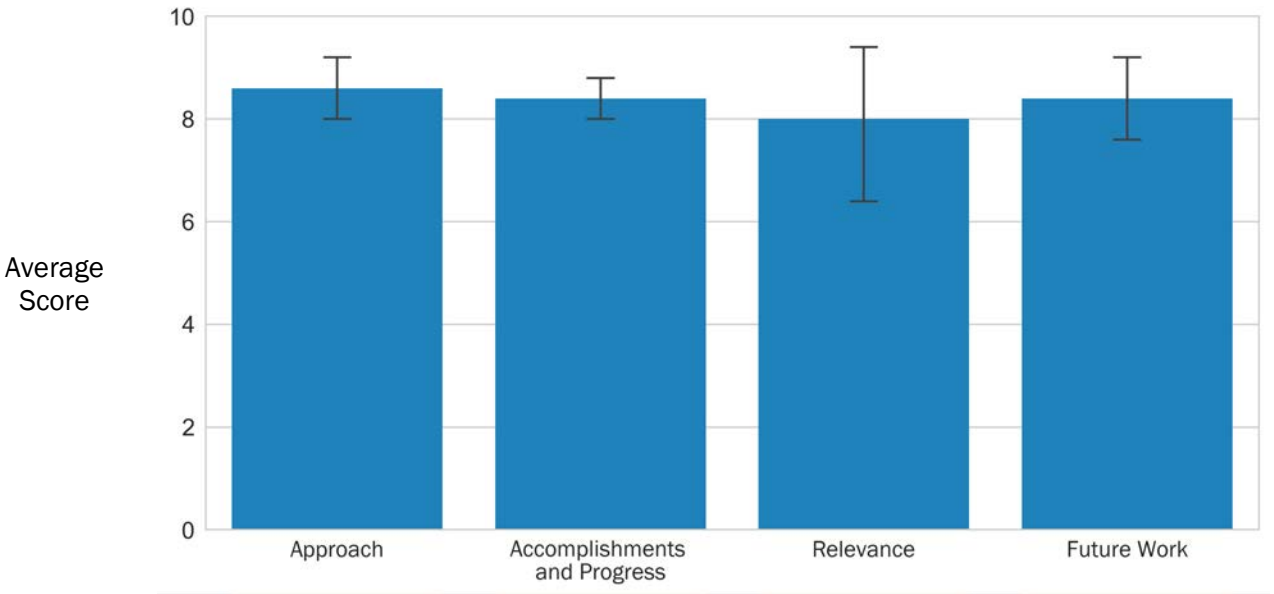
The objective of this project is to perform TEA to support and guide biochemical platform R&D efforts, through use of process and economic models which translate key process parameters into overall economics for purposes of setting future R&D targets and tracking performance progress against those targets. Outcomes of integrated TEA modeling are utilized by BETO to guide program plans, as well as by other NREL/partner projects to quantify the impact of research on key technology barriers and to prioritize future efforts.

WBS:	2.1.0.100
CID:	NL0008202
Principal Investigator:	Dr. Ryan Davis
Period of Performance:	10/1/2016–9/30/2019
Total DOE Funding:	$2,702,000
DOE Funding FY16:	$750,000
DOE Funding FY17:	$750,000
DOE Funding FY18:	$852,000
DOE Funding FY19:	$350,000
Project Status:	Ongoing

This work is highly relevant to BETO program goals, in that "bottom-up" conceptual modeling conducted under this project serves as a basis for understanding the technical feasibility to meet "top-down" program cost targets set by BETO. By providing a framework to translate technical performance to cost reductions within a biorefinery, our TEA models may be leveraged to direct R&D towards the most economically impactful priorities. This helps to maximize the efficiency of research funding, ultimately in support of demonstrating 2030 fuel cost targets below $2.50/GGE.

Weighted Project Score: 8.3

Weighting for Ongoing Projects: Approach - 25%; Accomplishments and Progress - 25%; Relevance - 25%; Future Work - 25%



This project has made significant achievements since the 2017 BETO Peer Review, including: (1) a go-no-go decision to downselect for the most economically promising pathways for sugar fermentation to hydrocarbon fuels, (2) establishment of a new Biochemical Design Report to highlight paths to achieving $2.50/GGE fuel cost targets in the future (based on two separate integrated process approaches reflecting the prior downselect guidance), and (3) providing state-of-technology (SOT) updates to benchmark progress towards those targets based on current experimental performance data. This work demonstrated the technical potential to achieve BETO's fuel cost goals and associated key gaps/barriers that must be overcome relative to current benchmarks, most notably including the need to maximize biomass utilization through opportunities to valorize lignin for value-added coproducts.

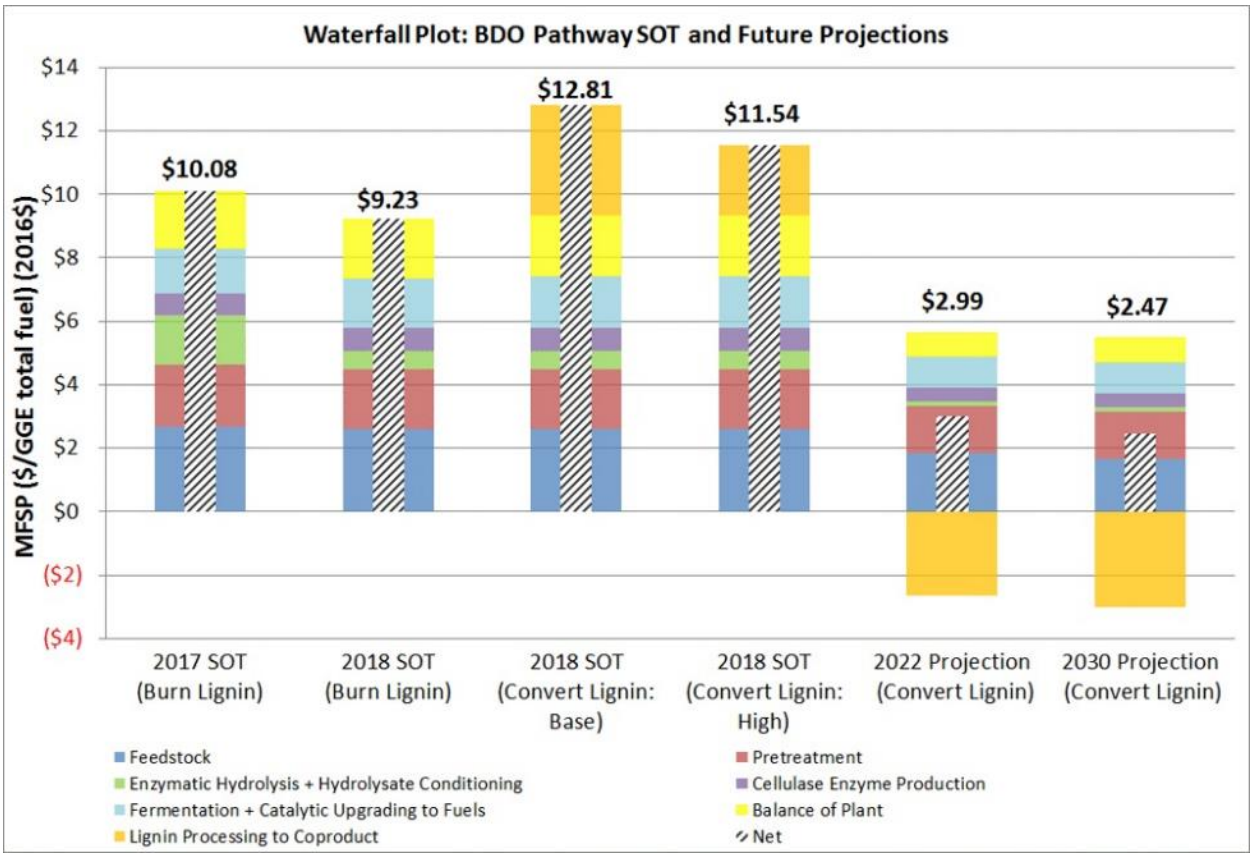


Photo courtesy of National Renewable Energy Laboratory

OVERALL IMPRESSIONS

- The objective of this project is to conduct process modeling, TEA, and sustainability assessment to support all biochemical platform R&D activities. This is an essential function towards reaching the BETO Multi-Year Plan (MYP) goals, as it enables choice of target molecules and processes with the highest probability of successfully meeting cost targets and defines performance metrics needed to accomplish them. The team is well integrated with other projects, regularly communicating with experimentalists to understand technical challenges. This project underscores BETO's increasing commitment toward ensuring commercialization potential of the funded work. One of this project's key accomplishments was to determine that anaerobic processes should be emphasized over the aerobic lipid pathways, and as a result developed case studies for two anaerobic processes: 2,3-BDO and butyric acid. Both can be converted to fuel molecules. It was also clear that lignin-upgrading technology must be improved to be cost advantaged relative to burning it. In addition, the team can serve as a valuable

resource among all projects to ensure economic viability and sustainability. Future projects are well defined and are focused on activities with the best impact on cost. It is recommended to increase funding to this effort so they can work on more projects, including those done externally.

- This project provides process designs and economic analysis for many existing BETO projects. This analysis is then used to identify worthwhile projects, in particular helping identify go-no-go decision points. A key success of this analysis is the identification of two promising processes (2,3-BDO and organic acids) and to deemphasize aerobic processes focused on fatty alcohol production. Overall, the team is making excellent process and addresses a key need within the BETO portfolio. The project would benefit from more input from industry and an increased focus on the costs of product recovery, which will influence upstream design decisions.

- This project addresses a critical ongoing need across the biochemical conversion platform to incorporate process/cost modeling into the decision making that guides research prioritization and project tracking. The project has already demonstrated success by guiding downselection of pathways and shift of focus toward anaerobics. Moreover, its Design Report Update work product is a valuable resource for BETO, and an exemplar study of how to pursue comprehensive, quantitative strategic guidance. Finally, the project's goals to extend its purview to other BETO projects, develop new models, and disseminate models to the community will greatly add to the impact of the work.

- Biochemical platform analysis is key to establishing commercial viability of a process, as it provides TEA that is necessary to evaluate uses of biomass-derived feedstocks for the bioproduction of chemicals and drop-in fuels, as well as the use of thermochemically treated products and residues as biocrude or upgraded advanced fuels. This activity provides BETO with the tools to establish progress in funded projects as well as greater diversification of the portfolio of products made from biomass.

RECIPIENT RESPONSE TO REVIEWER COMMENTS

- We thank the reviewers for their positive feedback in recognizing the impact of this project for BETO and the utility in guiding R&D priorities for NREL and the community. We welcome the suggestions to increase collaborations with and feedback from industry to help refine the TEA models, particularly for more new/novel process operations, including key separation and product recovery steps. Two such mechanisms for this include subjecting our draft design reports to a thorough peer-review vetting process (including stakeholders from industry) and subcontracting with engineering firms or other expert consultants to improve process understanding for such operations. For example, at present we are working with one such consultant from industry to improve current design and cost estimates for carboxylic acid recovery via membrane pertraction, to be incorporated into future TEA model iterations.

COPRODUCTION OF CHEMICALS AND FUELS ENABLED BY HYBRID CONVERSION OF LIGNIN AND BIOCONVERSION INTERMEDIATES

Pacific Northwest National Laboratory

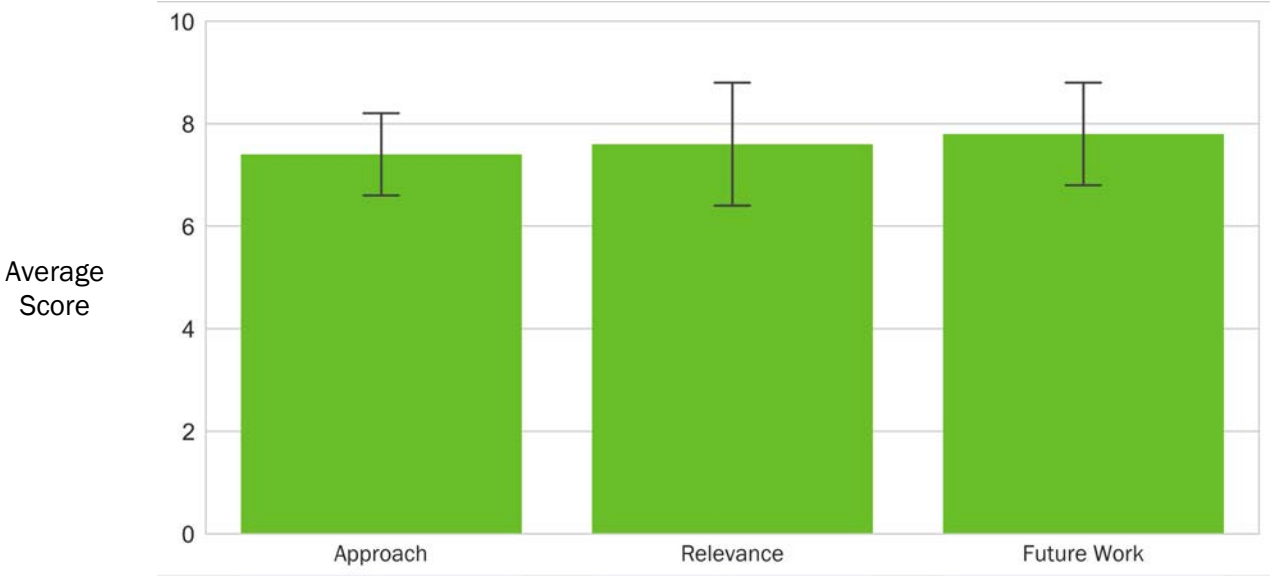
PROJECT DESCRIPTION

WBS:	2.2.2.501
CID:	NL0034399
Principal Investigator:	Dr. Jim Collett
Period of Performance:	10/1/2018–10/1/2020
Total DOE Funding:	$300,000
DOE Funding FY16:	$0
DOE Funding FY17:	$0
DOE Funding FY18:	$0
DOE Funding FY19:	$300,000
Project Status:	New

Contemporary biorefinery designs for bioconversion of lignocellulosic feedstocks to fuels and chemicals treat residual lignin and microbial cell mass as waste streams, and route them to combustion and wastewater treatment operations for production of heat and power. The opportunity and challenge are to optimize the value obtained from all streams within the biorefinery to increase total profitability, while retaining a high yield of liquid fuel from biomass. To meet this challenge, we have developed a new "hybrid conversion" biorefinery concept in which hydrolysate sugars are used to produce high-value commodity chemicals via bioconversion. Residual lignin and microbial cell mass are then routed to continuous hydrothermal liquefaction (HTL) for the production of biocrude oil, which may be upgraded via hydrotreating to a distillate blendstock for diesel and jet fuel. BETO has already played a major role in bioconversion R&D on commodity chemical production from biomass, and in the reinvention of HTL for continuous conversion of wet-waste streams such as mixed algae and sewage sludge. Combining these approaches provides a genuinely innovative and more efficient conversion process. As such, the goal of this new, two-year seed project is to demonstrate the potential for profitable bioconversion of corn stover hydrolysate sugars to a model commodity chemical (e.g., carboxylic acids), and co-conversion of the residual cell mass with corn stover lignin via HTL

Weighted Project Score: 7.7

Weighting for New Projects: Approach - 25%; Relevance - 25%; Future Work - 50%



I One standard deviation of reviewers' scores

to biocrude oil for the production of distillate fuels with an MFSP of $3/GGE. This seed project builds upon preliminary experimental work and TEA of the hybrid conversion biorefinery concept, which projected a distillate fuel yield of 63 GGE/ton of dry corn stover without coproduction of a commodity chemical. Achieving the objectives of this project will decrease risk in the BETO portfolio by providing a new, less complex route for lignin valorization in cellulosic biorefineries with lower installed costs compared to other current biorefinery designs, while maintaining a high yield of high-quality distillate blendstock for use in diesel and jet fuels.

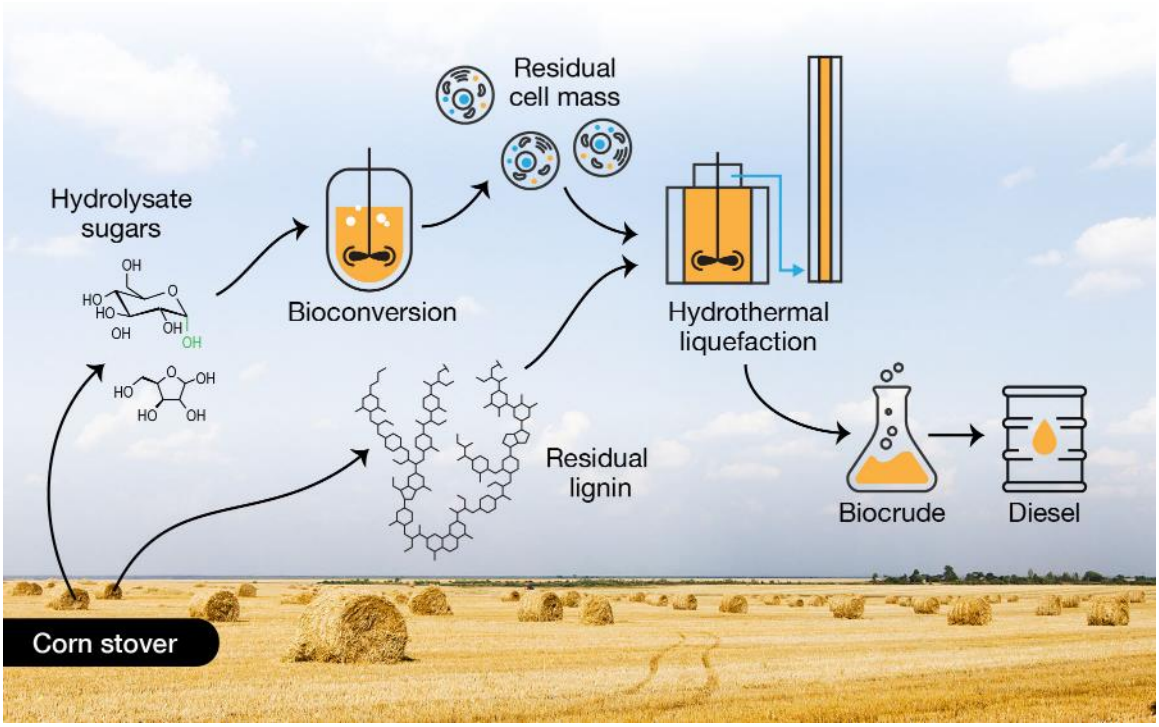


Photo courtesy of Pacific Northwest National Laboratory

OVERALL IMPRESSIONS

- Overall interesting concept; more work is needed to work through logistics in large-scale plants and how the outcome of this study will/can be manufacturable. PIs are encouraged to consider applying proper project management with relevant milestones and go-no-goes.

- New project aims to apply HTL of yeast and lignin biomass to enable coproduct production, which should approximately double yields. Cost estimates based on modeling and prior HTL work supports the aims of this project.

- The goal of this project is to develop a proof-of-principle biorefinery process, where fuels will be made from lignin, biomass, and other leftovers. This is a new seed project. The process will involve the oleaginous yeast *Lipomyces starkeyi* and HTL of lignin. The work looks promising, and clearly addresses key needs within the BETO portfolio.

- Organic acids are currently produced by fungal fermentations (yeast and mold) from cost-effective refined streams of sugars (molasses from cane/beets or corn dextrose syrups).The refining of sugars for production of food-grade chemicals would add cost to nonconventional feedstocks, as in this case. A similar challenge is faced for recovery of organic acids for non-food applications unless a cost process is possible. This is usually possible only using a low-pH/high-acid-tolerant strain. The aerobic production

of organic acids represents a significant cost. Titer, rates, and yields, as well as CAPEX considerations, need to be addressed further.

- This project seeks to use HTL to convert combined lignin and waste-cell mass to diesel-fuel molecules. The biorefinery concept presented is to use the higher-quality sugars to make a higher-value product, while using these lower-quality mixed streams to make the fuel. Task 1 is to characterize the residual broth from a model organism/product fermentation so it can be used as a substrate for HTL. Task 2 is to run the HTL process using the model biomass to understand the critical process parameters. Task 3 is to perform TEA to determine sensitivity of product/fuel ratio. The milestones for the next year are well defined, but the technical risks and challenges are not presented.

RECIPIENT RESPONSE TO REVIEWER COMMENTS

- The recipients choose not to respond to the reviewers' overall impressions of their project.

LOW-TEMPERATURE ADVANCED DECONSTRUCTION

National Renewable Energy Laboratory

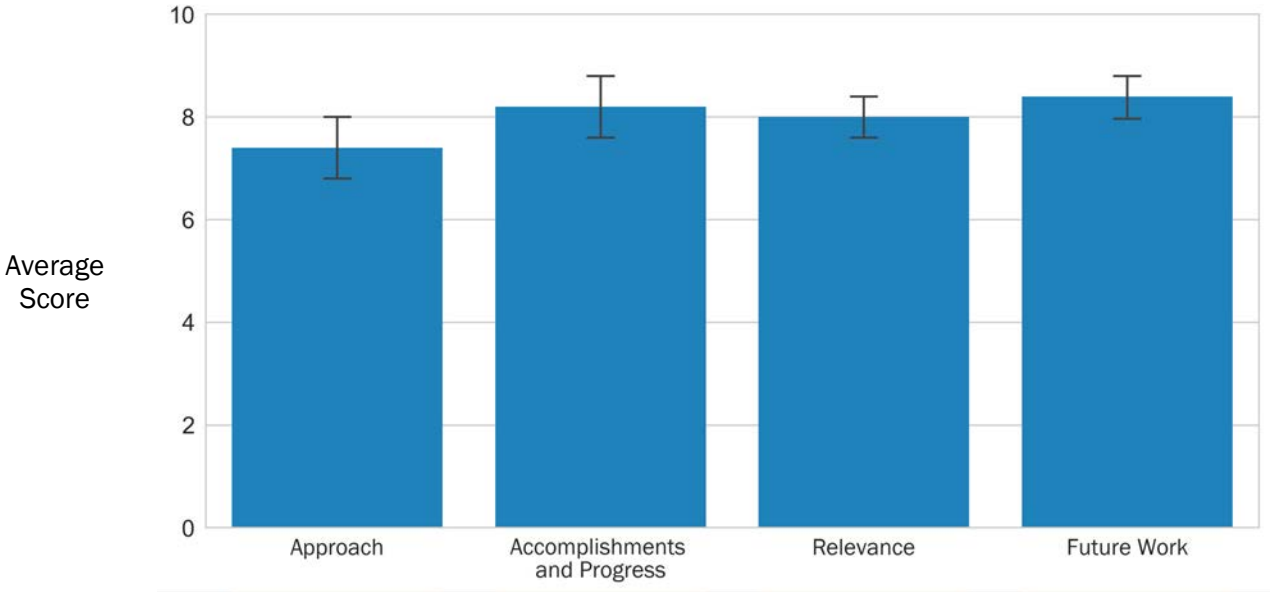
PROJECT DESCRIPTION

WBS:	2.2.3.100
CID:	NL0006288
Principal Investigator:	Dr. Mel Tucker
Period of Performance:	10/1/2015–9/30/2021
Total DOE Funding:	$5,915,368
DOE Funding FY16:	$1,400,000
DOE Funding FY17:	$1,400,000
DOE Funding FY18:	$1,615,368
DOE Funding FY19:	$1,500,000
Project Status:	Ongoing

The overarching Low-Temperature Advanced Deconstruction project goal is to develop industry-relevant low-temperature biomass deconstruction and fractionation processes that produce low-toxicity, high-concentration sugar syrups, and reactive lignin streams at low CAPEX and operational expenditures (OPEX) using relevant feedstocks for biological and catalytic upgrading to meet BETO's 2022—and beyond—targets and goals. We have demonstrated high-solids (33 wt % insoluble solids), enzymatic hydrolysis (EH) reactions with the NREL-developed DMR pretreated corn stover substrates. This process has achieved titers of over 270 g/L of fermentable monomeric sugars at greater than 80% yields (at an enzyme loading of 20 mg protein per g of cellulose). These sugars are highly fermentable using recombinant *Zymomonas* to produce ethanol or 2,3-BDO at high titers and yields. In recent developments, we have achieved over 80% monomeric sugar yields at an enzyme loading of 12 mg of protein per g of cellulose using least cost formulation (LCF) blended (corn stover, switchgrass, sorghum) feedstocks provided by INL. A differential deacetylation process option was applied to the LCF biomass feedstocks, increasing overall process sugar yields as high as 9% compared to the single-severity deacetylation process control. A continuous counter-current deacetylation (CCCD) process option further increased EH sugar production rates and yields, as well as reduced xylan losses and lignin condensation reactions, using a shaftless screw reactor to replace the current

Weighted Project Score: 8.0

Weighting for Ongoing Projects: Approach - 25%; Accomplishments and Progress - 25%; Relevance - 25%; Future Work - 25%



I One standard deviation of reviewers' scores

batch deacetylation process. The improved mass and heat transfer and decreased residence times in the CCCD process option resulted in lowering xylan solubilization and losses by 66%, while sugar yields in EH increased over 15%. Furthermore, a low-consistency disk-refining process option has been introduced to replace Szego milling in a multistage DMR process to solve the potential equipment scale and vibration issues. Low-consistency disk refining, which has been practiced at the commercial scale, shows similar sugar yield improvements as compared to Szego milling, with sugar yields increased by as much as 13% compared to EH of single-stage DMR pretreated corn stover substrates. Collaborations with the University of Colorado at Boulder and Princeton University have developed novel microbial electrochemical technology (MET) systems shown to recover 90% of the salts (especially sodium ions [Na+]) and 60% of the lignin using electrons generated by degradation of the dilute organic waste in the deacetylation black liquor (after removing the solubilized lignin), saving chemicals and reducing the environmental and life cycle assessment (LCA) footprints. Collaboration with Washington State University has shown successful upgrading of the technical lignin isolated from the black liquor waste stream to high-energy, high-density jet fuel blendstocks using super Lewis acid metal triflates combined with ruthenium on carbon or ruthenium on aluminum oxide metal catalysts, increasing the carbon efficiency and decreasing the quantity of waste streams at a biorefinery.

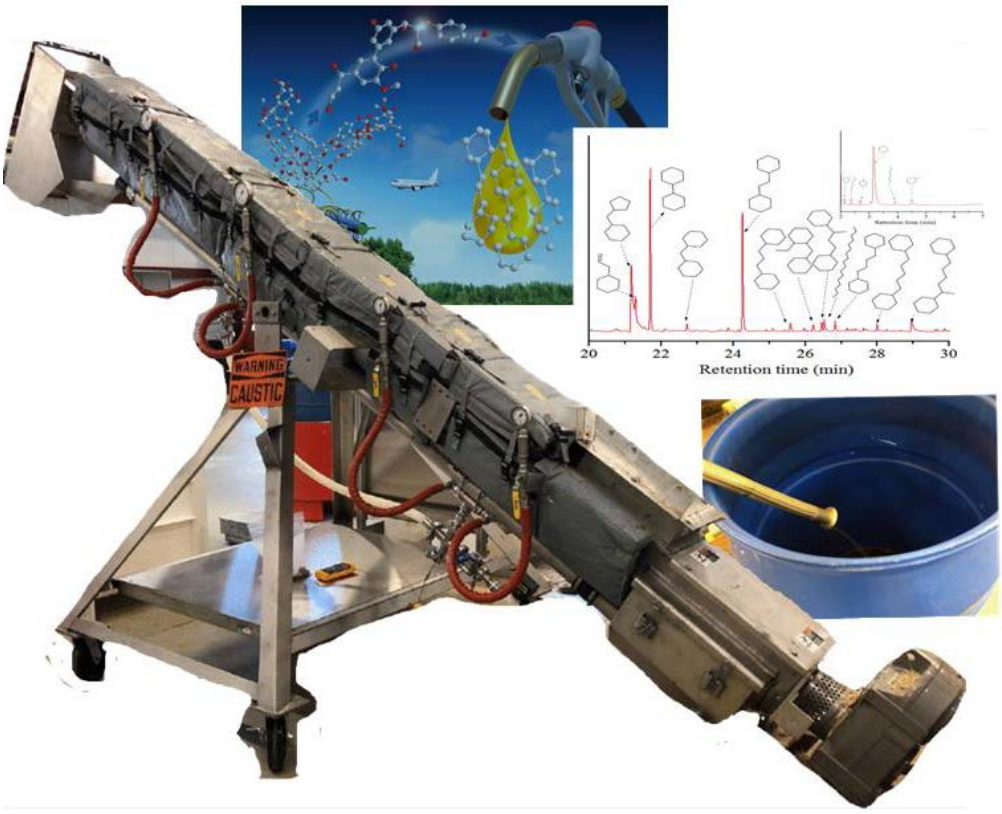


Photo courtesy of National Renewable Energy Laboratory

OVERALL IMPRESSIONS

- This project seeks to develop a biomass deconstruction process that produces high-quality, cost-effective sugars as well as a tractable lignin stream that is more amenable to processing than that resulting from conventional high-temperature methods. The project is divided into two tasks, one for biomass

deconstruction and the other for black liquor (deconstruction waste stream) recovery and upgrading. Work has proceeded on track, and they have a solid plan for future work.

- The goal of this project is to develop a cost-effective, low-temperature deconstruction process for sugar and lignin recovery. A key advance is the transition to continuous operation, as this will provide better yields and require less sodium hydroxide (NaOH). Overall, the team is making excellent progress. The project would benefit by comparing their process with existing ones. In particular, competitive benchmarks are lacking. In addition, more emphasis should be placed on NaOH recovery, as this will be critical for commercial viability.

- This project seeks to develop a low-temperature deconstruction process by combining a novel DMR with a novel method to utilize black liquor (waste). So far, the researchers have developed several interesting innovations to address technical barriers they have encountered, enabling them to make considerable progress toward both of these aims. Still, in order to attain direct industrial relevance, more progress is needed over the remainder of the performance period to attain the project's desired sugar yields and waste utilization targets.

- The project outlined progress in the development of a low-alkali process for deacetylation and two-step mechanical refining from corn stover that is less toxic to fermenting organisms to produce a DMR slurry that can be readily digested by enzymes and used as a fermentation feedstock. Good progress has been made in this process, but additional work is necessary for commercial viability.

RECIPIENT RESPONSE TO REVIEWER COMMENTS

- We thank the reviewers. Our project will continue developing novel, industry-relevant, low-temperature biomass deconstruction processes to improve sugar yields while reducing enzyme, chemical, energy, and water usages. In an earlier BETO FY 2017 Peer Review, we directly compared the enzymatic hydrolysis performance of the DMR process in head-to-head competition with deacetylation followed by dilute acid pretreatment on a variety of feedstocks (corn stover, switchgrass, and sorghum) and feedstock blends, but time constraints prevented presenting those data here. The DMR process achieved equivalent, or better, enzymatic hydrolysis and process sugar yields when compared to deacetylation followed by dilute acid pretreatment on various corn stover lots and blends. We also agree with the reviewers that NaOH recovery is one of the keys to the commercial success and application of the DMR process to biorefineries where we are continuing our research efforts in METs to recover water, sodium, lignin, and other chemicals for recycling back into and use by the biorefinery. To improve the DMR process, we are moving away from batch processes to developing continuous process options that are scalable. We chose to investigate low-temperature deconstruction processes to mitigate many issues that result from high-temperature pretreatments (e.g., hydrolysate toxicity, lignin condensation, operational reliability of feeding biomass into high pressure reactors, etc.). In order to achieve BETO's 2022 and beyond yields and targets, we will continue our research into scalable approaches as outlined in the presentation with guidance from TEAs and LCAs .

ALKALINE-OXIDATIVE PRETREATMENT OF WOODY BIOMASS FOR OPTIMAL COPRODUCT

Michigan State University

PROJECT DESCRIPTION

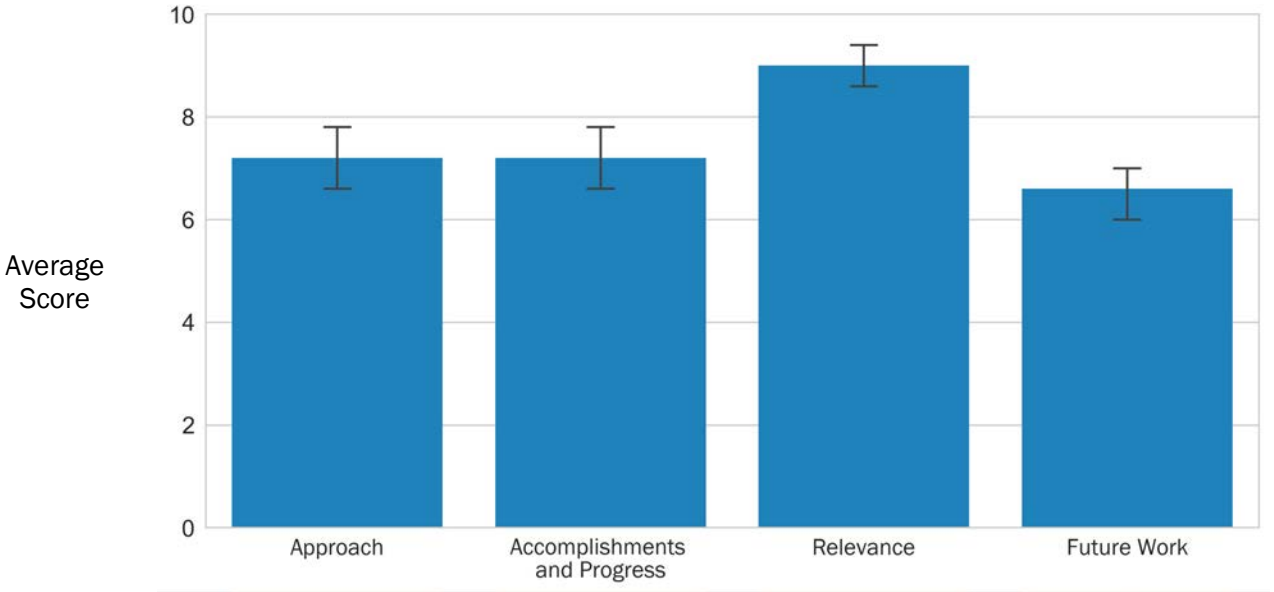
WBS:	2.2.3.601
CID:	EE0008148
Principal Investigator:	Dr. Eric Hegg
Period of Performance:	10/1/2017–12/31/2020
Total DOE Funding:	$1,800,000
Project Status:	Ongoing

Woody biomass represents a vast source of carbohydrates and aromatics and it is envisioned as a key feedstock for the sustainable production of biofuels and bioproducts. We recently demonstrated that combining an alkaline pretreatment with an alkaline-oxidative post-treatment is a promising strategy for the delignification of hybrid poplar to generate (1) sugars in high yields that can be converted to hydrocarbon biofuels, as well as (2) a largely unmodified lignin stream suitable for valorization to bioproducts, including aromatic monomers and polyurethane coatings. In addition, we identified a novel approach to lignin depolymerization that resulted in high yields of aromatic monomers from lignins, and we demonstrated that hybrid poplar lignins generated by our mild catalytic alkaline-oxidative pretreatment resulted in the highest monomer yields obtained from the many process-derived lignins screened.

Motivated by this promising route for integrated deconstruction and lignin valorization, the goals of this project are to (1) optimize the two-stage alkaline-oxidative deconstruction approach to generate both a sugar stream and a lignin stream from hardwoods, (2) understand how pretreatment conditions impact lignin properties and how these properties can be linked to lignin suitability for coproduct applications, (3) employ TEA and LCA to inform the experimental work and to ascertain the tradeoffs between processing costs and

Weighted Project Score: 7.5

Weighting for Ongoing Projects: Approach - 25%; Accomplishments and Progress - 25%; Relevance - 25%; Future Work - 25%



I One standard deviation of reviewers' scores

environmental costs associated with generating high-value coproduct streams at high yields, and (4) identify strategies to decrease the MFSP. Successful completion of this project will demonstrate the possibility of using this process to achieve the target of $3/GGE by generating both a low-cost sugar stream and a relatively clean lignin stream that is ideally suited to depolymerization and coproduct production.

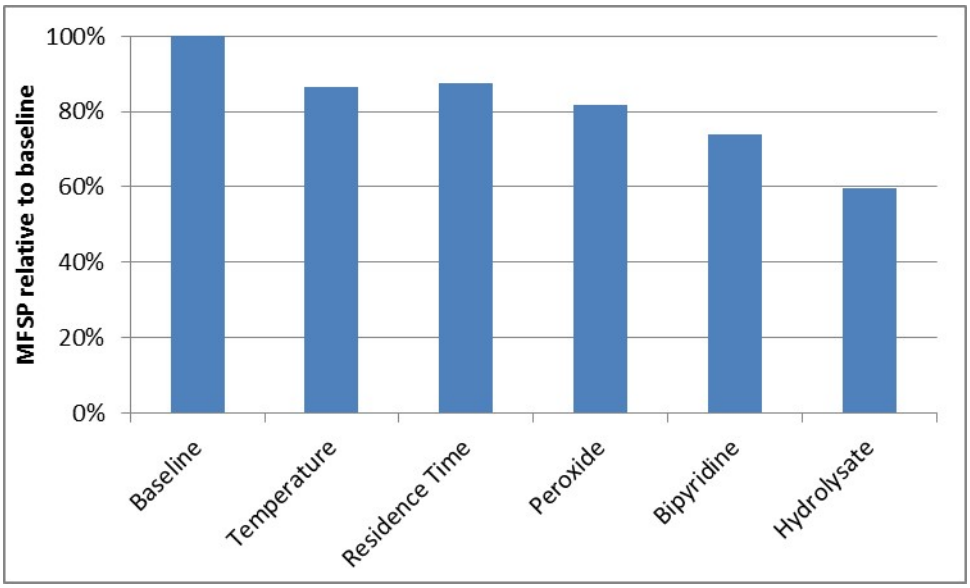


Photo courtesy of Michigan State University

OVERALL IMPRESSIONS

- The goal of this project is to produce pure sugars and lignin from woody biomass using a two-stage alkaline-oxidative deconstruction process. The project focuses on optimizing the process. The main advance and innovation are that the process can recover clean lignin, which can subsequently be depolymerized. The project appears to be on schedule. It is not clear if the process will be commercially viable. Competitive benchmarks and more transparent cost estimates would be useful. In addition, it is not clear how milestones relate to commercial viability.

- The team is developing a two-stage deconstruction process (called alkaline hydroperoxide [AHP]) to generate clean sugars along with lignins suitable for valorization from woody biomass. Thus far, they have established an initial system and optimized conditions for each of the two steps of the AHP process. It is too early to evaluate the eventual success of the project, but they have achieved all of their intermediate milestones to date. TEA/LCA is in progress and process integration is part of their future plans.

- The stepwise depolymerization of woody poplar with conversion of sugars post-enzyme treatments to hydrocarbon fuels and the alkaline oxidation of lignin to yield at least a target of 25% monomer is promising. The targeting of a range of products from lignin should aid in developing a cost-effective lignin biorefinery.

- This seems to be a great project. Having clear milestones, timelines, and a well-described project plan enables the project to be more accountable and the accomplishments more relevant to the overall goal. PIs are encouraged to work on those items more.

- The goal of this project is to optimize a biomass deconstruction process to generate both a clean sugar stream for fuel production and lignin suitable for coproducts. The technology is intended to produce a

higher-quality lignin stream that is very compatible with the oxidation/depolymerization process, which provides monomers to be used for upgrading. The two-stage process was optimized by evaluating the influence of process conditions on both yield and quality of the products. The two stages have to be optimized in parallel, because they are interdependent. Good progress has been made on both overall yield and monomer generation, estimated to reduce total cost by 25%. The project seems to be going in the right direction, but challenges and goals for the future are not clearly defined.

RECIPIENT RESPONSE TO REVIEWER COMMENTS

- We appreciate the reviewers' time and thoughtful comments. Our milestones and go-no-go decision points are linked to decreasing the MFSP. Progress on reducing the MFSP as well as other TEA data is reported to DOE quarterly.

- Our goals for the future include (1) scaling up the process, (2) integrating the different components, and (3) continuing to lower MFSP.

FUNGAL GENOMICS – GENETICS

Pacific Northwest National Laboratory

PROJECT DESCRIPTION

WBS:	2.3.2.103
CID:	NL0011937
Principal Investigator:	Dr. Jon Magnuson
Period of Performance:	10/1/2015–9/30/2021
Total DOE Funding:	$4,333,275
DOE Funding FY16:	$1,650,000
DOE Funding FY17:	$1,500,000
DOE Funding FY18:	$743,275
DOE Funding FY19:	$440,000
Project Status:	New

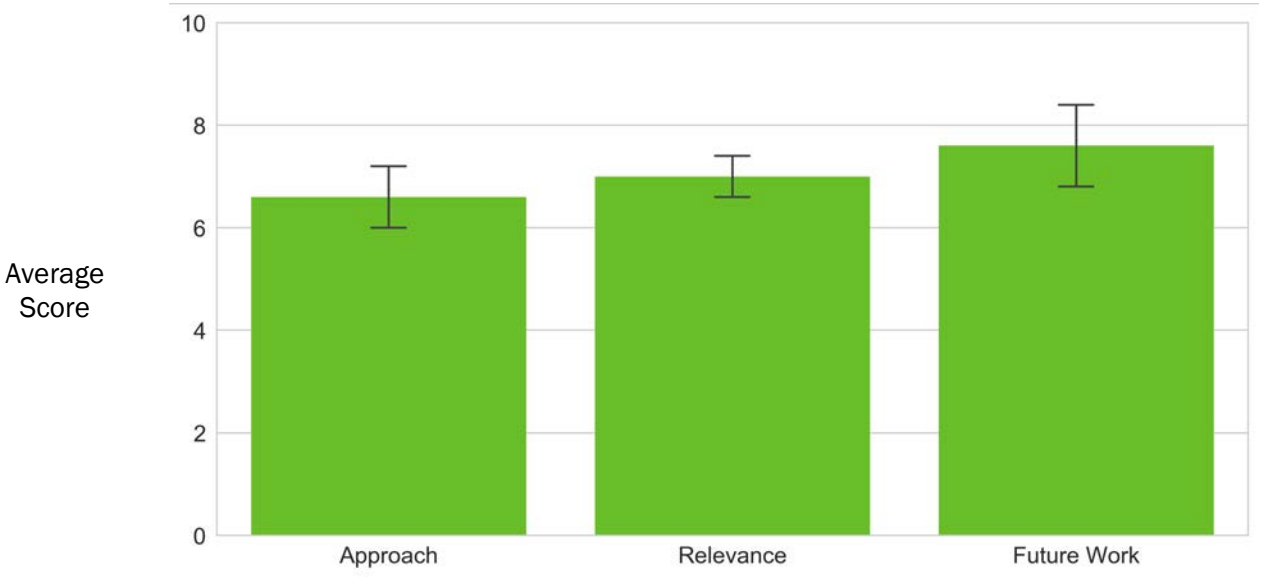
The challenge facing biological conversion routes from carbohydrates to hydrocarbon biofuels is that they are inherently carbon inefficient, because oxygen must be removed as carbon dioxide (CO_2) or water, or both. Our goal is to engineer the yeast *Lipomyces starkeyi* to maximize carbon efficiency by conversion of biomass sugars to the easily separated hydrophobic phase biofuel zingiberene and aqueous phase bioproduct malic acid, in parallel.

In a previous project we developed genetic engineering tools for *L. starkeyi* that enable us to accomplish the task of coproduction of fuels and chemicals for greater carbon efficiency. In true biological fermentation processes, a sugar is converted into one highly oxidized and another highly reduced molecule (e.g., glucose to ethanol and CO_2). We are applying that biological principle, coupled with carbon-fixing enzymatic steps, to generate a more carbon-efficient process for production of the highly reduced hydrocarbon biofuel zingiberene and the oxidized product malic acid.

In the first quarter of the project we succeeded in introducing the zingiberene synthase gene into *L. starkeyi* and obtained an initial titer of 18 mg/L in the best strain. Further media and culture condition optimization has increased the titer to 100 mg/L of zingiberene in a hydrophobic phase overlay. A strain with both the zingiberene synthase and farnesyl pyrophosphate synthase genes has been generated, and initial results indicate

Weighted Project Score: 7.2

Weighting for New Projects: Approach - 25%; Relevance - 25%; Future Work - 50%



an increase in titer relative to the control strain with only the zingiberene synthase gene. By the end of FY 2019 our milestone is to produce 300 mg/L of zingiberene. In addition, in FY 2019 we will overexpress the pathway to convert three-carbon central metabolites and CO_2 (released during zingiberene synthesis) into our four-carbon target bioproduct L-malic acid. Subsequent years will focus on increasing titers of both compounds and merging the two pathways into a single strain for coproduction.

Another challenge addressed in this project is driving down the gas-handling requirements of the bioprocess through two approaches. First is to continue bioprocess development with our engineered *Lipomyces* strains to determine the minimal aeration requirement throughout the process. Second is identifying and characterizing additional yeast species that may perform better than *Lipomyces* in regard to microaerobic growth. We started with a diverse set of 28 different yeasts for which we had sequenced genomes in collaboration with the DOE Joint Genome Institute from across the fungi kingdom. We have selected the 10 most promising candidates from initial screens for a final selection of the two most promising species by March 31, 2019. The selected strains must have microaerobic growth characteristics superior to *Lipomyces*, as well as a wide substrate range, and antibiotic sensitivities to enable genetic tool development.

TEA will be utilized throughout the project to guide our research toward the most impactful challenges and identify likely paths forward, during, and beyond the 36-month tenure of the project. One measure of project success at 36 months will be the production of 5 g/L of both the biofuel and the bioproduct in a single strain, preferably under much reduced aeration relative to the starting case of two vessel volumes per minute. This will be a significant milestone indicating the modified strain is worthy of further development, driving toward transfer of the technology and commercial development by industry.

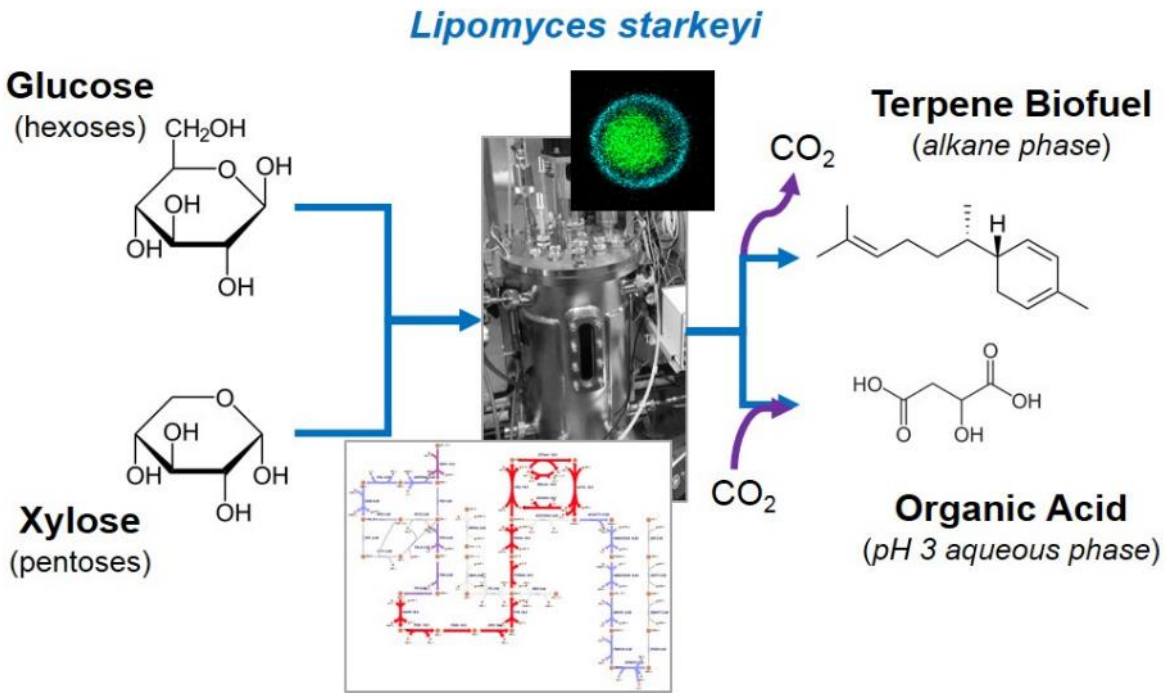


Photo courtesy of Pacific Northwest National Laboratory

OVERALL IMPRESSIONS

- This project leverages extensive research at PNNL in fungal genomics to develop a process for production of an oxidized bioproduct and reduced biofuel in the same fungal organism. By fixing CO_2 in

the reductive tricarboxylic acid (TCA) cycle branch, it maximizes carbon efficiency. They have chosen a terpene fuel molecule that can also be a valuable flavor compound in the short term. The acid tolerance of *Lipomyces* is leveraged to make the product, malic acid, in the free-acid form. The team has a clear path to a proof-of-concept demonstration, and milestones are clearly defined. A combination of metabolic and process modeling is used to inform the design, indicating a preference to get a microaerobic or anaerobic organism. This is an early-stage effort, with a goal to produce 5 g/L of each compound. If this proof of concept is successful, a larger project will be required to de-risk commercial viability. This will be particularly difficult if a new anaerobic yeast is used, requiring development of new tools. Although an anaerobic strain will have lower operating costs, it is not clear this would be worth the setback associated with a new organism.

- The goal of this project is to improve the carbon efficiency of fermentation processes by reducing losses due to CO_2 production. The team specifically proposes to produce terpenes and organic acids using *Lipomyces*. Overall, this is a high-risk proposal that is still far away from commercialization. In particular, it may not be possible to introduce a nonoxidative pathway into *Lipomyces*, because the genetic tools are still very limited. In addition, the team will likely run into issues associated with compartmentalization within the cell. Aside from *Lipomyces*, the team also proposes to look at alternate yeasts capable of growing during microaerobic conditions. This aspect of the project is not well developed or well motivated. Moreover, it is not clear whether such yeasts provide good platforms for making the proposed compounds. In general, this is a high-risk, potentially high-reward project still in the early phases of development.

- This new project has made good initial progress toward its goals to build the necessary capabilities to demonstrate a system that maximizes carbon utilization efficiency by producing oxygen-utilizing phase-separated coproducts. Already, they have established initial production strains.

- This is a highly challenging project that aims at the coproduction of a solvent-soluble terpene with a maleic acid that partitions to the aqueous phase. Targets for commercial viability need to be established once proof of concept can be demonstrated in a single strain that is adapted to use corn stover DMR.

- Great project and plan. Milestones need more clarification, organizing future work is needed with providing more detail on upcoming tasks and only high-level vision for far-future tasks. The project was great during the technical slides. It would be great to see more balance between the project management and technical slides.

RECIPIENT RESPONSE TO REVIEWER COMMENTS

- We agree that this is a higher-risk and high-reward project that is just beginning. The genetic tools are modest in comparison to a model organism, thus requiring more effort, but fortunately they allow us to manipulate the organism effectively. Compartmentalization is always something we consider in working with fungi and it is built into our metabolic model and design considerations. The strategy for increasing production of malate will be primarily directed at increasing the cytosolic pool. Mitochondrial transporters to address metabolic species imbalances are present in the organism. NADPH/NADH imbalance may be an issue and a transhydrogenase would be one possibility for addressing that issue. We agree that the task on assessing and developing alternative microaerobic yeasts accompanied by genetic tool development is high risk. In recognition of this risk, it was made the subject of the 18-month decision point. The LCA suggesting reduced carbon emission impacts by decreasing the need for gas handling suggests these alternative platforms are worthy of exploration.

- The terpene biofuel and malic acid coproduct will be the subject of a TEA, as we collect sufficient data in the latter two-thirds of the project. We recognize that the target titers, though ambitious for a project of this size, are not commercial titers. We anticipate they would need to be at least 10 times greater for

commercial viability, but the TEA will help assess that assumption and focus out-year efforts on the highest-impact parameters.

- With the first half year of the project behind us, we will add second-year milestones for increased clarity of the project direction. We fell into the familiar trap of scientists excited to talk about our early technical achievements but will seek greater balance at the next BETO Peer Review. We note that the project is of an intermediate size, involving a team at PNNL that has worked together for many years, so the project management is relatively straightforward.

BIOLOGICAL UPGRADING OF SUGARS

National Renewable Energy Laboratory

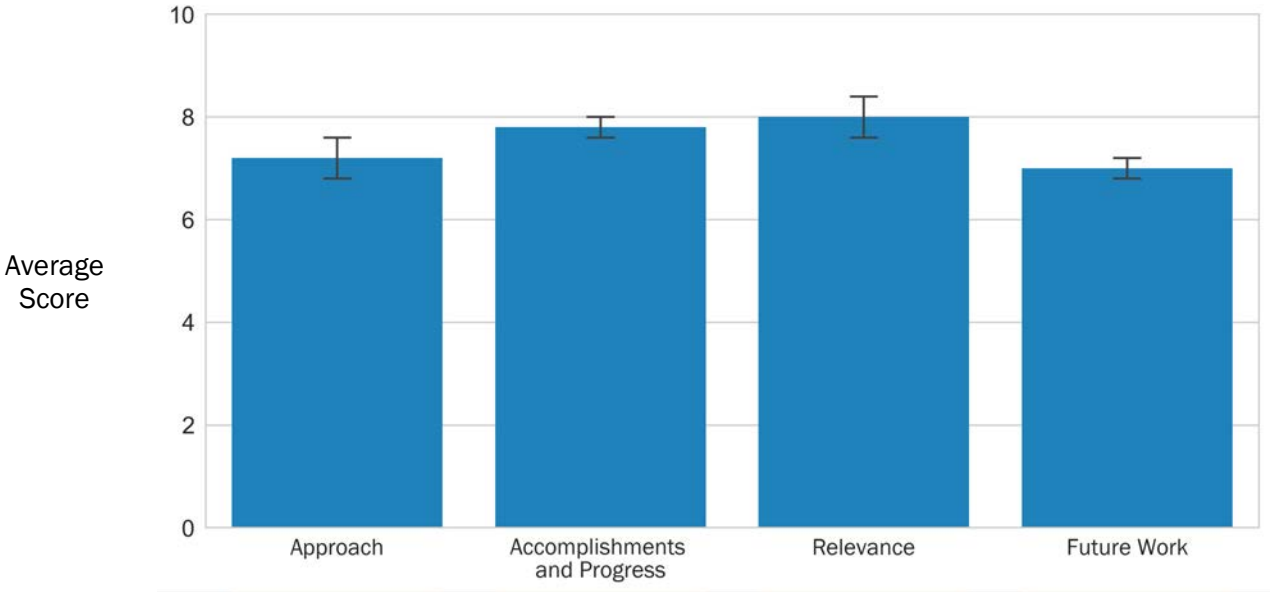
PROJECT DESCRIPTION

WBS:	2.3.2.105
CID:	NL0028598
Principal Investigator:	Dr. Jeff Linger
Period of Performance:	10/1/2015–9/30/2020
Total DOE Funding:	$6,781,256
DOE Funding FY16:	$1,800,000
DOE Funding FY17:	$1,800,000
DOE Funding FY18:	$2,031,256
DOE Funding FY19:	$1,150,000
Project Status:	Ongoing

The goal of the Biological Upgrading of Sugars project is to develop robust microbial strains to convert lignocellulosic sugars to fuel precursors at titer, rate, and yield targets set by TEA and which are fully integrated into a complete process to produce biofuels at the BETO 2022 goal of $2/GGE and future MYP targets. Our technical focus is on the anaerobic production of carboxylic acids using non-model bacteria and yeast that exhibit beneficial phenotypes for industrial bioprocesses. Carboxylic acids are readily catalytically upgraded to fuel molecules through known, well-studied chemo-catalytic transformations being developed in collaborating projects. Specifically, we focus on holistic process development and integration via collaborations with the BETO-funded Bioprocessing Separations Consortium (for separations of intermediates) and the Chemical Catalysis for Bioenergy Consortium (for catalytic upgrading of intermediates to hydrocarbon jet fuel and diesel), along with industrial and academic partners. Regarding carboxylic acids, we are primarily focused on the biological production of butyric acid. *Clostridium butyricum* and *Clostridium tyrobutyricum* are bacteria that naturally produce butyric acid from the principal sugars found in corn stover hydrolysate. In addition to native butyric-acid-producing bacteria, we are also exploring the use of acid-tolerant yeasts that demonstrate great potential for economically viable routes towards carboxylic acid production.

Weighted Project Score: 7.5

Weighting for Ongoing Projects: Approach - 25%; Accomplishments and Progress - 25%; Relevance - 25%; Future Work - 25%



I One standard deviation of reviewers' scores

OVERALL IMPRESSIONS

- In order to develop industrially relevant yeasts, the research team screened 32 strains against acidic, hydrolyzed feedstocks to find new acid-tolerant strains, and subsequent omics analysis identified a gene that might be responsible for acid tolerance. In parallel, the team has developed *Clostridium* strains capable of achieving high titers of organic acid via pertractive fermentation using mock hydrolysates. Demonstration of complete conversion (from hydrolysate to organic acids) has not yet been fully demonstrated for yeast but should be coming soon.
- The goal of this project is to produce butyric acid from cellulosic sugars, which can be catalytically converted to diesel and jet fuel. Two processes are being considered, one involving bacteria and the other involving yeast. Overall, the team is making excellent process with the bacterial route. Excellent titers and productivities are being achieved. Developing a process involving continuous distillation will be an important step for establishing feasibility and connecting with TEA. The route involving yeast is far less developed and does not integrate well with the bacterial work. It is also not clear whether these strains can produce butyric acid at high rates and titers. This work is still in the discovery phase. As such, this part of the project is not very compelling.
- Developing a robust production organism, whether bacterial or yeast, for production of 150 g/L butyrate is challenging. There are drawbacks for both systems, as well as challenges inherent in both. Focusing on one system would allow for more rapid progress. For all organic acid production, the cost associated with recovery of the acid should be considered in choosing the production strain. Based on that, the choice of organism can be different from an anaerobic *Clostridium*.
- This program develops microbial strains to convert cellulosic sugars to fuel molecule precursors. Due to input from the TEA performed by the integrated analysis team, the focus was shifted in the last two years from fatty acids to short-chain carboxylic acids, in particular butyric acid. They are currently evaluating two options, a yeast and bacterial strain. The bacterial (*Clostridium*) fermentation requires protractive fermentation to remove acid, while yeast can operate at low pH. However, the yeast strain tends to produce ethanol, does not readily take up C5 sugars, and is very sensitive to the organic acid products. Significant progress was made with strain and fermentation development of the bacterial process, and the pertractive fermentation gave reasonable titer, rate, and yield. Progress with the yeast strain has been slower, but they were able to achieve xylose utilization. Tolerance to the product remains a key challenge in yeast. If this is not solved soon, all resources should be shifted to the bacterial process.

RECIPIENT RESPONSE TO REVIEWER COMMENTS

- The reviewers have provided valuable guidance, which is well aligned with our own self-assessment of the project. When we pivoted from fatty-acid-based routes towards carboxylic routes, we opted to pursue two complementary approaches: (1) using top-candidate *Clostridium* strains, which natively produce butyric acid but have limited ability to grow at low pH; and (2) a higher-risk but potentially game-changing approach using acid-tolerant yeast, which can grow at low pH but do not natively produce carboxylic acids. TEA analysis suggests that the cost savings from employing bioprocesses with low-pH carboxylic acid production could be dramatic, thus we performed early work to determine the feasibility of this approach. While our top yeast strains are indeed tolerant to low pH (2.0), and to high concentrations of butyric acid (0.4 M) at neutral pH, the combination of high acid concentrations at low pH has thus far proven too harsh of conditions to present a viable path forward. Accordingly, once we publish this research, we will intensify efforts exclusively on the Clostridial platform, which has yielded promising results thus far. Notably, our lab-directed evolution campaign and multiomics data sets should enable development of these yeasts for production of a wide array of organic acids, and we have had discussions with commercial and academic entities related to future collaborations. Thus, while we will be focusing efforts on Clostridial production of butyric-acid-derived fuels moving forward, we still view this research pursuit as a worthwhile endeavor that will help move the field as a whole in a positive direction. Finally, regarding the comment that our targets of 150 g/L butyrate will be challenging, we agree that we still have some hurdles to overcome, but we have already demonstrated this titer via pertraction in a glucose medium with *Clostridium*. We are currently transitioning our process to hydrolysate and are ultimately aiming for even higher titers.

PET UPCYCLING – NREL

National Renewable Energy Laboratory

PROJECT DESCRIPTION

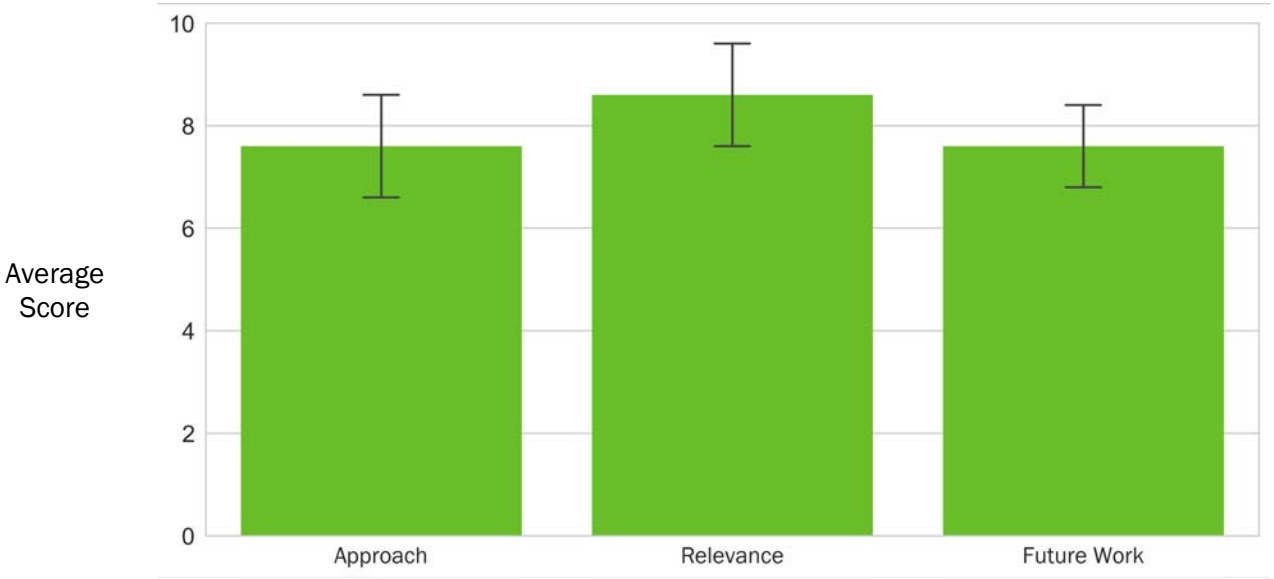
More than 300 million metric tonnes of plastics are produced each year. The volume of waste generated from plastics use is an enormous global problem, as plastics wreak massive environmental damage on terrestrial and aquatic ecosystems. Moreover, recycled products are most commonly lower in value than the original plastics due to a reduction in key material properties upon recycling, creating a disincentive for increased recycling rates relative to the use of virgin plastic materials. Accordingly, drastic changes in plastics recycling technologies will be essential to enable a more circular materials flow in plastics.

WBS:	2.3.2.109
CID:	NL0034397
Principal Investigator:	Dr. Gregg Beckham
Period of Performance:	10/1/2018–10/1/2020
Total DOE Funding:	$350,000
DOE Funding FY16:	$0
DOE Funding FY17:	$0
DOE Funding FY18:	$0
DOE Funding FY19:	$350,000
Project Status:	New

To that end, the aim of this project is to deliver cost-effective solutions to deconstruct and upcycle the massive plastic waste stream, polyethylene terephthalate (PET). PET is comprised of ethylene glycol (EG) and terephthalic acid (TPA) and is used for single-use beverage bottles, carpet, and other textiles. PET recycling rates are low around the world, and PET bottle recycling results in a loss in advantageous material properties, such that recycling is "downcycling." This work will leverage BETO investments in overcoming biomass recalcitrance, interfacial biocatalysis, metabolic engineering of aromatic-catabolic bacteria, and bioprocess development to ultimately enable the Circular Materials Economy for this plastic.

Weighted Project Score: 7.8

Weighting for New Projects: Approach - 25%; Relevance - 25%; Future Work - 50%



I One standard deviation of reviewers' scores

Specifically, we are targeting upcycling of PET carpet fibers as a nonrecyclable plastic. The challenges in PET biological or chemical deconstruction closely mirror that of cellulose depolymerization. Namely, the PET polymer is crystalline and is connected by C-O (ester) bonds that are difficult for (bio)catalysts to access. Like cellulose, there are natural enzymes (esterases) active for PET degradation to TPA and EG. Two potential strategies to upcycling are the enzymes that degrade PET, namely PETase and mono-2-hydroxyethyl terephthalate (MHET)ase, and chemo-catalytic solutions using easily recyclable catalysts (such as catalyzed glycolysis). We are pursuing two parallel, closely related aims to convert PET into higher-value chemical building blocks using a wholly biological strategy (Task 1) and a hybrid chemo-catalytic-biological strategy (Task 2). For both aims, we will leverage an engineered strain of *Pseudomonas putida* KT2440 that is able to readily consume EG, developed with BETO support in the Biological Conversion of Thermochemical Aqueous Streams project. We are actively engineering this strain to secrete two PET bis-hydroxyethyl terephthalate (BHET)-degrading enzymes, PETase and MHETase, and engineered variants of these enzymes. We are also engineering the baseline strain to consume the aromatic monomer TPA, first for growth and ultimately for conversion and upcycling to an exemplary performance-advantaged monomer, namely ß-ketoadipic acid, which is being researched heavily in the Performance-Advantaged Bioproducts mini-consortium. *P. putida* is an excellent chassis strain for both strategies because it already harbors genes for robust aromatic catabolism and for EG consumption, it is genetically tractable, both NREL and ORNL have extensive metabolic engineering experience with the strain, and it has previously been industrially scaled up for several applications by multiple companies. In addition, we are also pursuing enzyme discovery, evolution, and engineering. It is likely that more efficient enzymes exist in nature already, and they are waiting to be discovered and characterized. Working with colleagues at Montana State University and ORNL, we are prospecting existing metagenome libraries from various thermal and pH extrema across known microbiological samples. To date, we have already developed a set of putative PETases from these databases that are taken from thermal environments above the glass transition of PET, which represents an excellent starting point for enzyme screening. The purpose of this component of the project will be to discover more effective enzymes for PET degradation and BHET degradation, as well for the hybrid process.

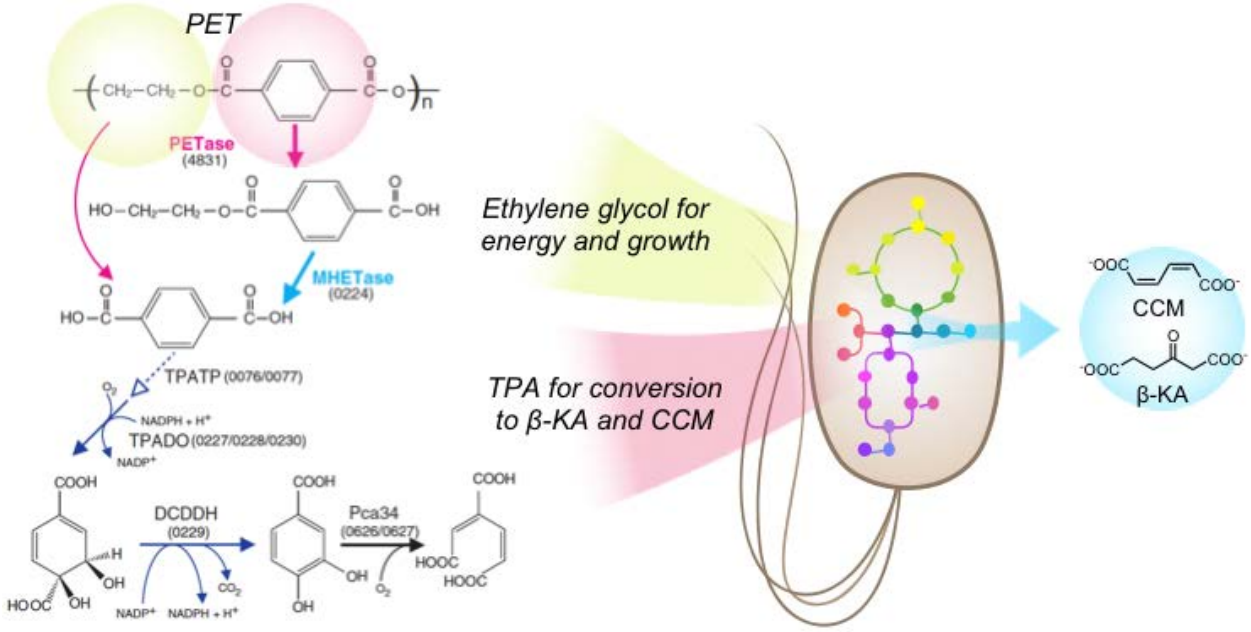


Photo courtesy of National Renewable Energy Laboratory

OVERALL IMPRESSIONS

- Overall relevance of the project to BETO and how the project plan can tackle the issue is not clear. There are a lot of technical challenges in the proposed work and the timing of the project might not be sufficient to address these challenges. But good start.

- This exciting new project seeks to leverage discoveries of PET-metabolizing microbes to develop processes for industrial-scale PET upcycling. Thus far, the team has demonstrated production and secretion of key enzymes in a *P. putida* host and has built genetic constructs for transport. If successful, this project will have a broad impact by converting plastic waste into a new feedstock for industrial production.

- The goal of this exploratory project is to develop processes for the upcycling of PET plastic. This project represents a new and promising direction for BETO. Overall, this is a fantastic project that is exploring both biological and chemical approaches. Many exciting ideas are proposed. The team is also outstanding. More importantly, it addresses a key environmental problem of concern to the general public.

- Overall, this a good project; while not a perfect fit for bioenergy sector, it is focused on using a biological approach to treat a recalcitrant material and in the process produce a chemical by bioconversion. Most short- to mid-term opportunities for chemical production can follow a similar approach and help establish bioconversion-based manufacturing as a viable industry while seeking greater deployment of plant-derived materials, as is the case for lignocellulosics.

- This project is aimed at breaking down PET and then using the resulting carbon monomers to make small molecules by fermentation and/or catalysis. Both chemical and biological processes will evaluate PET breakdown. The conversion to the exemplary product, ß-ketoadipate, will be done using *P. putida*, leveraging tools developed for engineering this organism. This is a very challenging program because it requires heterologous pathways to consume novel substrates, as well as producing a product. It should be considered high risk, high reward. However, this team is very experienced, and if anyone can be successful with it, they can. This will likely take many years to develop, but if successful could be revolutionary in the conversion of waste to value-added products.

RECIPIENT RESPONSE TO REVIEWER COMMENTS

- Plastics are a major component of municipal solid waste, which is relevant to the BETO portfolio. Moreover, many process and scientific concepts that are core elements of the BETO portfolio can be leveraged towards this key environmental problem, which is essentially the conversion of waste, polymeric, recalcitrant, low-value, diffusely available solids into small-molecule intermediates that can then be upcycled to higher-value materials. In terms of the timing, we agree that our milestones are aggressive, but we have assembled an excellent team of researchers from NREL and ORNL to tackle this problem and have been able to deliver and exceed our milestones thus far.

CONTINUOUS MEMBRANE-ASSISTED IBE FERMENTATION FROM AVAP CELLULOSIC SUGARS

American Process Inc.

PROJECT DESCRIPTION

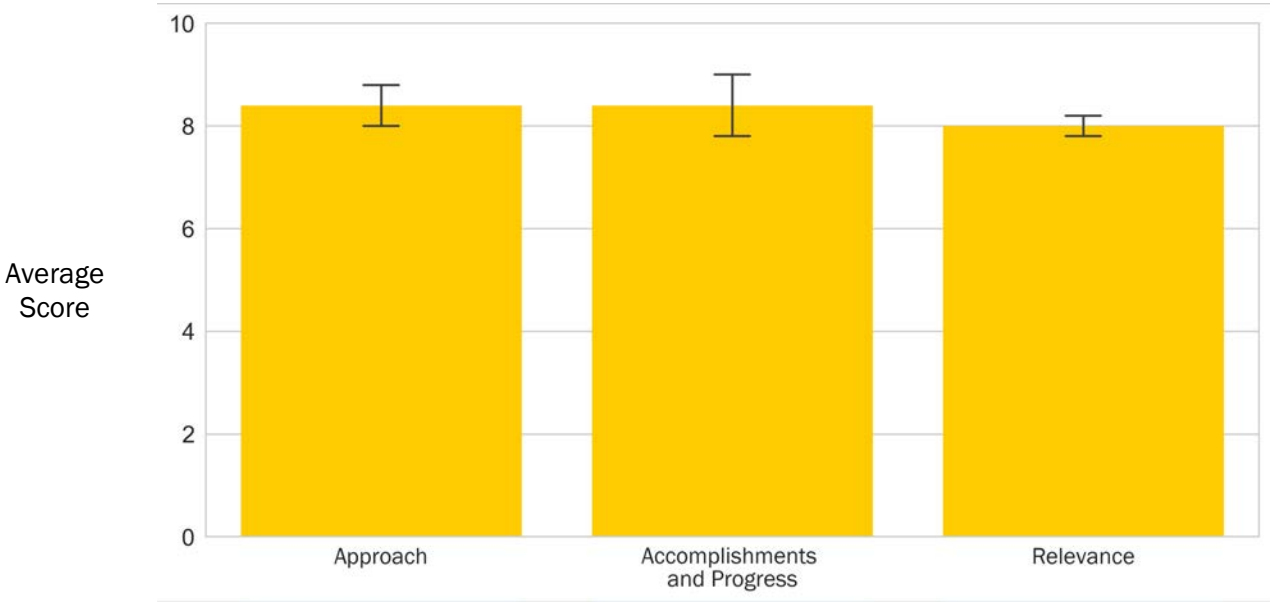
The process demonstrated in this project consisted of continuous membrane-assisted isopropanol, butanol and ethanol (IBE) fermentation of American Value Added Pulping (AVAP) cellulosic sugars and recovery of the IBE fuel mixture. The overall goal of the project was to demonstrate the process in an integrated pilot plant so as to collect data that demonstrates the ability to scale up production of IBE as a transportation fuel in an economical manner. It was further demonstrated that the targeted final MFSP of $3/GGE (corresponding to $2.27/gallon, or $745/ton of IBE) can be achieved in a plant using 1,200 bone dry (BD) tons per day of corn stover feed stock.

WBS:	2.3.2.202
CID:	EE0006879
Principal Investigator:	Dr. Vesa Pylkkanen
Period of Performance:	7/1/2015–3/31/2018
Total DOE Funding:	$3,088,632
Project Status:	Sunsetting

The proprietary AVAP pretreatment technology, based on fractionating biomass in a sulfur dioxide-ethanol-water fractionation was used to produce cellulosic and hemicellulosic sugars. A conditioning step was developed for the hemicellulosic sugars, which allowed the utilization of the xylose fraction to increase to 50%. The membrane-assisted recycle fermentation process developed increased the fermentation volumetric solvent productivity to 8.85 g/L/hr with an average titer of 14.75 g/L, and a yield of 0.36 g/g of utilized sugar. The production of isopropanol by genetic modification of *Clostridium acetobutylicum* was demonstrated for short periods.

Weighted Project Score: 8.3

Weighting for Sunsetting Projects: Approach - 25%; Accomplishments and Progress - 50%; Relevance - 25%



A novel liquid-liquid extraction technology was applied for the recovery of the alcohols from the fermentation broth. A butanol recovery of 90% was achieved at a low organic-to-aqueous ratio—as low as 0.4:1 during integrated operation. Reactive distillation to produce butyl butyrate extractant was also demonstrated.

Pinch analysis was conducted on the fundamental process to increase the energy efficiency though heat integration. Following the Pinch analysis, an LCA showed that equivalent CO_2 emission was about 85% lower than gasoline blend stock and 70% lower than the first-generation butanol from corn. The coproduct acetone-replacing petroleum equivalent generated a carbon credit that was larger than the emissions during IBE manufacture (e.g., a carbon-negative fuel).

The commercial plant scale-up was simulated using an American Process Inc. proprietary biorefinery simulator, apiMAX™, and was further optimized using value engineering and heat integration. The TEA established that in the base case, an MFSP for the IBE mixture of $4.88/gallon is necessary in order to obtain positive net present value (NPV) for the project. A number of realistic improvement assumptions were then superimposed in the base case scenario to develop an optimized case scenario. In the latter, a plant making about 57 kilotons per annum (kta) of IBE fuel and 22 kta of acetone coproduct from 420 BD kta of corn stover priced at $60/ton would have a positive NPV at an MFSP of $3/gallon of IBE. The NPV can be further significantly increased if the capital cost were further decreased through integration to an existing facility with infrastructure and boiler house.

OVERALL IMPRESSIONS

- The goal of this project is to develop a process to produce butanol from various domestic cellulosic feedstock sources with economics that do not require any subsidy. Continuous membrane-assisted fermentation and liquid-liquid extraction were novel methods used to increase productivity and remove butanol *in situ* so concentration remains low. The team engineered *Clostridium* to produce isopropanol instead of acetone for a higher-value coproduct. Acetone conversion to isopropanol was not as high as projected, so this requires more work on the strain. Productivity and yield goals were achieved, and most of the challenges associated with parallel fermentation of two sugar streams and butanol separation were solved at the lab scale. Overall, this was a very successful project. The process should be de-risked at a larger scale when possible.

- The goal of this project was to create a continuous IBE process using membrane separations. The team achieved most of their milestones. In these regards, the project was successful. The team is now looking to license their technology. Many innovative ideas were explored in this project; key among them was the use of reactive distillation to make the extractant for product separation. Much was also learned from project failures, such as the need for better biomass sensors, better foam control, and less process intensification, particularly at this stage of process development.

- The novelty of this project is that it combines in-depth process optimization with continuous membrane-assisted fermentation to attain up to 20-times increases in productivity of the generation of alcohols from cellulosic sugars. The process is enhanced by further introducing a low-temperature, recycling-friendly liquid-liquid extraction system, which could cut thermal energy use in half. The team has produced several compelling technical achievements, including getting the model system operational, demonstrating continuous fermentation, and nearly optimal extractant recovery, but the acetone-to-isopropanol conversion is still lagging and requires additional enhancement, which might include strain engineering.

- On the overall, the project progressed reasonably well in spite of the challenges of integrating fermentation, cell recycle, and solvent recovery. Improvement in solvent recovery in g/L/hr looks good for a continuous system and the 500 hours of continuous operations is a step in the right direction.

- The project was clear on technical and financial targets and designed project plans to test the targets. The projects worked both on upstream process improvement (fermentation) as well as downstream recovery and fractionation.

RECIPIENT RESPONSE TO REVIEWER COMMENTS

- The recipients choose not to respond to the reviewers' overall impressions of their project.

ENGINEERING *CLOSTRIDIA* FOR *N*-BUTANOL PRODUCTION FROM LIGNOCELLULOSIC BIOMASS AND CO_2

The Ohio State University

PROJECT DESCRIPTION

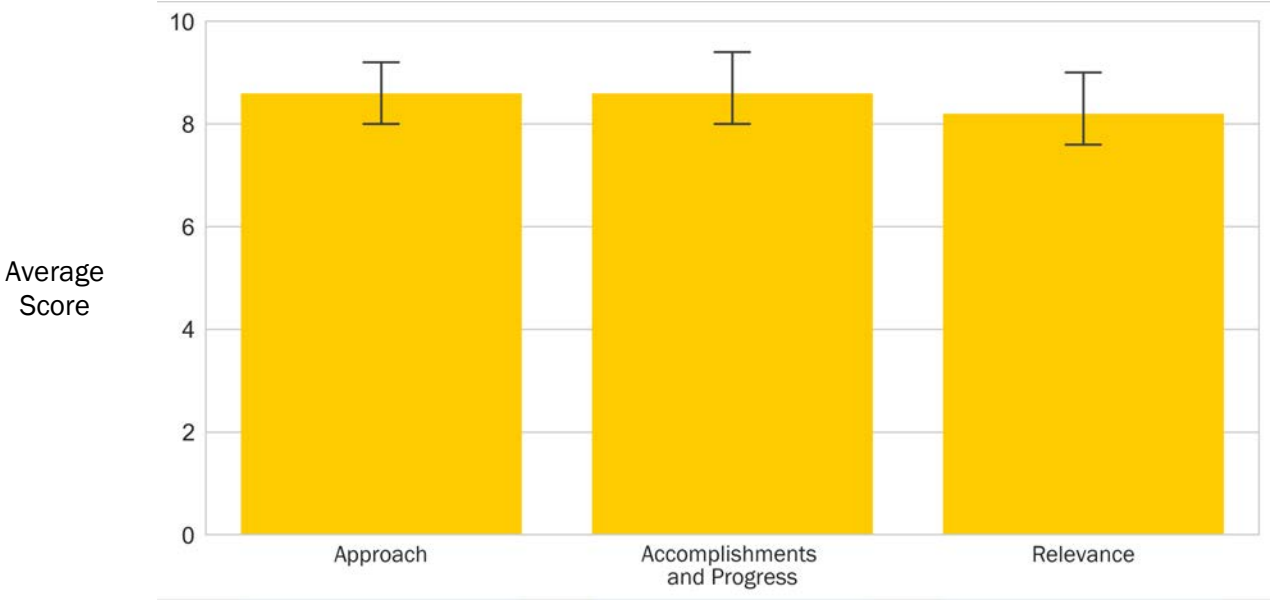
WBS:	2.3.2.203
CID:	EE0007005
Principal Investigator:	Dr. Shang-Tian Yang
Period of Performance:	10/1/2015–9/30/2018
Total DOE Funding:	$1,232,148
Project Status:	Sunsetting

The goal of this project was to develop engineered *Clostridia* strains and fermentation process that can directly utilize cellulose and fix CO_2 for *n*-butanol production from lignocellulosic biomass. To achieve this goal, the project included the following specific objectives: (1) engineering *Clostridia* for *n*-butanol production from cellulose and CO_2 hydrogen (H_2) (years 1 and 2); (2) fermentation kinetics studies and process optimization; (3) omic analysis of mutants in fermentation; and (4) process design and cost analysis.

More than 20 engineered strains of *Clostridium cellulovorans* and *Clostridium carboxidivorans* were generated and evaluated for butanol production from cellulose and CO_2 H_2. Among all engineered strains, *C. cellulovorans* co-expressing the gene for aldehyde–alcohol dehydrogenase (adhE2) with thl and hbd genes showed the greatest potential for butanol production from cellulose, with the highest butanol titer (5.5 g/L), yield (0.27 g/g), butanol-ethanol ratio (6.63 g/g), alcohol-acid ratio (1.32 g/g), and C4-C2 ratio (2.84 g/g) in batch fermentation with glucose and the addition of methyl viologen (MV). The strain overexpressing adhE2 and fnr genes produced 5.28 g/L butanol and 1.93 g/L ethanol from cellulose, with a high butanol yield of 0.33 g/g, alcohol-acid ratio of 5.30 g/g, and C4-C2 ratio of 2.35 g/g in batch fermentation with MV. These mutant strains meet our target of 5 g/L butanol with a yield of 0.3 g/g cellulose in batch fermentation. However, butanol production was inhibited by butanol and impaired by sporulation under butanol stress. It is thus

Weighted Project Score: 8.5

Weighting for Sunsetting Projects: Approach - 25%; Accomplishments and Progress - 50%; Relevance - 25%



I One standard deviation of reviewers' scores

necessary to further engineer the strain to improve its butanol tolerance. Metabolic flux analyses for selected mutants and fermentation conditions also suggested the necessity to further improve NADH availability and to knock down acid production. Comparative proteomics and metabolomics analyses, along with the fermentation kinetics data, provided the basis for developing a novel dynamic model, which can be used to guide further metabolic engineering strategies. Through further process and medium optimization, butanol production from lignocellulosic biomass and CO_2 H_2 would be economically viable, as indicated by the cost model developed for the consolidated bioprocess. A well-to-pump LCA shows that the integrated biobutanol production process can potentially reduce CO_2 and greenhouse gas (GHG) emissions by ~75% compared to a traditional chemical process.

Strain development via metabolic engineering and fermentation process optimization, assisted by omic analysis of the mutation and process effects, helped us to achieve our goal of producing *n*-butanol and ethanol (biofuel) from lignocellulosic biomass at a cost of $3/GGE or less. The co-fermentation technology using both cellulose and CO_2 H_2 for biofuel production can greatly increase product yield from the biomass feedstock while also reduce GHG emissions, and is novel and advantageous compared to existing ethanol and acetone-butanol-ethanol fermentation technologies.

OVERALL IMPRESSIONS

- This project is a collaboration with Green Biologics to improve butanol titer, rate, and yield using both strain engineering and process development. They converted an acetogenic *Clostridium* into an ethanol/butanol producer and used a novel approach to have a second strain to fix CO_2 using the Wood-Ljungdahl pathway. Using two organisms rather than putting both pathways in a single organism is a good approach, since the Wood-Ljungdahl pathway is very difficult to engineer into other organisms. Cocultures with organisms using different substrates should be stable, and results were demonstrated at lab scale. Industry involvement indicates there is confidence in scale-up ability of the process. Milestones were clearly defined, and most were accomplished.
- The goal of this project was to increase butanol production from cellulose using engineered strains of *Clostridia*. In addition, they also proposed to capture the CO_2 produced in the fermenters using homoacetogens. The team was able to increase butanol production and achieve most of their milestones. In addition, they were able to capture some of the CO_2 produced. However, they will need to supplement with hydrogen or potentially syngas. Overall, the project was successful. The TEA appears overly optimistic and would have benefited from external evaluation.
- This project sets a high bar for thorough strain development and process development for biobutanol production from lignocellulosic biomass. By merging gene mining, metabolic engineering, and omics/flux analysis, they demonstrate a coculture system capable of producing high levels of butanol. Their success appears to be aided by the employment of a coculture technique to instantaneously reclaim CO_2 produced during fermentation, although the evidence of this is indirect.
- On the overall strain development, work is good but not sufficient for commercial deployment, as there are several challenges. A critical review of this work and TEA would help in assessment and cost sensitivity analysis with side-by-side comparison to the current Green Biologics acetone-butanol-ethanol process.
- If the project continues, it would be great to see plans on further production cost reductions (profit margin is still low) and learn more about scalability and manufacturing success of the proposed process/strain.

RECIPIENT RESPONSE TO REVIEWER COMMENTS

- The recipients choose not to respond to the reviewers' overall impressions of their project.

PRODUCTION OF HIGH-PERFORMANCE LUBRICANTS FROM CELLULOSIC SUGAR

Cargill

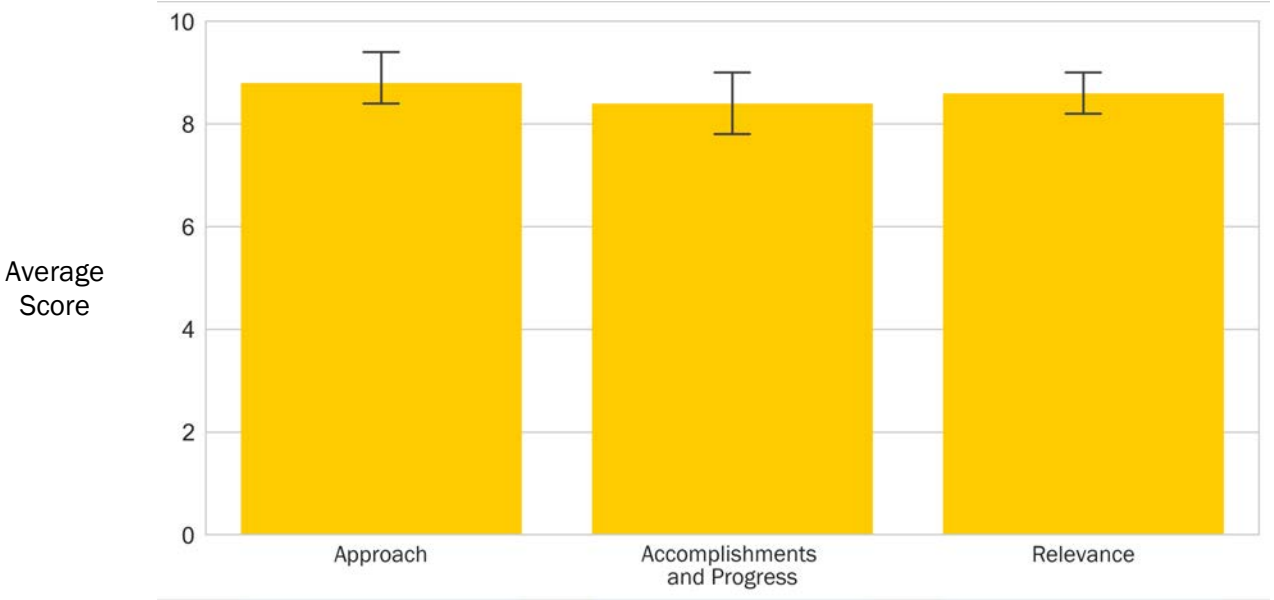
PROJECT DESCRIPTION

WBS:	2.3.2.204
CID:	EE0007007
Principal Investigator:	Dr. Tom McMullin
Period of Performance:	1/1/2016–11/14/2017
Total DOE Funding:	$2,000,000
Project Status:	Sunsetting

Certain classes of fatty acids are valuable raw materials that can be used to produce bio-based lubricants; however, their broader use has been limited by high cost and limited availability. This project was focused on producing a fatty acid methyl ester (FAME) of a specific chain length from cellulosic sugars. Development of a biological pathway and engineered organisms for production of low-cost FAMEs from cellulosic sugar is a novel and potentially disruptive technology that is supportive of BETO's mission to displace all fractions of a barrel of crude oil. The project was organized around four tasks that focus on (1) production pathway engineering, (2) host strain engineering, (3) assembly of a production pathway in the host strain, and (4) fermentation process development. Pathway engineering focused on discovery and improvement of two key pathway enzymes, namely 3-ketoacyl coenzyme A (CoA) synthase and wax ester synthase. Both enzymes were improved using a variety of enzyme engineering and mutagenesis approaches and assembled into a host strain with relevant additional pathway genes and genome modifications. Assembled strains were evaluated in two-liter benchtop fermenters using a commercially produced cellulosic hydrolysate to supply carbon source for FAME production. These evaluations demonstrated that performance targets and final milestones were met or exceeded for sugar uptake rate, product titer, product specificity, and production rate.

Weighted Project Score: 8.6

Weighting for Sunsetting Projects: Approach - 25%; Accomplishments and Progress - 50%; Relevance - 25%



I One standard deviation of reviewers' scores

OVERALL IMPRESSIONS

- The goal of this project was to produce lubricants from cellulosic sugars. The project was successfully completed. The technical approach was very impressive. The team exceeded their final milestones by 2.5-fold, which is outstanding. Future work is unfortunately on hold due to changes in the caprylic acid market and business priorities.

- Cargill has developed pathways in *E. coli* capable of processing hydrolysates to C8 caprylic fatty acids, which can be used in a variety of industries. Impressively, they paired screening of hundreds of natural enzymes with rational protein design to more than double the activity of the needed enzymes *in vivo*. It is also noteworthy that they pursued process development through to the fermentation process, successfully exceeding their production targets. The work is relevant to BETO in that it successfully transforms renewable biomass into a bioproduct with commercial viability.

- Overall, the research expands uses of a hydrolysate for production of lubricants of commercial value. It is hard to judge from the titers, rates, and yields, as well as the process parameters necessary for commercial scale-up production.

- Great progress even with lower-than-expected 3-ketoacyl-CoA synthase activity. It would be great to include TEA.

- The team is developing a process for producing caprylic methyl ester from cellulosic hydrolysate. The product and others of different chain lengths have a potential application as high performance lubricants that are biodegradable. The use of an acyl carrier protein-independent fatty acid pathway removes product synthesis from native fatty acid regulation and esterification with methanol reduces toxicity of the C8 fatty acid. The project is well structured with specialized functional teams, and goals are well defined. Much of the effort was focused on improving the pathway enzymes to achieve needed specific activity and chain specificity so cycle stops at C8. This was accomplished by a combination of natural enzyme screening, rational modifications, and random mutagenesis. With the improved enzymes combined with fermentation optimization, production targets were reached. Progress was made on cellulosic sugar utilization, but there was not full co-consumption of glucose and xylose.

RECIPIENT RESPONSE TO REVIEWER COMMENTS

- The recipients choose not to respond to the reviewers' overall impressions of their project.

SECOND-GENERATION MIXOTROPHY FOR HIGHEST YIELD AND LEAST EXPENSIVE BIOCHEMICAL PRODUCTION

White Dog Labs Inc.

PROJECT DESCRIPTION

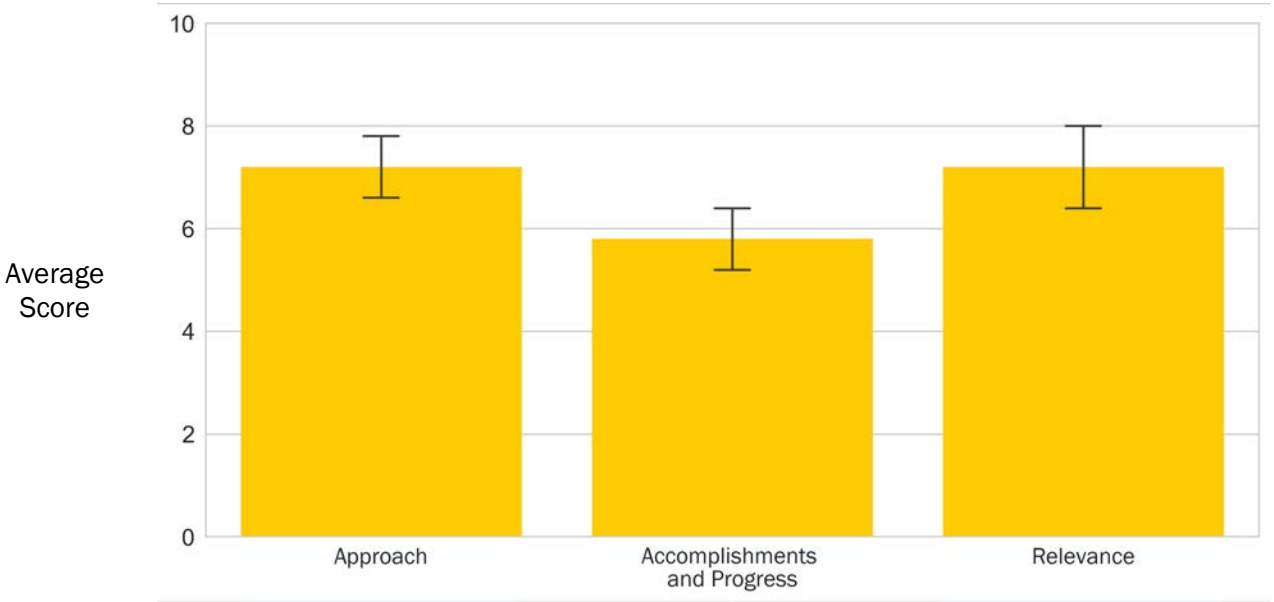
WBS:	2.3.2.205
CID:	EE0007564
Principal Investigator:	Dr. Shawn Jones
Period of Performance:	9/1/2016–12/31/2018
Total DOE Funding:	$1,539,826
Project Status:	Sunsetting

The primary economic driver for second-generation biochemical biofuel processes is feedstock cost and cost associated with the conversion to fermentable carbohydrates, and therefore it is crucial to maximize conversion of the feedstock into a product of interest. However, biomass (particularly sugar) is less reduced than the majority of biochemical products of interest, particularly non-oxygenated fuels and alcohols. Accordingly, fermentation processes must oxidize a portion of the biomass, thus releasing CO_2 and decreasing the potential mass yield of the product of interest. White Dog Labs Inc. is developing a technology to help mitigate this loss to CO_2 termed Anaerobic Non-Photosynthetic (ANP) Mixotrophy, or just Mixotrophic Fermentation. This technology consists of using specific microorganisms that can concurrently consume both a sugar feedstock and the CO_2 evolved, thus limiting the amount of CO_2 released from the process.

As a first demonstration of this technology, White Dog Labs is targeting acetone production, an important commodity chemical and a feedstock for poly(methyl methacrylate) (PMMA) production. Using traditional fermentation technology, the theoretical maximum conversion of sugar into acetone is only 32 wt %, thus wasting a significant fraction of the carbon feedstock. However, applying a Mixotrophic Fermentation process will increase the mass yield to 45 wt %, a similar yield to commercial ethanol facilities, which dramatically changes the economic outlook for a bio-acetone facility. Importantly, this is a technology that can be applied to

Weighted Project Score: 6.5

Weighting for Sunsetting Projects: Approach - 25%; Accomplishments and Progress - 50%; Relevance - 25%



I One standard deviation of reviewers' scores

a wide variety of biochemical biofuel molecules, making many of these molecules economically viable when produced through a biological route.

OVERALL IMPRESSIONS

- The goal of this project is to produce acetone from cellulosic sugars with increased mass yields. Increased yields would be achieved by capturing CO_2 produced during fermentation using acetogenic microorganisms. This is an interesting idea that, if successful, would greatly improve yields. Acetone was chosen as a proof-of-principle molecule, which is a good target given the organisms being explored. While the team was able to produce acetone using an engineered microorganism, they failed to meet their final milestones due to a number of technical problems. The project was technically ambitious, and the performers were unable to achieve their goals. Nonetheless, they developed a promising platform of organisms for future work.

- The team attempted to beat theoretical yield maximums by recovering CO_2 during fermentation. Data from plasmid-transformed strains indicate that the approach has promise, but unfortunately difficulties associated with integration into the genome prevented the team from realizing a strain to demonstrate industrial relevance.

- This project is technically and commercially challenging. Acetone is a commodity chemical and is produced commercially. The fermentation route requiring engineering of a *Clostridium* strain that co-metabolizes C5/C6 sugars in hydrolysates to produce commercially viable quantities is a major undertaking. Green Biologics is already producing acetone by acetone-butanol-ethanol fermentation. The advantage of Mixotrophic Fermentation resides in combining aspects of both conventional fermentation and gas fermentation into a single microbe. The microbes can fix CO_2 produced during catabolism of sugar to produce more product, thereby increasing carbon yields. This assumption is valid for a robust strain with high titer, rates, and yields that can be commercially competitive with other routes. It is not clear the limit of tolerance of the selected strain to acetone.

- It seems the project is continuing under a different program. If that is the case, PIs are encouraged to address the remaining issues in this project and complete evaluations/tests of the integrated system.

- The Mixotrophic Fermentation platform technology utilizes the CO_2 produced during glucose catabolism to increase product yield. The team constructed an acetogen that could consume all C5 and C6 sugars and evolved to cellulosic hydrolysates. However, this mutant was not readily transformable. Growth of the organism is slow, so a cell retention system was used to maximize productivity. After solving problems with membrane fouling, the team got stable continuous operation of the bioreactor, but production was low. They did not reach fermentation titer and yield goals, but progress was demonstrated. Overall, most of the individual challenges were addressed but could not be combined in a single strain. The concept is a great idea, though acetone may not be the most profitable target product. Given the challenges so far, it is going to be difficult to reach commercial metrics.

RECIPIENT RESPONSE TO REVIEWER COMMENTS

- The recipients choose not to respond to the reviewers' overall impressions of their project.

FERMENTATIVE PRODUCTION OF TRICARBOXYLIC ACID CYCLE-DERIVED CHEMICALS USING CELLULOSIC SUGARS

Lygos, Inc.

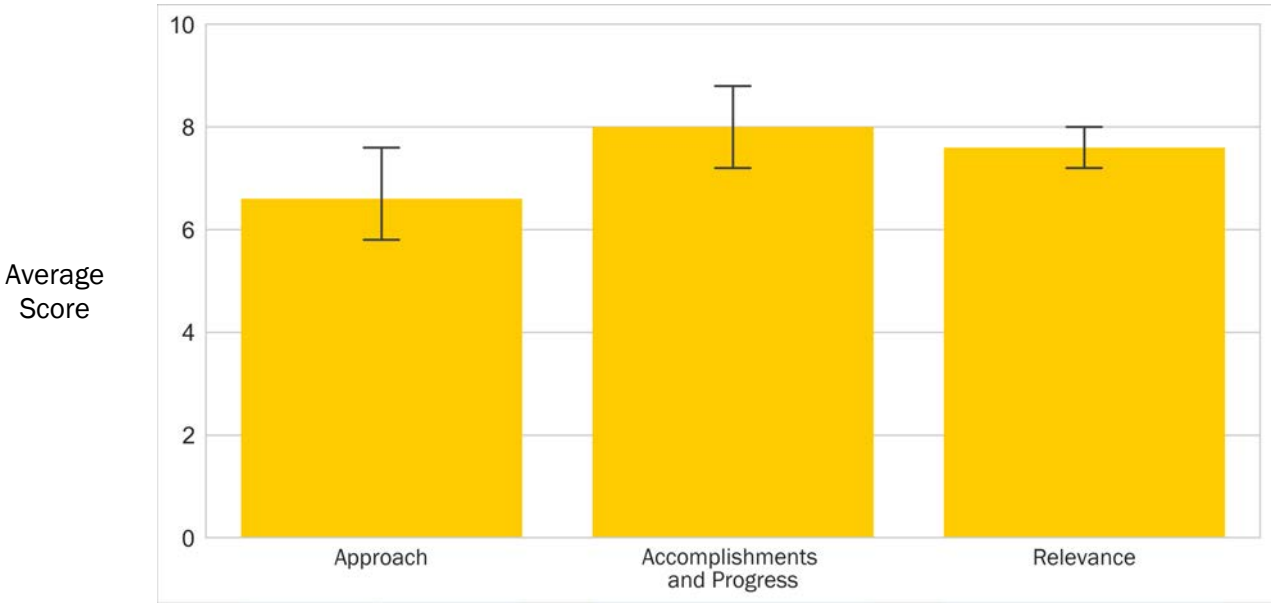
PROJECT DESCRIPTION

WBS:	2.3.2.206
CID:	EE0007565
Principal Investigator:	Dr. Jeffrey Dietrich
Period of Performance:	10/1/2016–1/1/2019
Total DOE Funding:	$1,709,464
Project Status:	Sunsetting

Lygos, Inc. has demonstrated the fermentative production of a tricarboxylic acid cycle-derived organic acid product from cellulosic glucose. Furthermore, we also demonstrated the ability to separate and purify this product from the fermentation broth. More recently, we made significant strides in the area of strain engineering and process optimization, as evident by the steady improvements in the overall production metrics from our engineered production host. Currently, successful scale-up is at the forefront of our engineering efforts to ensure that we are on a track to achieve the techno-economics that support cost-effective production from cellulosic sugar at commercial scales.

Weighted Project Score: 7.5

Weighting for Sunsetting Projects: Approach - 25%; Accomplishments and Progress - 50%; Relevance - 25%



I One standard deviation of reviewers' scores

OVERALL IMPRESSIONS

- The goal of the project is to produce aspartic acid from cellulosic glucose. The motivation for producing aspartic acid is that it can potentially be produced at high yields from glucose, in part by capturing the CO_2 produced during glycolysis. Initial work was done in *Pichia* yeast; the initial results were not promising. The team then switched to *C. glutamicum*, which is an industrial amino-acid-producing strain. The team was able to achieve target yields and titers using engineered *C. glutamicum*. Process was demonstrated at the two-liter scale. Overall, the team is on track to meet all of its project milestones. The project would benefit from TEA and comparison to existing amino acid processes given that a lot of work has already been done in this area.

- Lygos, Inc. has demonstrated a novel design for production of aspartate from TCA (via aspartate dehydrogenase) and achieved all performance targets. Lack of TEA makes it hard to judge the broader

- Great outcomes, clear plan, and clear progress. It would be great if the PIs can do a TEA to estimate cost of production, and if that would be a feasible process.

- This project is intended to develop a full process from cellulosic sugars to aspartic acid. The pathway is redox balanced and high yielding (2:1 molar from glucose), so it is a good choice for a low-cost, carbon-efficient process. Originally a *Pichia* strain was chosen, but this did not transport the product well, so they switched to *C. glutamicum*, which is well suited for amino acid production. All milestones were met, indicating that the pathway is de-risked. However, only the C6 stream of the cellulosic feedstock was utilized. It is also not clear how their work compares to the current state of the art in the amino acid industry.

RECIPIENT RESPONSE TO REVIEWER COMMENTS

- The recipients choose not to respond to the reviewers' overall impressions of their project.

INTEGRATED PROCESS FOR COMMERCIAL PRODUCTION OF FARNESENE, A VERSATILE PLATFORM CHEMICAL, FROM DOMESTIC LIGNOCELLULOSIC FEEDSTOCK

Amyris, Inc.

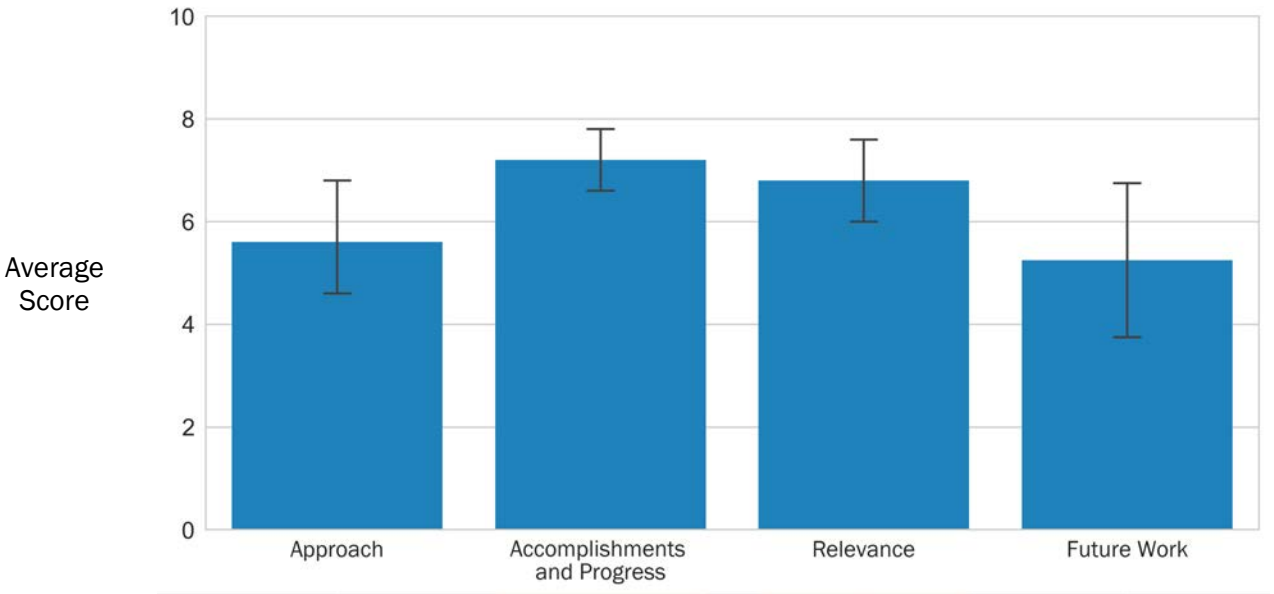
PROJECT DESCRIPTION

WBS:	2.3.2.207
CID:	EE0007729
Principal Investigator:	Dr. Joel Cherry
Period of Performance:	10/1/2016–12/31/2019
Total DOE Funding:	$7,000,000
Project Status:	Ongoing

The goal of this project has been to develop an engineered yeast strain and a scalable, lignocellulosic-based manufacturing process for the production of farnesene for fuel and bioproducts from woody feedstocks. This work has been carried out by Amyris, Renmatix, and Total, three commercial entities with complementary capabilities. Renmatix has employed its Plantrose® process using supercritical hydrolysis to fractionate hemicellulosic and cellulosic sugars from pine. Amyris has developed yeast biocatalysts and fermentation processes for conversion of Renmatix's cellulosic sugars into farnesene that is of equal quality to that produced from cane syrup feedstocks. This involved engineering a farnesene manufacturing strain to consume the xylose and to be resistant to growth inhibitors present in biomass-derived sugars. Total has conducted a thorough engineering study and TEA to provide production cost estimates and has developed a rigorous LCA to assess environmental impact. The final project goal was to develop a manufacturing-ready process to produce farnesene from cellulosic sugar in the United States for a manufacturing cost of $2/L. Such a production cost would allow immediate access to existing farnesene markets.

Weighted Project Score: 6.2

Weighting for Ongoing Projects: Approach - 25%; Accomplishments and Progress - 25%; Relevance - 25%; Future Work - 25%



I One standard deviation of reviewers' scores

Progress on the project has been very positive, but final cost targets now appear impractical. Amyris biocatalyst improvements have met milestone targets for xylose consumption, cellulosic hydrolysate tolerance, and consumption of organic acid growth inhibitors present in hydrolysates. Amyris has also adapted their fermentation processes for non-syrup feedstocks, and developed technologies for the analytical measurement and biological assessment of hydrolysate components. Renmatix has developed a viable and scalable strategy for fractionation of their sugar streams in case further purification is still required after biocatalyst improvement. Total has modeled a fully integrated U.S. manufacturing plant to assess reductions in carbon emissions and overall production costs. This last activity reveals unanticipated CAPEX costs that make final cost targets infeasible for now.

OVERALL IMPRESSIONS

- The goal of the project was to produce farnesene from lignocellulosic sugars. This work builds on Amyris's technology for producing farnesene from cane syrup. Farnesene is a promising target as it has many applications, from fuels to cosmetics. The strength of the project was that the milestones include realistic cost targets. The major challenge was that engineered strains did not grow well due to inhibitors in hydrolysates. The team was able to generate a strain that could partially tolerate inhibitors in hydrolysates. The project failed to achieve its objectives and was terminated early, mostly due to high capital costs associated with a planned process. That said, the project looked promising, and not all good ideas work. This work, though unsuccessful, may provide a useful benchmark for other processes with regards to commercial feasibility.

- This project revealed several important insights concerning technical risk in switching feedstocks on a highly engineered industrial production strain. After establishing growth inhibition, assays helped pin down organic acids as the major contributors, and liquid chromatography–mass spectrometry was used to identify specific toxins. This information was successfully used to guide the engineering of strains containing workaround pathways. This data-driven strain engineering was impressive, but it is unfortunate that the TEA estimates that ultimately triggered a "no-go" for the project were not performed at an earlier project phase.

- The assumptions used for the cost of production of farnesene were not sufficiently detailed to show cost sensitivity from higher OPEX and CAPEX for the added unit operations that would be needed for processing of pine hydrolysate and waste treatment required for a stand-alone facility. A more rigorous TEA at the outset may have helped in determining targets for commercial viability.

- Knowing when to kill a project or put a pause on it is as valuable as having a successful outcome. It is great that the PIs are making the decision to stop the project due to the high production costs.

- Amyris has a well-established process for producing farnesene from pure sugars, and the objective of this program is to adapt this process to lignocellulosic feedstocks. One of the main challenges is sensitivity of the strain to impurities in the feedstock. It appears that the magnitude of this problem, compared to conventional ethanol yeast, was not fully appreciated at the start. This issue was partially solved, but farnesene productivity suffered as a result. Both TEA and evaluation of tolerance should have been done much earlier in the project, before a significant amount of money was spent to continue. Although a go-no-go milestone on process economics was not reached, it is doubtful the strain performance metrics would have been reached by the end of the project. However, a number of accomplishments could have general applicability:

    - Characterization of biomass inhibitors
    - Synthetic inhibitor mix to match hydrolysis inhibition
    - Engineering inhibitor consumption into the yeast strain.

The reason economics did not work out was high cost of Outside Battery Limits (OSBL) infrastructure, so a possible solution is to co-locate multiple bioprocessing plants at the same facility. However, this result is rather surprising because it wouldn't seem that OSBL costs should vary too much across all the biochemical conversion projects. Thus, it is suggested that the team works with the biochemical platform analysis team at NREL to check assumptions compared to those used for evaluation of internal projects.

RECIPIENT RESPONSE TO REVIEWER COMMENTS

- The recipients choose not to respond to the reviewers' overall impressions of their project.

IMPROVING TOLERANCE OF YEAST TO LIGNOCELLULOSE-DERIVED FEEDSTOCKS AND PRODUCTS

Massachusetts Institute of Technology

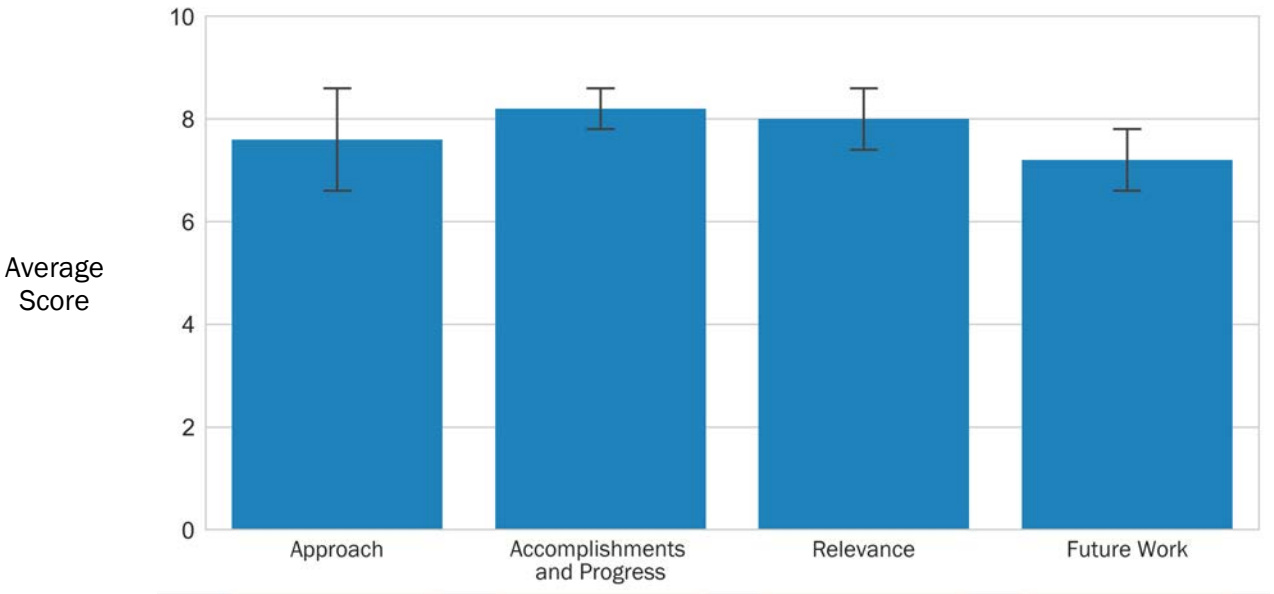
PROJECT DESCRIPTION

WBS:	2.3.2.208
CID:	EE0007531
Principal Investigator:	Dr. Greg Stephanopoulos
Period of Performance:	10/1/2016–9/30/2019
Total DOE Funding:	$1,500,000
Project Status:	Ongoing

Combined substrate-product toxicity in microbes is one of the major constraints hampering the scale-up and economic competitiveness of bioprocesses based on lignocellulosic feedstocks. Hydrolytic pretreatments of biomass release numerous compounds impinging on cell viability. The three most significant to yeast (the industry-dominant biocatalyst), and common to all plant sources, are furfural, hydroxymethylfurfural, and acetic acid. Furthermore, the desired end product, such as ethanol, typically attacks a multitude of host cellular functions via mechanisms yet to be fully understood. We propose to enhance lignocellulosic fermentations in yeast using nutrient adjustments proven previously to boost alcohol tolerance combined with genetic modifications aimed at alleviating hydrolysate toxicity. Thus far, we have systematically characterized the component toxicities in biomass hydrolysates and quantified their relative impacts on ethanol production. Currently, we are engineering tolerance to these inhibitory compounds using strategies combining enzymatic detoxification with viability-enhancing adjustments of the fermentation medium. Ultimately, we will assess the extent to which these tolerance methods are transferrable beyond ethanol, specifically to yeast processes synthesizing the antifreeze precursor monoethylene glycol.

Weighted Project Score: 7.8

Weighting for Ongoing Projects: Approach - 25%; Accomplishments and Progress - 25%; Relevance - 25%; Future Work - 25%



I One standard deviation of reviewers' scores

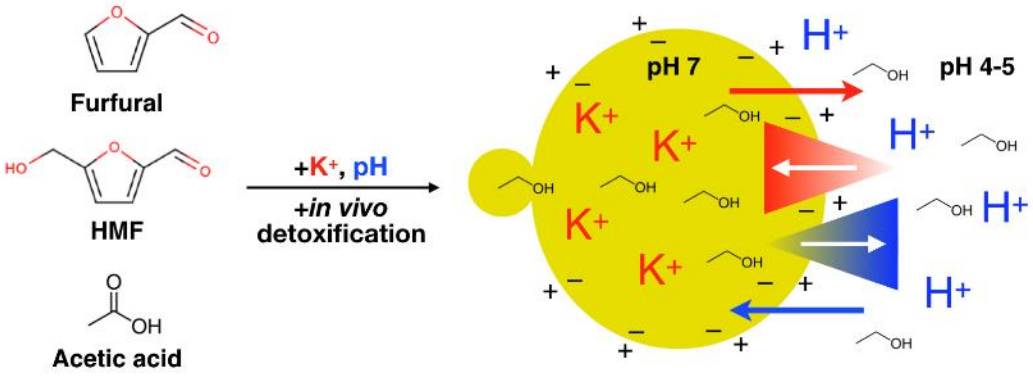


Photo courtesy of Massachusetts Institute of Technology

OVERALL IMPRESSIONS

- The goal of this project is to improve the tolerance of yeast to inhibitors to lignocellulosic hydrolysate. The team is making excellent progress. The project would be strengthened by the inclusion of competitive benchmarks, as similar work has been completed by many other groups. The team also needs to use real hydrolysates rather than synthetic ones, which they readily acknowledge. Finally, they also need to consider hydrolysate variability, which would improve the impact of the work.

- This team has made compelling progress toward engineering hydrolysate tolerance into *S. cerevisiae* via rational, stepwise metabolic engineering. To do this, they have established a system that uses media with defined composition of inhibitors that appear in feedstocks and have shown nearly complete recovery of ethanol production with their engineered strains. The resulting yeasts have the potential to provide a bridge between benchtop research and platform production systems grown on cellulosic biomass. The group has also demonstrated monoethylene glycol production in yeast and aims to combine these efforts to show production in the presence of inhibitors.

- This project aimed for the development of industrial yeast strains with increased inhibitor tolerance for second generation that can ferment both C5/C6 from biomass hydrolysate. The project also aims to engineer a yeast strain for the production of monoethylene glycol from xylose.

- The project provided sound technical approach, and the described technical approach presented the accomplishments so far and was clear on future work. Adding a go-no-go and more details into the percentage of work done for the last two remaining tasks would make the project even more clear.

- This project aims to engineer *S. cerevisiae* to produce ethylene glycol, then introduce lignocellulosic inhibitor tolerance. Tolerance is assessed by the ability to produce the product in fermentation using hydrolysate, and the targets are based on product titer from this substrate. The product pathway has been introduced to produce ethylene glycol from xylose, and 4 g/L were obtained. The team has also made good progress evaluating toxicity mechanisms and found that nonproductive cells were still alive. Improving tolerance has been the topic of numerous past studies, and some strategies discussed here have already been evaluated (e.g., drug efflux pumps, conversion of aldehydes to alcohols). The results presented here so far show improvement over wild type, but it is unclear how it compares to the current state of technology.

RECIPIENT RESPONSE TO REVIEWER COMMENTS

- Many thanks to the reviewers for the overall positive feedback and insightful comments. Spurred on by multiple suggestions to validate our approach on real hydrolysates, we are working to secure genuine pretreated biomass through our various BETO contacts. Furthermore, given the relatively high tolerances we have observed with our simulated feedstocks, we are hoping to ultimately source a variety of samples in order to evaluate the robustness of our strains to inhibitor variability. Regarding how our efforts compare to the current state of the art, we acknowledge that the lack of benchmarking against commercial strains is a weakness. However, it is one that remains difficult to address given the proprietary nature of these materials. While we continue seeking the partnerships to gain access to these reagents, the production numbers published in the literature continue to serve as our metric. Lastly, to our knowledge, our plans to make cellulosic monoethylene glycol will continue to be the first demonstration of a non-ethanol product to be fermented directly from un-detoxified hydrolysates.

ENGINEERING THERMOPHILES TO PRODUCE DROP-IN FUELS FROM SYNGAS

Kiverdi

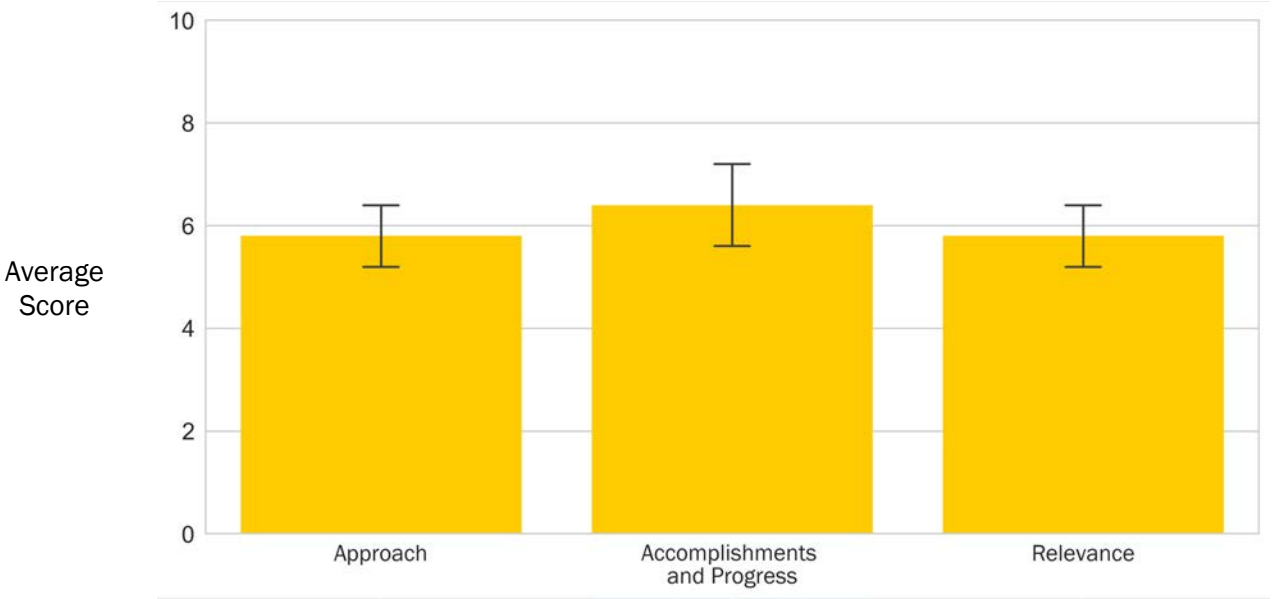
PROJECT DESCRIPTION

Develop recombinant thermophilic microbial strains capable of metabolizing syngas (carbon monoxide, CO_2, hydrogen) into monoterpenes to enable industrial production of fuels and solvents.

WBS:	2.3.4.204
CID:	EE0007008
Principal Investigator:	Dr. John Reed
Period of Performance:	9/1/2015–6/30/2018
Total DOE Funding:	$886,322
Project Status:	Sunsetting

Weighted Project Score: 6.1

Weighting for Sunsetting Projects: Approach - 25%; Accomplishments and Progress - 50%; Relevance - 25%



I One standard deviation of reviewers' scores

OVERALL IMPRESSIONS

- The goal of this project was to develop a thermophilic microorganism capable of converting syngas to monoterpenes. The team was not able to achieve the goals of this ambitious project. They did accomplish most of their intermediate goals. The team was able to produce terpenes from syngas using mesophiles, though not at target titers. Progress was stymied, in part, due to initial strain choice, which did not use syngas despite claims in the literature.

- This team sought to bring monoterpene biosynthesis online from syngas in a new hyperthermophile. The team was able to identify a new production host and to engineer in genes for monoterpene biosynthesis, as well as establish analytical methods for detection. Product was not observed, but the genetic design was validated in a separate mesophilic host. Relevance of targeting hyperthermophiles was not clearly explained, but bioconversion of syngas has clear implications for BETO.

- The basic premise for this project was to establish the growth of thermophilic mixotrophic green non-sulfur bacterium selected on syngas for the production of drop-in fuels from syngas.

- Presentation (with its current format) seems incomplete without clear flow, in a way that makes it look like more information is needed to assess this project. One main comment for the PIs—besides from presentation—is to comment on the scalability and relevance of the Air-Lift Syngas Bioreactor.

- This project is a collaboration between Kiverdi and NREL to develop a thermophilic microbial strain to convert syngas into monoterpenes. Challenges are obtaining a terpene synthase that can function at high temperature, development of genetic tools for the thermophilic strain, and the high demand of adenosine triphosphate for terpene synthesis. The originally chosen thermophile was not able to consume carbon monoxide, so much of the project focused on identifying a new strain that could consume syngas and was transformable. A suitable strain was found, as was a thermostable monoterpene synthase, but production was not obtained. Instead, terpenes were produced from syngas in a mesophilic organism. Anyway, the advantages of using a thermophilic strain are not really convincing.

RECIPIENT RESPONSE TO REVIEWER COMMENTS

- The recipients choose not to respond to the reviewers' overall impressions of their project.

DEVELOPMENT OF A SUSTAINABLE GREEN CHEMISTRY PLATFORM FOR PRODUCTION OF ACETONE AND DOWNSTREAM DROP-IN FUEL AND COMMODITY PRODUCTS DIRECTLY FROM BIOMASS SYNGAS VIA A NOVEL ENERGY-CONSERVING ROUTE

LanzaTech, Inc.

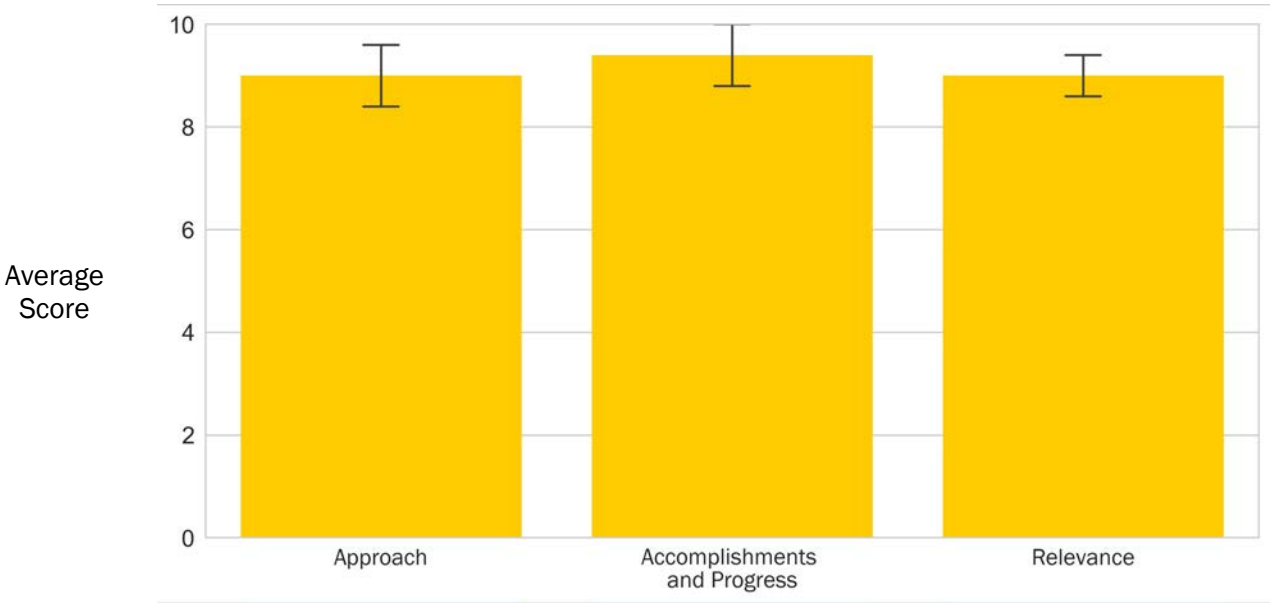
PROJECT DESCRIPTION

LanzaTech and ORNL have developed and scaled up a process for sustainable production of acetone and downstream drop-in fuel and commodity products directly from biomass syngas via a novel energy-conserving route in engineered acetogenic bacteria. This process offers a safer and more environmentally friendly production method for acetone production than the current phenol-dependent method, and the product has significantly lower GHG emissions. The developed process offers a cost-competitive route to acetone and enables biofuels at or below DOE's $3/GGE target. In addition, it also provides an attractive biological alternative to traditional sugar-based acetone-butanol-ethanol fermentation by enabling utilization of non-food biomass resources as fermentation feedstocks.

WBS:	2.3.4.205
CID:	EE0007566
Principal Investigator:	Dr. Michael Koepke
Period of Performance:	10/1/2016–9/30/2018
Total DOE Funding:	$736,544
Project Status:	Sunsetting

Weighted Project Score: 9.2

Weighting for Sunsetting Projects: Approach - 25%; Accomplishments and Progress - 50%; Relevance - 25%



I One standard deviation of reviewers' scores

Challenges overcome:

- **Byproducts formation:** Byproduct 2,3-butanediol and 3-hydroxybutyrate reduce yield and stability. Addressed by elimination of both pathways in our chassis acetone production strain.
- **Cost competitiveness:** Addressed by developing an integrated acetone strain and eliminating byproducts to increase yield and stability. Optimized co-selectivity and co-productivity of acetone and ethanol instead of titer, based on TEA.
- **Limited enzyme variety:** Addressed by genome mining of over 300 industrial strains in the LanzaTech collection, identifying unique enzyme sequences, and refactoring these unique enzyme variants through LanzaTech's engineering platform. Resulted in over 10-times improvement in acetone production from gas.
- **Continuous process (stability):** Identified bottleneck through detailed omics studies, addressed by integration of the acetone pathway on the chromosome.
- **Novel process to scale-up:** Addressed by demonstrating stable acetone production in 80-L pilot reactor, with acetone productivity and selectivity comparable to those observed under the same condition in 2-L reactors.

We have demonstrated stable acetone production for over seven days at commercial target rate and selectivity in 2-L reactors and have piloted the process.

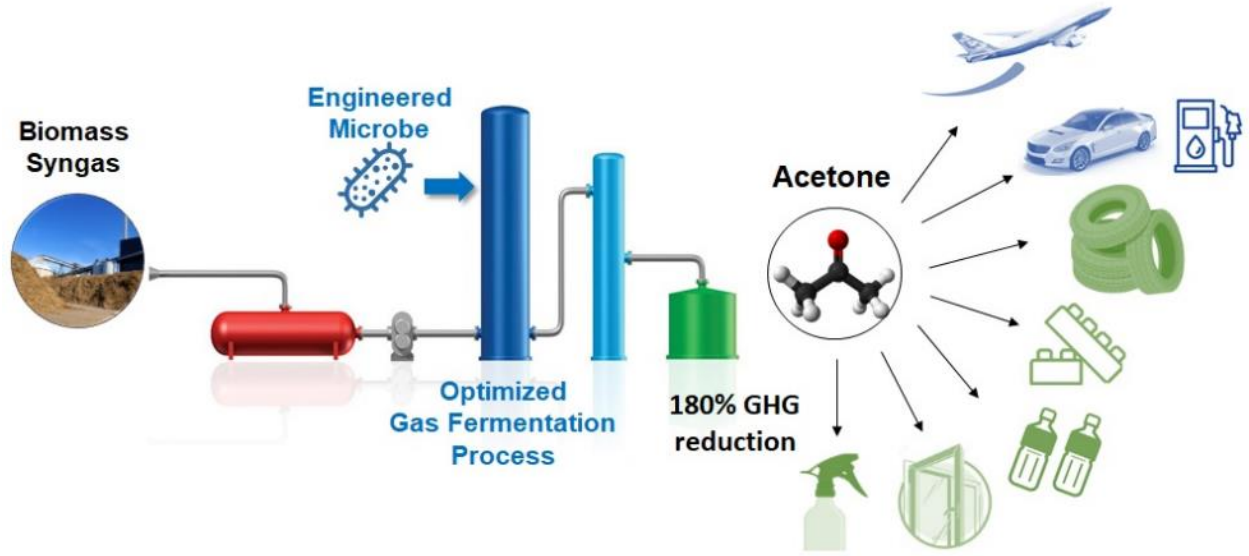


Photo courtesy of LanzaTech, Inc.

OVERALL IMPRESSIONS

- The goal of the project was to convert syngas to acetone using a gas-based fermentation process. This works builds on LanzaTech's syngas-to-ethanol process. The team was able to complete all of their technical milestones. A key milestone was engineering a strain with >10-times acetone production. Another key milestone was maintaining acetone productivity during 25-day continuous operations. In

addition, they were able to scale the process up to 80 L. Overall, the project was a success and lays the foundation for a commercial bio-based acetone production process.

- The team has demonstrated a commercially viable system for the production of acetone from syngas.
- An interesting approach to engineering a *Clostridium* strain for acetone production from syngases. There is a misconception about biomass feedstock pricing, supply, and costs. A viable biomass-to-chemical industry will compete as any other commodity in an open market that cannot be completely decoupled from fossil-fuel prices. A robust sensitivity analysis will also reflect this uncertainty.
- Great project that took a concept from lab scale to full product development and a great selection of partner and project allocation between the teams. It was great to see economic/cost analysis as well. Well done.
- This project is intended to develop a process for acetone production from syngas in a continuous bioreactor. An acetone pathway was introduced into an acetogen, then the strain and process were optimized to increase flux. Through a combination of pathway improvement, host modification, and process optimization, the goal of maintaining continuous production was achieved. Good use of modeling and omics analysis. Project is complete, and commercialization is planned. However, as there were no goals defined for rate or yield, it is hard to tell how far they are from commercialization.

RECIPIENT RESPONSE TO REVIEWER COMMENTS

- We appreciate the reviewers' concerns. Gasification is able to access biomass on the far end of the supply curve and thus access lower-priced feedstock. There is substantial analysis that negative externalities have been heavily discounted by society in the domain of energy production and use, which undervalues some alternative pathways, like the ones in the project. A competitive market would appropriately price all externalities (positive and negative).
- At the end of this project, we had acetone and ethanol co-productivity and co-selectivity within 5% of the commercial target goal. Exact rate, titer, selectivity, and yield are stated in the progress and public reports (nonpublic information).

BIOSYNGAS TO FATTY ALCOHOLS (C6–C14) AS A PATHWAY TO FUELS

Dow Chemical Company

PROJECT DESCRIPTION

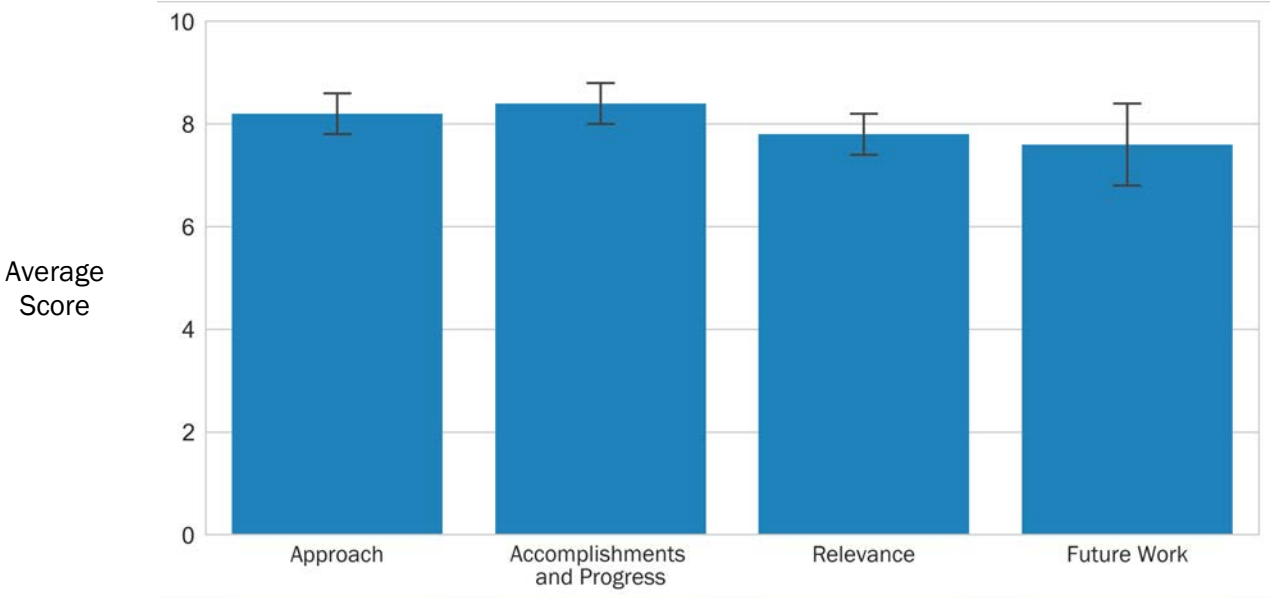
WBS:	2.3.4.207
CID:	EE0007728
Principal Investigator:	Dr. Devon Rosenfeld
Period of Performance:	1/1/2017–5/31/2019
Total DOE Funding:	$1,988,690
Project Status:	Ongoing

The Dow Chemical Company, in partnership with LanzaTech and Northwestern University, is developing a process for the bioconversion of biomass-derived syngas to fatty alcohols as a pathway to biofuels. The fermentation of biosyngas from lignocellulosic biomass will decouple the biofuel supply chain from the food chain. The production of intermediate fatty alcohols offers a unique opportunity to traverse the "valley of death" for biofuel process and infrastructure development by leveraging the robust chemical markets and high-margin applications of fatty alcohols and their derivatives. The process will change the paradigm for biofuels production, enabling the sale of fuel for less than $3/GGE while vastly improving sustainability.

Initial laboratory experiments produced fatty alcohols and demonstrated bottlenecks limiting overall alcohol yield. Our research project deploys the syngas fermentation and strain engineering expertise of LanzaTech, the computational modeling capabilities of Northwestern University, and the process development expertise of the Dow Chemical Company to remove bottlenecks discovered in the previously developed metabolic pathway maximizing the production of C6–C14 alcohols for channeling into the chemical derivative and fuel markets. The project team has delivered all of our milestones since the 2017 Project Peer Review, including our go-no-go milestone at the end of budget period two. With the target of completing our project in 2019, we are

Weighted Project Score: 8.0

Weighting for Ongoing Projects: Approach-25%; Accomplishments and Progress-25%; Relevance-25%; Future Work-25%



I One standard deviation of reviewers' scores

focused on overcoming key technical challenges through strain and fermentation optimization and conceptual flowsheet design.

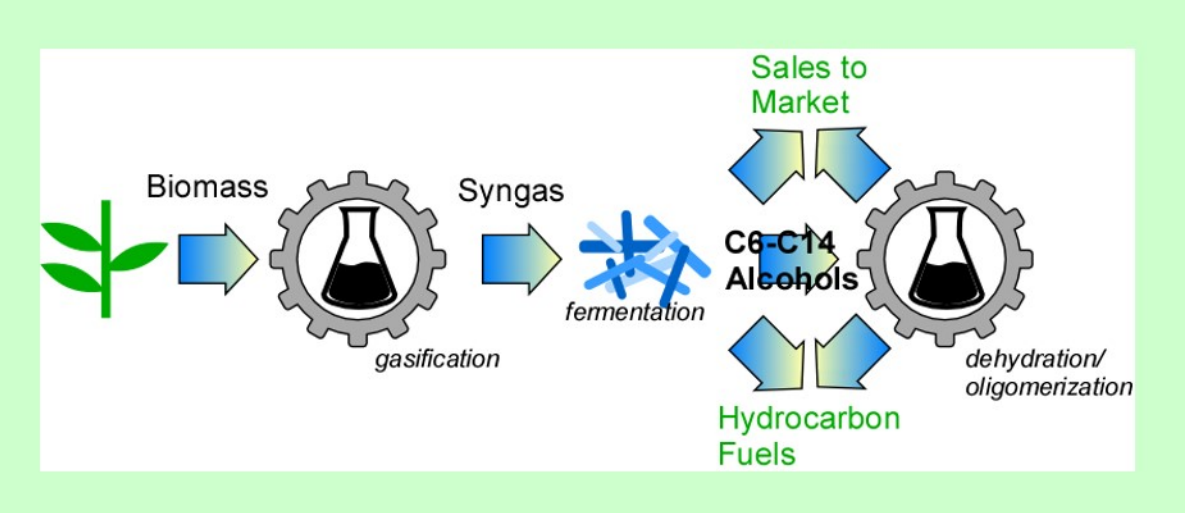


Photo courtesy of Dow Chemical Company

OVERALL IMPRESSIONS

- The goal of this project is to produce fatty alcohols from syngas. Fatty alcohols can be upgraded to numerous chemicals. The basic strategy involves producing fatty alcohols using the +1 iterative pathway. This process can potentially produce odd-chained fatty alcohols, which are not currently attainable using current approaches, with linear or branch chains. The team is making excellent progress. They have achieved their titer targets and have developed stable continuous fermentations but are still far away from achieving their productivity targets. Details are vague regarding how they will achieve these final targets.

- Dow presented results of a collaboration with LanzaTech and Northwestern University to produce a variety of fatty alcohols from syngas. The project is not yet complete, but the team has shown promising progress toward industrially relevant levels of bioconversion performance.

- This project aims for the engineering of bacteria for the production of high alcohol from syngas. The proper adaptation and selection of strains is critical to commercial viability. The project is optimistic in its projections for ease of engineering of a production strain with current cost constraints.

- Reallocating the work, when needed, and changing priority is a great approach. The project has a lot of compressed milestones to complete in the next few months; a no-cost extension might be good to consider, as the project is really interesting.

- This collaboration between Dow and LanzaTech is to develop a fermentation process for syngas conversion to intermediate fatty alcohols, produced concomitantly with ethanol. These molecules have a strong market as intermediates that are upgraded to specialty chemicals. The primary challenges of pathway limitations, byproducts, and fermentation stability are well understood, and the team is well suited to address them. Various modeling approaches were used to drive progress, including enzyme kinetics, metabolic network, and thermodynamics; these guided improvement of pathway flux and reduction in byproducts. The project is still far from commercial targets of less than $3/GGE. The tools are in place to achieve them, but it will be difficult to do so by the end of the project period, even with the extension.

RECIPIENT RESPONSE TO REVIEWER COMMENTS

- We appreciate the reviewer's feedback. To address the gap in productivity, we are pursuing multiple parallel paths. We are in the process of carrying out a combinatorial analysis of pathway genes with a large library we constructed to generate optimized strains. We are also carrying out optimization of fermentation parameters to find the optimal balance between titer and productivity guided by targeted metabolomics.

- Our team requested and was granted a six-month no-cost extension, changing the end of our program to November 30, 2019, and a strategy has been worked out to address the remaining gaps within this timeframe.

BENCH-SCALE INTEGRATION

National Renewable Energy Laboratory

PROJECT DESCRIPTION

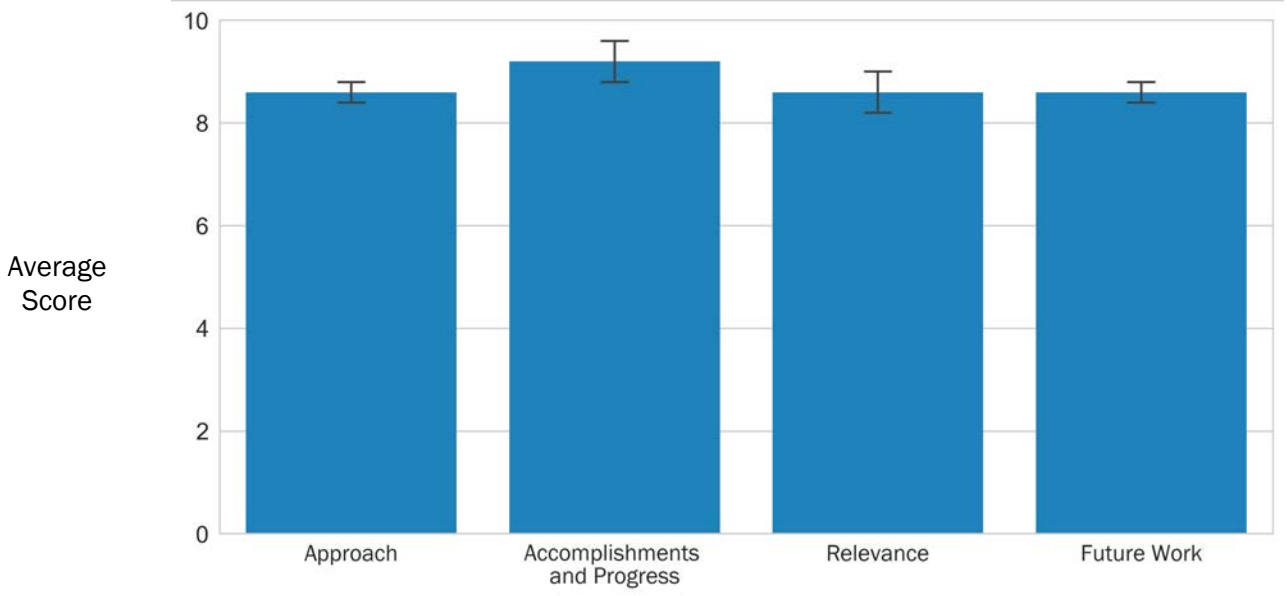
WBS:	2.4.1.100
CID:	NL0026683
Principal Investigator:	Dr. Nancy Dowe
Period of Performance:	10/1/2015–9/30/2020
Total DOE Funding:	$3,752,475
DOE Funding FY16:	$1,000,000
DOE Funding FY17:	$1,000,000
DOE Funding FY18:	$1,002,475
DOE Funding FY19:	$750,000
Project Status:	Ongoing

Producing renewable fuels and chemicals from biomass at a cost that is competitive with petroleum is the ultimate challenge. With many steps in the process—from feedstock handling, to pretreatment and conversion, to separations and upgrading—each unit operation has its own set of technical barriers to overcome. The technical barriers for the conversion step are many. Specifically, the fermentation step requires high titers, rates, and yields for a cost-competitive biofuel. This is what drives the research direction of the strain engineers and fermentation scientists. For the bench-scale R&D team, we are focused on development and optimization of fermentation processes with the outcome being an optimized, robust, and industrially relevant fermentation process. Our approach is to first assess the microorganism, product, and the TEA to determine the R&D approach. Then we apply fermentation science to develop and optimize conditions like aeration levels, feeding strategies, fermentation conditions (pH, temperature, redox), and nutrient management for high titers and production rates. Increasing titer and production rates have a direct impact on capital and operating costs of the conversion and downstream processing equipment.

The project made improvements in lipid and 2,3-BDO production, which directly impacted the TEA. These improvements are captured in yearly SOT reports. Lipids are produced in an aerobic fermentation utilizing the oleaginous yeast *Cryptococcus curvatus*. Because lipid production is not associated with cell growth, growth

Weighted Project Score: 8.8

Weighting for Ongoing Projects: Approach - 25%; Accomplishments and Progress - 25%; Relevance - 25%; Future Work - 25%



I One standard deviation of reviewers' scores

and lipid production required separate optimization. Our approach was to manipulate nutrients and employ a fed-batch process for fast growth and high cell density and to limit nitrogen after growth for high lipid production. The type of nitrogen source was also key to high lipid titer; an organic nitrogen source (yeast extract) was preferred over inorganic nitrogen (ammonium sulfate). Lipid titer and productivity increased from 50 to 60 g/L and 0.76 to 0.97 g/L/hour, respectively, during FY 2017, reducing MFSP by $0.50/GGE.

NREL's BDO process utilizes a different microorganism, a recombinant strain of *Zymomonas mobilis* developed by the Target Microbial Development (TMD) project. *Z. mobilis* is an anaerobic bacterium that produces ethanol as the primary product from sugars. TMD strain developers have successfully made a strain of *Z. mobilis* that no longer produces ethanol, only BDO. However, the modified strain of *Z. mobilis* suffers from a redox imbalance, which can be alleviated by adding small amounts of air to the process. Optimizing the micro-aeration conditions in the fermenter became the primary focus of the fermentation development research to increase titers. High titers are necessary to reduce the cost of downstream separations and catalytic upgrading. Over the past two years, we have used a fed-batch process using biomass liquor and optimized aeration levels to demonstrate increases in BDO titers by nearly 10 times (10.6 g/L in FY 2016 to 90 g/L in FY 2019). We have also successfully demonstrated scaling of the process from 0.5 L to 100 L. In addition, we have begun the transition from biomass liquor to whole slurry and have demonstrated 55 g/L BDO from whole slurry, which helped reduce the MFSP by $0.85/GGE. Future work will continue to focus on BDO titer and using biomass whole slurry. Our goal is to produce 125 g/L BDO titer by 2020 to further reduce upgrading costs and incorporate whole slurry to reduce solid-liquid separation costs. We will develop operational parameters and procedures for scaling fermentation so that we can produce pilot-scale quantities of fermentation broth for separations and upgrading R&D and prepare for pilot-sale demonstrations of the conversion process in 2022. As new strains are available, we will incorporate them into the fermentation process.

Photo courtesy of National Renewable Energy Laboratory

OVERALL IMPRESSIONS

- This project is focused on process development and optimization. The project was initially focused on lipid and BDO production; however, lipid production was dropped due to challenges associated with separations and oxygen transfer. Current work is focused on BDO production. The goal is to develop a process at the ~100-L scale. A key accomplishment is the incorporation of off-gas analysis; this will enable them to close the fermentation mass balance. Overall, the work in this project is central to many activities within NREL and BETO. The team is making excellent progress. The milestones are clear. The impact of the project would greatly improve if they explicitly considered product recovery rather than focusing on fermentation titers. In addition, the team should consider how they can generalize these results for new processes.

- This project has applied process integration to achieve impressive BDO titers from cellulosic biomass and appears to be on track to further increase these numbers. The project also demonstrated new capacities to test commercial enzymes and for off-gas analysis, both of which have general applicability.

- The team has had good successes in fermentation development and improvement and scale-up to the 100-L scale. They have done a very good job in attempting to have better fermentation control and analysis and the development of robust fermentation processes with two different microorganisms.

- This team works on fermentation development and optimization at the small scale from cellulosic sugars, leading towards scaling to the pilot scale. Bench-scale fermentation is a key de-risking step prior to scale-up and provides a standard platform for evaluating strains from different projects. Thus, it clearly plays a critical role in reaching the MYP objectives. The current focus is on 2,3-BDO, though they also collaborate with both internal and external projects in other areas. Historically, the team played a big role in meeting cost targets for cellulosic ethanol and reducing the cost of lipid production. This demonstrates their ability to work in a variety of organisms and inspires confidence in their ability to achieve the same with 2,3-BDO. These expectations were met, exceeding the goal and producing nearly 90 g/L BDO from relevant substrates. The team also works closely with the TMD team so that learnings from one can help to guide the other.

RECIPIENT RESPONSE TO REVIEWER COMMENTS

- We thank the reviewers for their positive comments and appreciate their acknowledgment of the importance of the bench scale R&D team's role in developing biofuel fermentation processes at bench scale to facilitate and de-risk scale-up. We recognize the importance of working closely with the strain development groups to evaluate strains in process-relevant conditions and providing important feedback on strain performance. The project has historically been aligned with pretreatment, pilot-scale integration and analysis. With the focus of the project on 2,3-BDO fermentation process development, we have begun to collaborate closely with the Bioprocessing Separations Consortium and the Chemical Catalysis for Bioenergy Consortium to provide material for separations and upgrading research and begin producing fermentation broth that aids the research and development efforts. We continue to maintain a close association with industry by providing information on biocatalyst performance in a process context, which we hope will aid in scale-up. For future work, we agree with the reviewers that developing a scaled-down fermentation model to evaluate new strains would accelerate the screening process, and we have plans to utilize the BioLector micro-fermentation system. We also recognize there are significant challenges in scaling a fermentation process with whole-slurry biomass and agree that engaging industrial fermentation experts on vessel design and micro-aeration would greatly help our development and scale-up efforts.

CONTINUOUS ENZYMATIC HYDROLYSIS DEVELOPMENT (FORMERLY SEPARATIONS DEVELOPMENT AND APPLICATION)

National Renewable Energy Laboratory

PROJECT DESCRIPTION

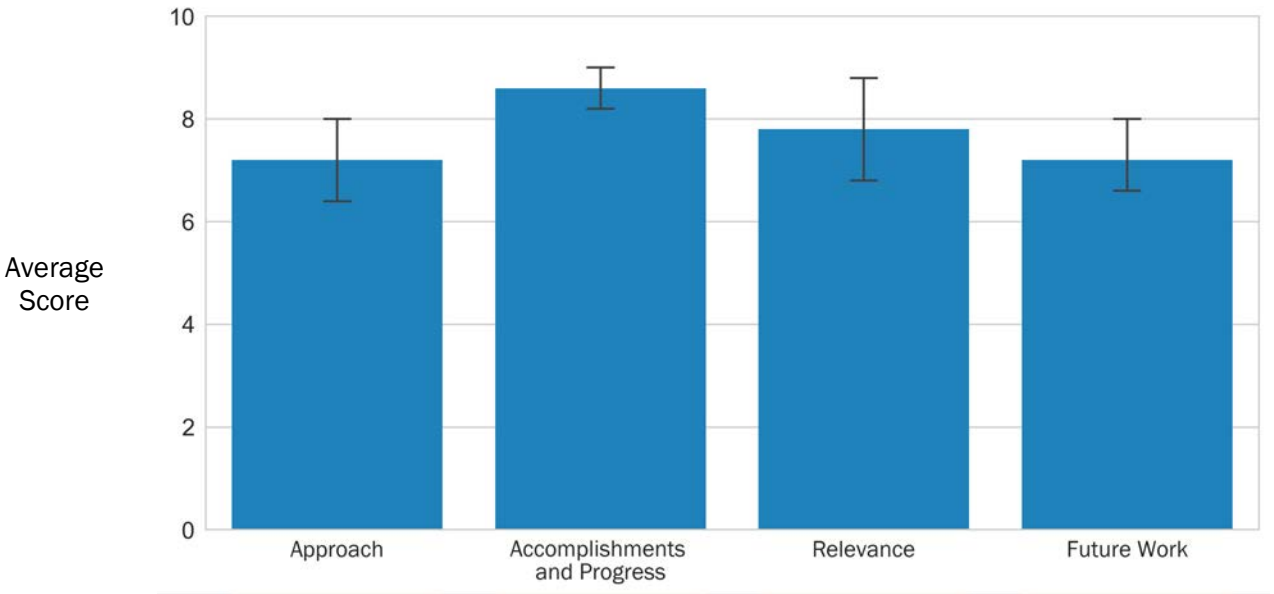
This project is focused on developing, demonstrating, and improving continuous enzymatic hydrolysis (CEH) as a transformational, process-intensified, and lower-cost method compared to batch enzymatic hydrolysis (BEH) for producing soluble clarified biomass sugars and insoluble lignin-rich streams—two essential biochemical platform intermediates. The project's goal is to apply combined reaction-separation technology to intensify the production and reduce the cost of producing and recovering these key intermediate streams at or above minimum yield and quality specifications. This scope complements the Bioprocessing Separations Consortium's emphasis on separations to recover upgraded products rather than process intermediates.

WBS:	2.4.1.101
CID:	NL0026682
Principal Investigator:	Dr. Jim McMillan
Period of Performance:	10/1/2015–9/30/2020
Total DOE Funding:	$2,774,214
DOE Funding FY16:	$750,000
DOE Funding FY17:	$750,000
DOE Funding FY18:	$774,214
DOE Funding FY19:	$500,000
Project Status:	Ongoing

Guided by TEA, the project's objective is to prove through a combination of experiments and cost modeling (joint with the biochemical platform analysis project) that CEH can pass relevant technical and economic performance go-no-go criteria (high yield, scalable, with potential to achieve BETO's MFSP [$/GGE] cost goal). Performance data on CEH are generated to inform and refine process TEAs and LCAs. Ultimately, we

Weighted Project Score: 7.7

Weighting for Ongoing Projects: Approach - 25%; Accomplishments and Progress - 25%; Relevance - 25%; Future Work - 25%



I One standard deviation of reviewers' scores

seek to produce compelling experimental data and descriptive physicochemical models to inform TEA calculations to show that MFSP can be significantly reduced using CEH. The project's stretch goal is to reduce projected MFSP by ≥$1/GGE through implementation of CEH.

Current SOT performance levels of BEH and CEH are as follows: For BEH, the 2016 SOT used a starting solids concentration of 20%, which equates to ~12% insoluble solids (IS), and an enzyme loading of 10-mg Novozymes Cellic® CTec3 enzymes per gram of cellulose. After 120 hr (5 days), a cellulose conversion yield of 85% is achieved, which after downstream solid-liquid separation losses (required to produce a solids-free sugar stream for upgrading) translates into a BEH SOT process yield to produce clarified biomass sugars of approximately 81%. The required enzymatic hydrolysis reactor size is approximately 720 L/kg insoluble solids fed.

In contrast, CEH is envisioned to be implemented in a multiple continuous stirred-tank reactors (CSTRs)-in-series-type process, although experiments are so far limited to showing proof-of-concept performance of a single CEH stage comprising an CSTR reactor and membrane unit. We have so far operated CEH using IS levels up to 8.5%. At this IS level, using an enzyme loading of 10-mg Novozymes Cellic CTec2 enzymes per gram of cellulose and a mean residence time of 15 hr, a single CEH stage achieved 58% cellulose conversion yield. The total conversion yield approaches 99% in a simulation model of four such CEH reactor stages running in series. After accounting for sugars lost in the spent (residual) solids, the process yield is 90%. Total required CEH reactor size is estimated to be 220 L/kg insoluble solids fed, less than one-third the size required for BEH. While CEH produces a more dilute sugar stream than BEH, both streams require further concentration if fed-batch production using concentrated sugar streams is intended, which it is in the current base case. Thus, additional concentration of a CEH-produced sugar stream to match what is produced in BEH must be included in comparative TEA. Or, conversely, clarification of BEH slurry to remove solids from the product sugar stream must also be included in TEA so that in comparative assessments, the two processes are producing equivalently clarified and concentrated sugar solutions.

The main goal of recent CEH research has been to overcome technical challenges encountered at bench mini-pilot scale in feeding and recirculating high IS concentration slurries (IS ≥10%). In FY 2019, new equipment better suited to higher-IS solids CEH operation is being purchased, installed, and tested. Once a higher IS-capable experimental system has been established, CEH performance at IS levels ≥8.5% will be benchmarked and the cost benefit of using CEH over BEH will be assessed through TEA (joint with the biochemical platform analysis project).

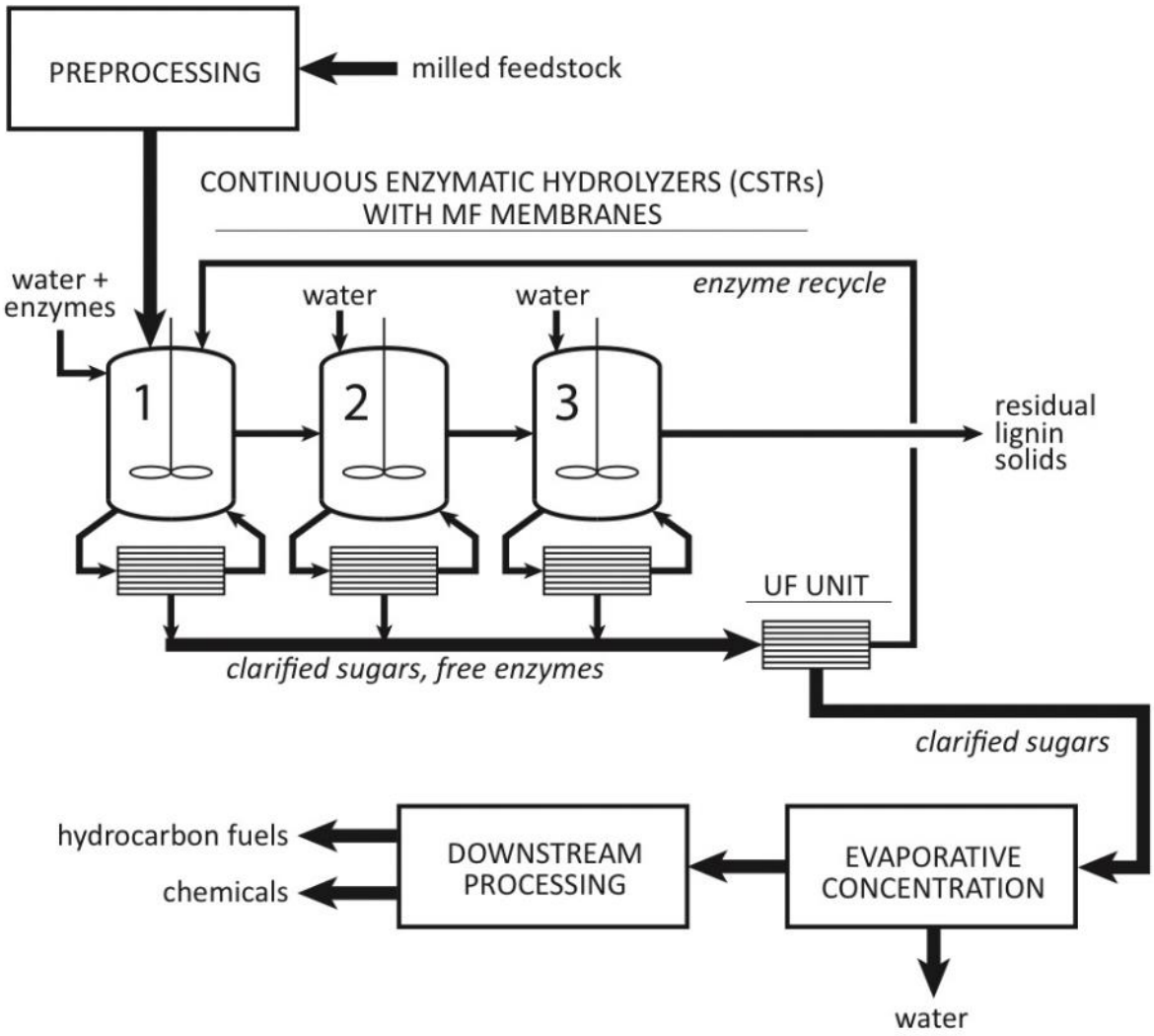


Photo courtesy of National Renewable Energy Laboratory

OVERALL IMPRESSIONS

- The goal of this project is to develop a continuous, multistage enzymatic hydrolysis process. The team is making solid process with regards to their overall goals. The focus on process intensification is especially appealing. One limitation of the project is that the work focuses on single-stage processes and does not address the ultimate goal of developing a multistage process. In addition, it is not clear how well membrane-based separations for enzyme recovery would scale, or whether these operations are economically feasible.

- The group has begun important work toward demonstrating a new method to reduce costs and improve manufacturability of a biomass hydrolysis. A central tenant of the project is to convert to a multistage system, in which residual lignin from each stage is passed to the next, which can, in principle, greatly reduce CAPEX. Thus far, the group has built a benchtop model of a first stage and has tuned and measured its performance. The project has interesting prospects if the performance can be made to match the TEA assumptions, but it has had to confront technical challenges in coping with insoluble solids. It will be important to see the updated TEA results that incorporate the proposed enhancements to the

method, as well as updated modeling predictions that incorporate expected data from simulating stages two and three using their single-stage system.

- On the overall, the project seems to be progressing in spite of some of the limitations in bench-scale equipment that have been identified by the project performers. There are numerous challenges inherent in this work from the choice of pumps, reactors, mixing equipment, and membranes.

- Continuous enzymatic hydrolysis in multiple stages will reduce cost and improve efficiency over the traditional batch process. At this stage of the project, the team is demonstrating using a single stage at insoluble solids that approach 10%. Clear progress has been made towards operating at higher insoluble solids content. The team takes a unique approach, combining TEA and reactor modeling to guide performance improvement. Overall, the team has demonstrated continuous progress in the past two years, and future work is also well positioned for success. Cost modeling indicated MFSP reductions that meet a go-no-go decision point if sustained performance can be achieved. Rather early in the project, they determined that final goals could not be reached with the current facility, so they acquired and tested new equipment for various unit operations. The team appears to have a good understanding of what is needed for success.

RECIPIENT RESPONSE TO REVIEWER COMMENTS

- Thank you. We agree that it is not yet clear to what level membrane-based separations for enzyme (and solids) recycle and sugar recovery can be scaled, or how economically competitive they can be. Reducing this knowledge gap is a key objective of this project, albeit it is still at a low technology-readiness level and not focused on scale-up per se for FY 2019–FY 2020, but rather on demonstrating compelling proof of concept that this approach to implementing CEH can be efficacious at high insoluble solid levels where substantial economic benefits can be realized. The experimental portion of the project's focus is constrained by available budget and existing equipment to studying a single CEH reactor stage. However, we are considering approaches to extend experiments to explore system performance in a second or further CEH reactor stage, as this will be key to validating modeling results for later states. Two methods conceived to date are: (1) running at extended mean residence times in the single CEH reactor system to achieve greater extents of reaction, as would occur in subsequent stages; and (2) collecting and storing the first-stage reactor's solids output stream or partially batch enzymatically hydrolyzed material (how to stabilize such materials and for how long they can be stored must also be explored). This partially enzymatically hydrolyzed material could then be used as the input feed into the single CEH reactor in a subsequent experiment to simulate second- or later-stage operation.

- Thank you. We agree, and a key objective of FY 2019–FY 2020 work is to develop performance data at higher insoluble solids levels (10% or higher targeted) that can be used to further validate the kinetic model, as well as to update the TEA. To clarify potential confusion about the model development and validation cycle used to date: the CEH model is developed based on batch kinetics data and model projections are used as input for Aspen Plus software-based TEA, with experimental CEH results used to validate the model.

TARGETED MICROBIAL DEVELOPMENT

National Renewable Energy Laboratory

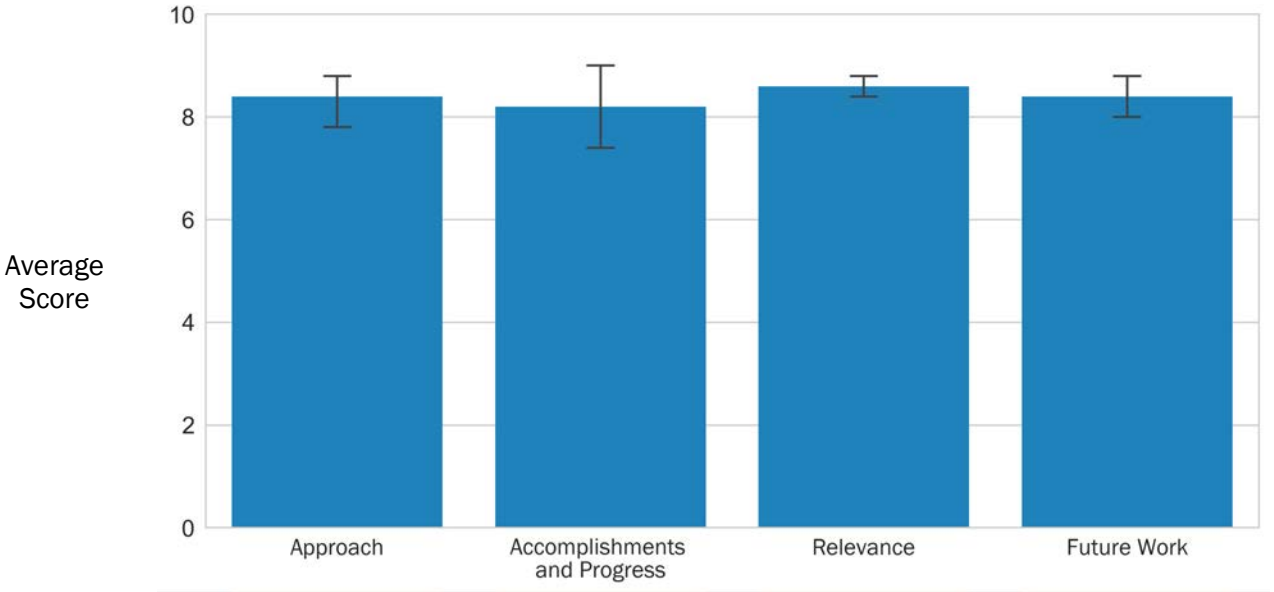
PROJECT DESCRIPTION

WBS:	2.4.3.102
CID:	NL0026684
Principal Investigator:	Dr. Min Zhang
Period of Performance:	10/1/2015–9/30/2021
Total DOE Funding:	$6,061,559
DOE Funding FY16:	$1,900,000
DOE Funding FY17:	$1,900,000
DOE Funding FY18:	$1,461,559
DOE Funding FY19:	$800,000
Project Status:	Ongoing

The goal of this project is to develop microbial pathways capable of producing high-carbon-efficiency intermediates amenable to economic separation and catalytic upgrading to hydrocarbon fuels and chemicals. This work will substantially contribute towards achieving BETO's 2022 cost target of $3/GGE ($2.50/GGE in 2030). By applying metabolic engineering and synthetic biology tools, we are working to engineer *Zymomonas mobilis* to efficiently utilize sugars for 2,3-BDO production. 2,3-BDO can be chemically upgraded to fuels and other chemicals as coproducts. Moreover, *Z. mobilis* is known for its high specific glucose uptake rate, rapid catabolism, and high ethanol yield. It has been engineered to efficiently convert the second- and third-most abundant plant-derived sugars—xylose and arabinose—to ethanol at high yield. With its ability to utilize most biomass sugars, even in toxic hydrolysate environments, it is now important to enable this microorganism as one of the leading platforms for biomass conversion. It is therefore essential to turn down or knock out the ethanol-producing pathway to redirect carbon flow to other chemical syntheses. We recently demonstrated success in redirecting carbon flow by deleting the ethanol synthesis pathway, which enabled the organism to produce 2,3-BDO exclusively. The outcome of this project will be to provide leading technologies to produce high-carbon-efficiency intermediates suitable for catalytic upgrading to hydrocarbon fuels at reduced cost. We also intend to identify future sugar-upgrading technologies, as well as the critical

Weighted Project Score: 8.4

Weighting for Ongoing Projects: Approach - 25%; Accomplishments and Progress - 25%; Relevance - 25%; Future Work - 25%



I One standard deviation of reviewers' scores

knowledge base needed to support the bioenergy industry and research community to further R&D working towards production of third-generation hydrocarbon biofuels from biomass.

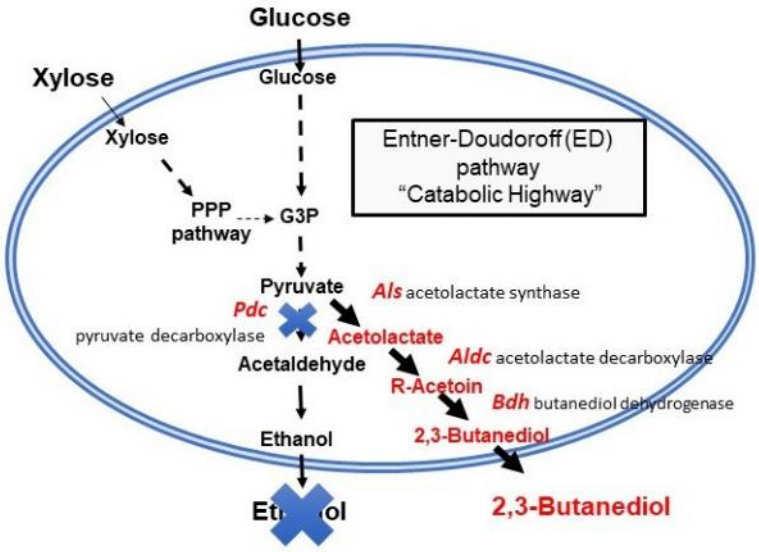


Photo courtesy of National Renewable Energy Laboratory

OVERALL IMPRESSIONS

- The goal of this project is to covert lignocellulosic sugars to 2,3-BDO. The team is accomplishing this task by genetically engineering *Zymomonas mobilis*. Engineering *Z. mobilis* to produce 2,3-BDO and not ethanol is a significant technical achievement. Moreover, they are able to achieve good productivity numbers with their engineered strain. Future work is devoted to improving pentose sugar utilization and addressing issues associated with redox balancing. The plan is compelling and there is no better team capable of achieving these goals. One question concerns the use of *Zymomonas*. Many other microorganisms can produce 2,3-BDO, including yeast. In addition, even though *Zymomonas* is an excellent ethanol producer, it has never displaced yeast. More justification would be useful, especially comparative TEA.

- The team has successfully engineered *Zymomonas* strains to produce industrially relevant titers of BDO from hydrolysate liquor, increasing yields from 20 g/L to 80 g/L.

- The strain development and fermentation groups need to be congratulated on a good effort. They have demonstrated over the past three years that they can improve fermentation titers and strain performance.

- The objective of this program is to develop a biochemical conversion strategy that is carbon efficient. In particular, they are focused on producing 2,3-BDO in *Z. mobilis*. The project originally had three tasks, but one was discontinued because of a low technology-readiness level and the other was spun out as a separate project. The team has focused on solving the key issues of ethanol byproduct and redox balancing, which will reduce manufacturing cost due to easier separations and a lower aeration requirement, respectively. The team works closely with the bench-scale integration team to evaluate their strains in fermentation. Through a combination of strain engineering and process development, significant progress has been made toward reaching commercially relevant metrics. Little work has been done on improving pentose and arabinose utilization, but this work is planned for the coming year.

RECIPIENT RESPONSE TO REVIEWER COMMENTS

- We thank the reviewers for the positive comments about the progress the project has made over the past three years with regard to achieving the high 2,3-BDO titers from hydrolysate liquors using engineered *Zymomonas* strains. We appreciate the reviewers' supportive feedback on our future research plan to improve pentose sugar utilization and addressing issues associated with redox balancing. As noted by the reviewer, there are many native and engineered microorganisms, including yeast, that are capable of producing high titers of 2,3-BDO from glucose. However, many of them do not ferment xylose well, including the engineered yeast strains. In addition, to reach high productivity, these organisms usually grow under highly aerobic conditions to produce cell mass in the first stage. Carbon is therefore used for cell mass growth rather than product formation, resulting in overall lower yield. *Zymomonas* has a threefold higher specific sugar uptake rate compared to yeast and it does not have an active TCA cycle; therefore, carbon is mostly directed to product formation, thus achieving high rates. It would be interesting to conduct comparative yeast TEA as suggested.

- Due to resource constraints in FY 2018, we reprioritized our research by downselecting to one pathway focusing on engineering *Z. mobilis* for producing 2,3-BDO. This decision was based, in part, on our success in making 2,3-BDO exclusively from mixed ethanol and BDO products, as well as TEA assessments.

PROCESS INTENSIFICATION FOR THE REDUCED COMMERCIAL CAPEX OF BIOFUELS PRODUCTION (PRICE CAP) USING DYNAMIC METABOLIC CONTROL

Duke University

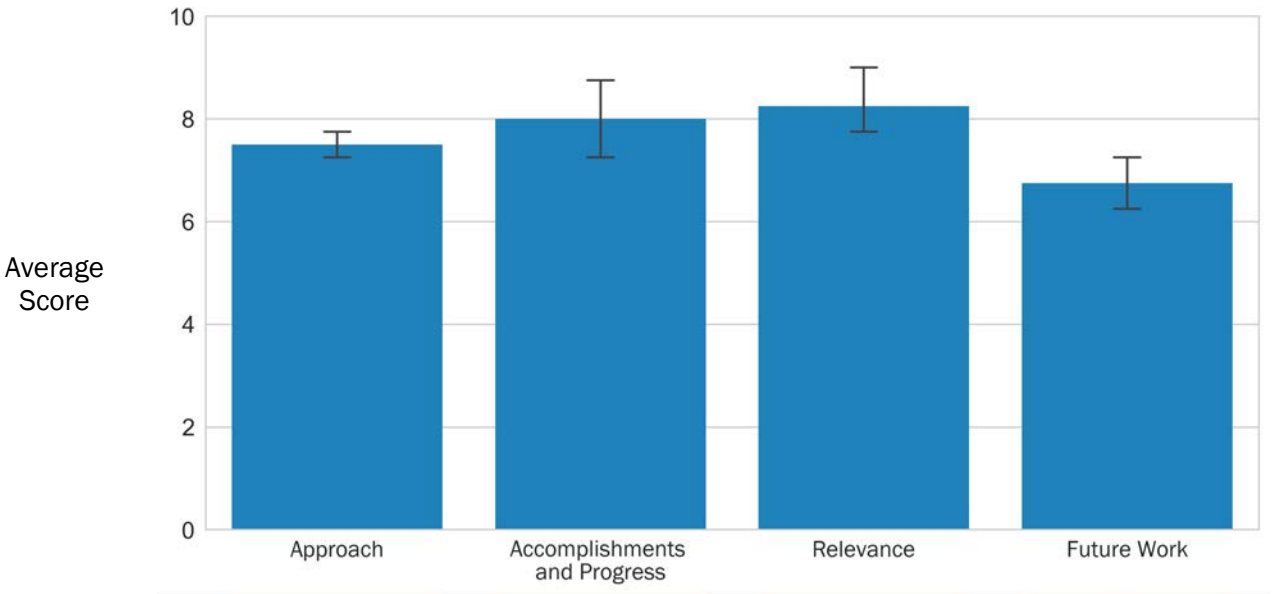
PROJECT DESCRIPTION

WBS:	2.4.3.200
CID:	EE0007563
Principal Investigator:	Dr. Mike Lynch
Period of Performance:	10/1/2016–12/31/2020
Total DOE Funding:	$1,691,595
Project Status:	Ongoing

Major barriers currently impede the successful commercialization of integrated biorefineries and large-scale industrial bioprocesses for the production of biofuels and value-added chemicals. One of the most challenging barriers is the large capital requirement needed to scale these technologies (CAPEX per plant capacity). These challenges persist despite the numerous advances in strain and pathway engineering that have resulted in attractive product yields using fermentation-based approaches. Large capital costs are a major challenge to the realization of the potential of numerous sustainable bioconversion technologies. The focus of the proposed program will be to develop a first ever semi-continuous fermentation process producing the fuel precursor farnesene from cellulosic sugars at currently unprecedented titers, rates, and yields, resulting in greatly reduced commercial CAPEX requirements. The result will be a technology enabling a commercial-scale bioprocess (100 million gallons of fuel per year) with capital costs less than that of a current demonstration plant ($40 million). This translates to a cost less than $0.50 per gallon of fuel production capacity and a step change in capital cost reductions compared to the current state of the art.

Weighted Project Score: 7.6

Weighting for Ongoing Projects: Approach - 25%; Accomplishments and Progress - 25%; Relevance - 25%; Future Work - 25%



I One standard deviation of reviewers' scores

OVERALL IMPRESSIONS

- The goal is to produce farnesene with reduced capital expense. The key innovation involves two-stage metabolic control, where cell growth is decoupled from production. One strength of the proposal is that it is motivated by TEA. Reduced CAPEX will be achieved by high-density continuous fermentation. The team has accomplished a lot work. However, it is not clear that the team will meet their final productivity milestones. Nor is it clear how close the team is toward reaching these milestones.

- Duke University presents a compelling case for an innovative approach to cutting production costs by combining process intensification with dynamic metabolic control. The group first validates its approach via TEA, and then pursues intensification technology, strain development, and process integration. The project is not yet complete, but the initial results from these efforts look promising.

- The project outlines the use of a dynamic metabolic control process to reduce the CAPEX of manufacturing biofuels. This process relies on recycling of cellular biomass and the use of stationary cells as the biocatalyst.

- Great basic research project. Adding project management will help the project track progress better.

- This project uses dynamic metabolic control and cell recycle to increase cell density and reduce CAPEX, enabling the construction of smaller plants. Farnesene is the model product. The team has improved C5 utilization by *E. coli*, and reasonable co-utilization was obtained. TEA was used to guide the optimization of process conditions, finding the best biomass concentration that reduces fermenter size without introducing unreasonable heat removal and oxygen transfer problems. This demonstrated that CAPEX of <$0.50/gal for the product could be achieved if the target metrics could be reached. The team achieved high specific rates of farnesene production and implemented a semi-continuous process. Project management was very loose, which could have had more serious consequences if significant problems had arisen.

RECIPIENT RESPONSE TO REVIEWER COMMENTS

- The recipients choose not to respond to the reviewers' overall impressions of their project.

BIOCHEMICAL PROCESS MODELING AND SIMULATION

National Renewable Energy Laboratory

PROJECT DESCRIPTION

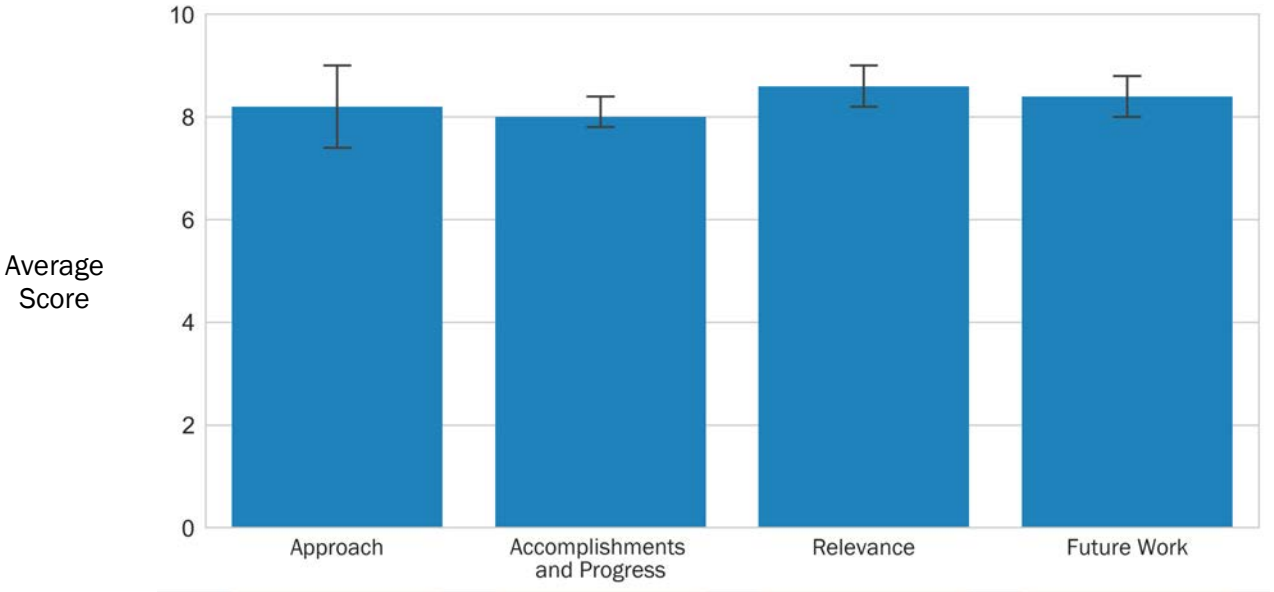
This project aims to reduce the cost and time of research by applying theory, modeling, and simulation to the most relevant bottlenecks in the biochemical process. We use molecular modeling, quantum mechanics, metabolic modeling, fluid dynamics, and reaction-diffusion methods in close collaboration with pretreatment, hydrolysis, upgrading, and TEA. The project's outcomes are increased yields and efficiency of the biochemical process, added value to products, and reduced price of fuels by specifically targeting catalytic efficiency, reactor design, enzyme efficiency, and microbial design.

WBS:	2.5.1.100
CID:	NL0013377
Principal Investigator:	Dr. Mike Crowley
Period of Performance:	10/1/2015–9/30/2021
Total DOE Funding:	$5,432,276
DOE Funding FY16:	$1,500,000
DOE Funding FY17:	$1,500,000
DOE Funding FY18:	$1,382,276
DOE Funding FY19:	$1,050,000
Project Status:	Ongoing

We work closely with experimental projects to identify problems and iterate with experiments to find and refine solutions. By working with experimentalists, we decide on problems that can be solved with simulation that could otherwise not be solved or would take too long with experiment alone to reach BETO's targets. We have produced solutions that have resulted in determining the most likely fatty-acid derivative for passive transport out of bacteria that upgrade biomass, and we have also designed enzyme mutations for enhanced lignin upgrading. Metabolic models have been developed to tune the activity of BDO production for the 2022 target. A computational method to deliver understanding of how the complex metabolomics and proteomics data can be interpreted in the metabolic pathways of organisms used in the Agile BioFoundry consortium. We have found methods to overcome

Weighted Project Score: 8.3

Weighting for Ongoing Projects: Approach - 25%; Accomplishments and Progress - 25%; Relevance - 25%; Future Work - 25%



I One standard deviation of reviewers' scores

specific barriers and continue to develop those methods. Our reactor studies have guided the design of both the microbes and reactors for aerobic and microaerobic production at all scales and have been instrumental in improving the accuracy of TEA models. This project is essential in the process of selecting the final processes for 2022 and 2030 biofuel production targets.

OVERALL IMPRESSIONS

- This team uses modeling, theory, and simulation in conjunction with experimental data to examine solution space that cannot be accessible easily by experiment. This enables the projects to downselect targets, pathway options, knockouts, enzymes, fermentation conditions, etc. Three tasks are molecular modeling, metabolic modeling, and mechanistic process modeling, thus spanning scales from molecular to cellular to macroscopic. The team has demonstrated impact on project teams; for example, leveraging models of membrane transport to prevent unproductive research. In the future, the team is using a unique combination of metabolic modeling and computational fluid dynamics to design a microaerobic process for BDO production. This is a well-managed program that has demonstrated very relevant work and will likely continue to do so in the future. It is recommended to increase funding to this effort so they can work on more projects, because this project has demonstrated clear benefits so far.

- The goal of this project is to provide modeling support for various NREL and BETO activities. The activities range from atomistic simulations to fermenter models. A key strength is the connection to experimental work. Most of the progress in the past year was related to membrane permeability and enzyme design. In addition, there has been some work related to aerobic fermenter design. This is the most promising and unique aspect of this project, as it directly supports the goal of commercialization. In other words, it can help de-risk scale-up. More resources should be directed towards these efforts. Overall, the team is productive, supporting key activities within BETO and doing quality work.

- This reviewer especially appreciates the characterization of the role of modeling to help reduce the experimental search space. This team is developing a multiscale portfolio of approaches to address this challenge, including molecular, metabolic, and process-level modeling. They have produced impressive models in all of these areas and have shown that they have a positive impact on experimental outcomes. However, it should be noted that this kind of model validation is very indirect (perhaps out of necessity), which limits the direct feedback available to further refine these models in the future.

- The 2,3-BDO titer and/or increased yield of 10% represents a major milestone and challenge to meet. It is a good test for the use of modeling to drive process improvements by identifying process bottlenecks.

- Bringing together a metabolic model, dynamic process modeling, and reactor design is a great idea and will help reduce the timeline from concept to product. The project will benefit from focusing on one of the three tasks instead of working on all three together.

RECIPIENT RESPONSE TO REVIEWER COMMENTS

- We thank the reviewers for all their effort to carefully evaluate this project, its strengths, and its weaknesses. The feedback is valuable and appreciated. It is very clear that these reviewers carefully considered our project and took the time to understand and provide valuable feedback.

- Regarding validation, we agree that validation is essential to the value of modeling and prediction and appreciate the comments of the reviewers. Our work is always connected with an experimental effort and our predictions are always tested in experiments and provide, perhaps not directly, a means of validating our models. Rarely, if ever, are the main takeaways of a modeling effort directly measured in an experiment. We are always using all experimental feedback for improving models in an iterative fashion.

- Our history has shown that the combination of multiple models has been very successful in all three tasks and allows us to provide a multiscale approach to experimental bottlenecks from reaction

mechanism to industrial-scale reactors. Often these scales overlap in a question and require expertise at each scale to work together, such as in the BDO work. This project does not require the direct attention of the PI in all three tasks. Each task lead has the responsibility of concentrating on achieving the goals of each task and collaborating with experimental efforts in BETO.

ANALYTICAL DEVELOPMENT AND SUPPORT

National Renewable Energy Laboratory

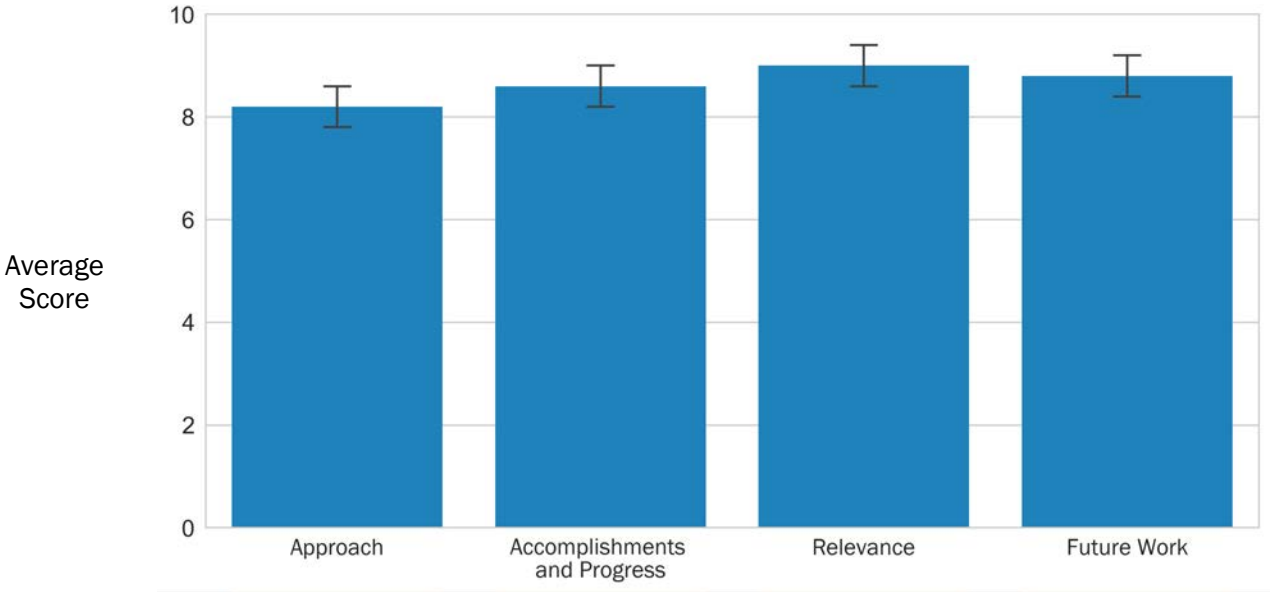
PROJECT DESCRIPTION

WBS:	2.5.1.101
CID:	NL0026685
Principal Investigator:	Dr. Ed Wolfrum
Period of Performance:	10/1/2015–9/30/2021
Total DOE Funding:	$4,396,516
DOE Funding FY16:	$1,200,000
DOE Funding FY17:	$1,200,000
DOE Funding FY18:	$1,246,516
DOE Funding FY19:	$750,000
Project Status:	Ongoing

The goal of this project is to enable biofuel and bioproducts R&D at NREL by ensuring high-quality analytical data, and to advance the tools available to the wider community through method development and globally adopted procedures. Our project is divided into two tasks: one task to improve existing analytical methods and to develop and implement new methods, and one task to maintain existing analytical facilities at NREL and to provide outreach to external stakeholders. We actively cultivate partnerships with industry, academia, and other government laboratories, based largely on our reputation for excellence in analytical chemistry. This project is best known for our publicly available laboratory analytical procedures, which provide detailed methods for the summative analysis of biomass materials. Our work is relevant to the overall goals of the program because robust, accurate, and precise analytical methods that can be easily and widely implemented directly support and help decrease the costs associated with analytical measurements. This is a critical enabling activity both for other NREL researchers and for the larger biofuels research community.

Weighted Project Score: 8.7

Weighting for Ongoing Projects: Approach - 25%; Accomplishments and Progress - 25%; Relevance - 25%; Future Work - 25%



I One standard deviation of reviewers' scores

OVERALL IMPRESSIONS

- This is a necessary project (both from running and keeping labs and developing new methods) and it will provide more predictability to biofuels and bioproducts. It would be great to see a comparison between methods for PI-developed fermentation parameter monitoring and on-the-shelf equipment.

- The goal of this project is to provide analytical services for various NREL and BETO projects, along with a large group of external collaborators. Overall, the project and performance are outstanding. A key strength is that they are posting procedures for biomass analysis and standards. The proposed transition to low-cost near-infrared (NIR) spectroscopy is very promising. The only recommendation is to keep doing what you are doing.

- This project plays a vital role in developing, providing, and standardizing analytical services for the national laboratories and collaborators in academia and industry. They perform tens of thousands of analyses a year, supporting dozens of research groups, and have emerged as an important repository of knowledge regarding laboratory analytical procedures. The greater community increasingly relies on this important resource.

- This project is need driven. NREL should continue to focus on the development of cost-effective methods that can be deployed by research, testing, and industrial labs. More attention should be paid to cost-effective, user-friendly methods that can be successfully implemented by potential industrial end users.

- This project helps ensure the generation of high-quality data for both internal and external stakeholders. The team develops and improves analytical methods that are used across projects and manages sample workflow so that the project teams can operate efficiently without having to wait on data. There is a strong emphasis on standardization, consistency, and quality control. The project team is also developing new methods and has reported development of a low-cost at-line NIR method for real-time analysis of a fermentation broth that is nearly as accurate as a much higher-cost instrument. One gap in their tools is the analysis of intracellular metabolites for metabolomics and 13C metabolic flux analysis applications.

RECIPIENT RESPONSE TO REVIEWER COMMENTS

- We thank the reviewers for their comments. We will "keep doing what we are doing" and we will try to improve our performance with closer collaborations with internal and external collaborators.

- We strongly believe that there is substantial value in establishing, supporting, and communicating common analytical procedures to ensure a "common language" among biomass researchers, and this is a unique role that BETO and NREL can play in moving the community forward.

- Analytical method development is core to this project, and we are continually developing methods that will improve the ease of sample analysis and will share these improved methods with the community. However, recent programmatic shifts to focus on a diversity of product pathways has necessitated a shift to an increased breadth of methods over improvement of existing methods. To share method improvements with the community and ensure that methods developed are relevant to industry end users, we collaborate through avenues such as ASTM International standards. We ultimately hope to help implement robust secondary methods, such as NIR partial least squares (PLS) prediction, to provide cost-effective alternatives to more labor-intensive primary methods.

ADVANCED SCADA FOR BIOCHEMICAL PROCESS INTEGRATION (WITH BEND)

Pacific Northwest National Laboratory

PROJECT DESCRIPTION

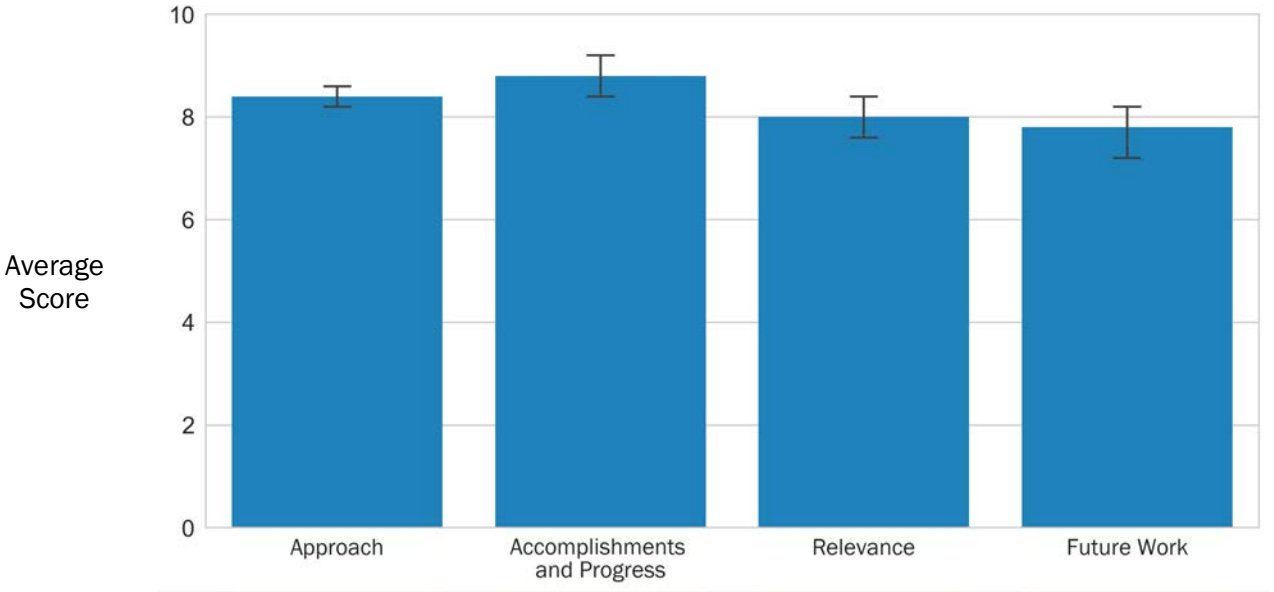
Bioconversion pathways for hydrocarbon fuels and biochemical are expected to deploy fed-batch control systems, systems that can discern sugars, viable cell mass, and products in crude, high-solids hydrolysates. This will require real-time sensor systems that are not yet commercially available for tracking hydrolysate chemical components. PNNL, in collaboration with industrial partners, is advancing process analytical technologies (PAT) for bioreactor control systems to support profitable bioconversion of biomass feedstocks to advanced biofuels and commodity chemicals.

WBS:	2.5.1.102
CID:	NL0026718
Principal Investigator:	Dr. Jim Collett
Period of Performance:	10/1/2015–9/30/2020
Total DOE Funding:	$1,177,000
DOE Funding FY16:	$300,000
DOE Funding FY17:	$300,000
DOE Funding FY18:	$327,000
DOE Funding FY19:	$250,000
Project Status:	Ongoing

Dielectric, NIR, and Raman spectroscopy tools and methods are being developed to support fed-batch and chemostat controls that will scale from the lab up to biorefinery supervisory control and data acquisition (SCADA) systems. During the present three-year project cycle, we have demonstrated Raman spectroscopy methods and equipment configurations that enabled prediction of both glucose and xylose concentrations during bioconversion of DMR hydrolysate with respective root mean errors of prediction of 3.39% and 3.77%.

Weighted Project Score: 8.2

Weighting for Ongoing Projects: Approach - 25%; Accomplishments and Progress - 25%; Relevance - 25%; Future Work - 25%



I One standard deviation of reviewers' scores

Further work during the present project cycle will include development of:

- Raman methods for real-time prediction of bioconversion products within bioreactors
- Multiblock PLS models that fuse data from multiple spectroscopy modes to improve accuracy
- Chemometric data-processing workflows within the free and open-source, web-enabled LabKey database platform to support faster, more efficient development of PLS calibration models
- PAT-enabled chemostat controls to support directed evolution of microbial biocatalysts.

This project responds to industry stakeholder interests and reduces risk in BETO R&D by working with equipment manufacturers to develop efficient, scalable PAT tools for biomass conversion that can be predictably scaled up or down to reliably extrapolate biorefinery unit operation performance from bench- and pilot-scale data.

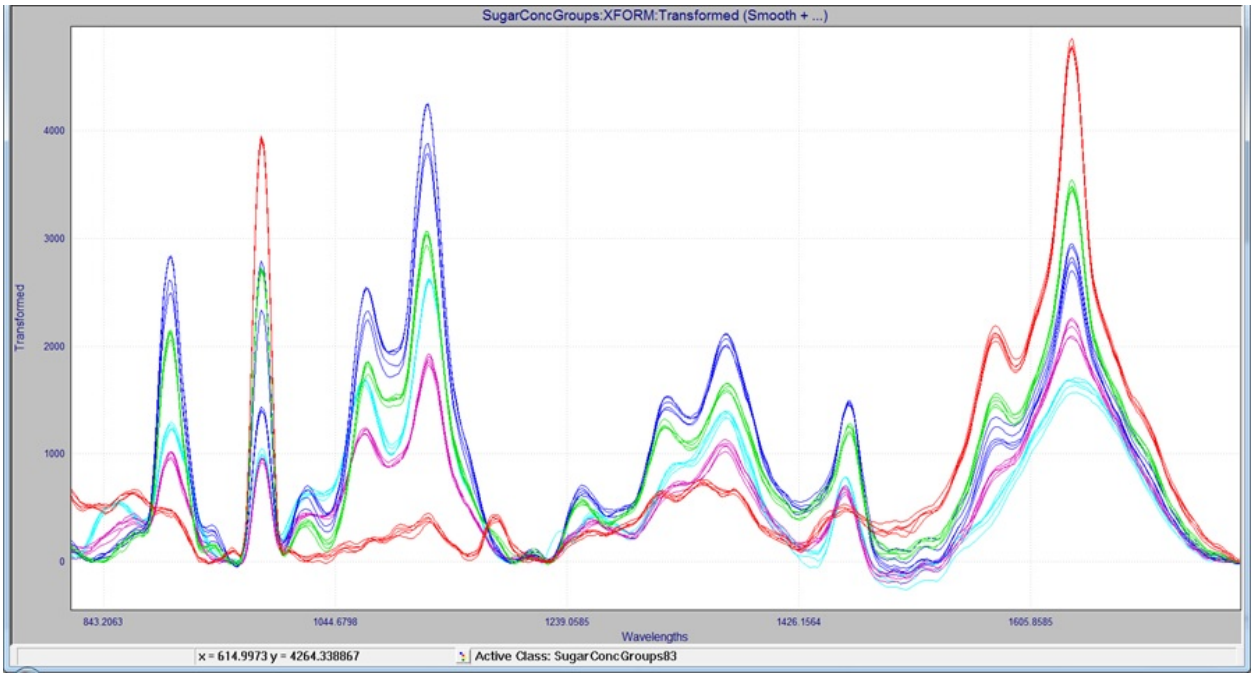


Photo courtesy of Pacific Northwest National Laboratory

OVERALL IMPRESSIONS

- Great progress so far (achieved set milestone, clear plan for 2019). Needs to better define how "integrated spectroscopy methods and the LabKey open-source chemometric informatics platform" fits with the overall project and what are the risks and critical success factor for these efforts.
- This team has been developing important new technologies to enable at-line (potentially on-line) monitoring and controls, as well as software to facilitate data integration. Their results in using dielectric spectroscopy to monitor growth and NIR (FY 2017) and Raman (current) techniques to monitor and control glucose levels in hydrolysate with insoluble solids is very promising. Active industry and national laboratory engagement help ensure relevance.
- The goal of this project is to provide process analytic technologies for fermenter control and monitoring. The team is making excellent progress. Much of the work is focused on using Raman spectroscopy to

measure sugar concentrations within DMR hydrolysates. In addition, they propose to develop fusion data models that incorporate multiple measurements. Overall, the team is making excellent progress. This project is well integrated with other BETO projects and external stakeholders. Clear project milestones are provided for the next year. If successful, this project can have real impact with regards to commercialization.

- The overall project has been successful in demonstrating the use of dielectric, infrared, and Raman spectroscopy to monitor and control a fed-batch fermentation process for substrate and biomass production. This has been demonstrated in presence of a high level of lignin that may interfere with spectroscopic analysis. The team approach and involvement of several labs, startups, equipment manufacturers, and end users has provided the framework that enables a good and successful outcome.

- The team is using new spectroscopy tools to support advanced control systems for fed-batch bioreactors. Great progress has been achieved so far using Raman spectroscopy to measure composition of complex fermentation broths, and to integrate all measured data with metadata in a software platform. Use of the data for process control has not yet been demonstrated but is a goal for the end of the project. Also, it is not clear how well the analytical methods will scale to commercial fermentation vessels.

RECIPIENT RESPONSE TO REVIEWER COMMENTS

- We appreciate the Peer Review panel's experience and broad perspective on industrial bioconversion operations, and their endorsement of our approach for developing new spectroscopy tools and methods for real-time control of bioreactors. Commercial deployment of such tools requires not only the increasingly affordable spectroscopy hardware that we have adapted for use in biomanufacturing in the course of our project; it will also be necessary to reduce the complexity of joining process sensor, event, and sample analysis data that have different formats and varying timestamp intervals into integrated training data sets for the construction of multivariate calibration models. Moreover, tracking the performance of calibration models over the course of many process runs is necessary to ensure their robustness in predicting concentrations of bioreactor broth components during bioconversion of crude biomass feedstocks that may vary considerably in composition with changes in season and market conditions. As such, we are customizing the open-source LabKey R&D data management system to provide a user-friendly software environment that will support chemometric data integration and calibration model development by relatively skilled biorefinery personnel such as senior operators. Indeed, although we are encouraged by the advances we have made in our laboratory in developing process analytical technologies for the production of advanced biofuels and bioproducts, we are keenly aware that empowering plant personnel to build and maintain calibration models themselves (rather than having to rely on outside vendor specialists) will be critical for cost-effective deployment of these new tools within the biorefinery.

ENGINEERED REVERSAL OF THE BETA-OXIDATION CYCLE IN *CLOSTRIDIA* FOR THE SYNTHESIS OF FUELS AND CHEMICALS

Northwestern University

PROJECT DESCRIPTION

Rapid population growth, a rise in global living standards, and economic competitiveness have intensified the need for sustainable, low-cost production of biofuels and bioproducts. Industrial biotechnology using microbial cell factories is one of the most attractive approaches to address this need, particularly when large-scale chemical synthesis is untenable.

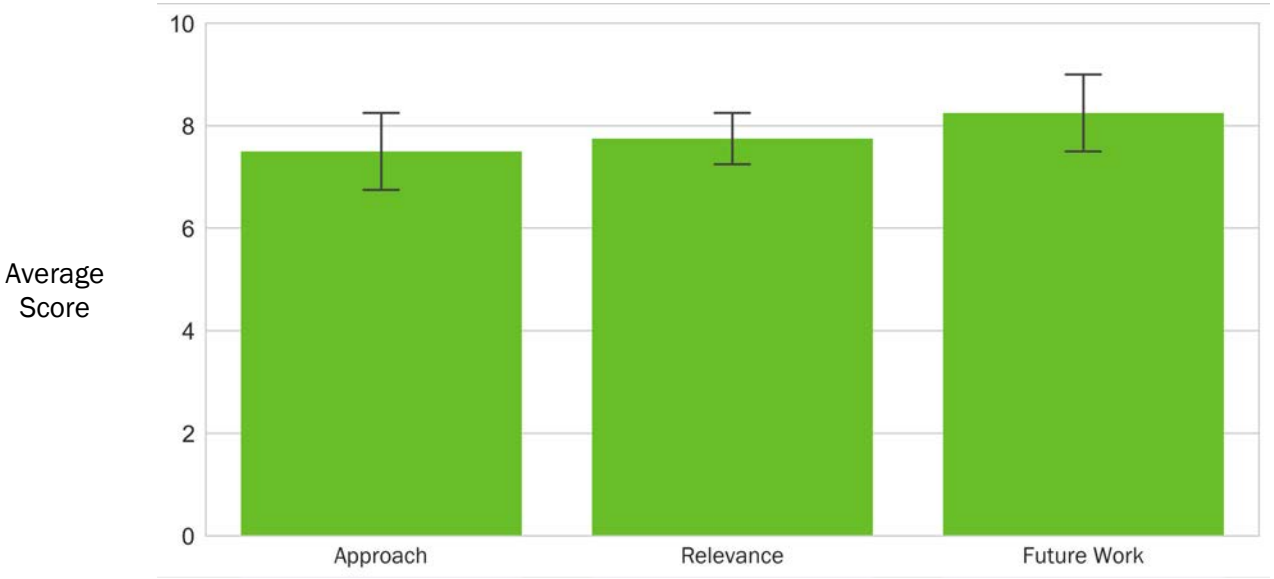
WBS:	2.5.3.206
CID:	EE0008354
Principal Investigator:	Dr. Michael Koepke
Period of Performance:	10/1/2018–9/30/2021
Total DOE Funding:	$1,600,000
Project Status:	New

Unfortunately, designing, building, and optimizing biosynthetic pathways in cells remains a complex and formidable challenge for many reasons. First, Design-Build-Test cycles for optimizing a given biosynthetic pathway can take on the order of weeks to months, requiring hundreds of person-years of R&D time to bring a new bioproduct to market. Second, most high-throughput platforms for testing engineered organisms focus on *Escherichia coli* or yeast, which limits the platform organisms, accessible feedstocks and target molecules, and stable operating environments for development. Third, a focus on linear heterologous pathways limits co-development of multiple products.

With support from DOE, we will address these limitations via unique pathway tools and engineering strategies that enable rapid synthesis of next-generation biofuels and bioproducts from lignocellulosic biomass in *Clostridia*. The core of our unique approach is to reconceptualize complex biological systems engineering by combining *in vitro* and *in vivo* work to advance state-of-the-art pathway design, prospecting, and validation in

Weighted Project Score: 7.9

Weighting for New Projects: Approach - 25%; Relevance - 25%; Future Work - 50%



I One standard deviation of reviewers' scores

an integrated framework. This framework will diversify the breadth of both products and platform organisms to meet DOE and USDA bio-based industry goals. We specifically aim to develop a new platform for engineering reversal of the β-oxidation cycle (r-BOX) in *Clostridia* for synthesis of advanced fuels and bioproducts from biomass syngas produced by established gasification technologies. The targeted products are worth $1,600–$3,000/metric ton and have a multibillion-dollar market, showing the potential economic impact of our proposed technology. The environmental, community, and rural economic development impacts will be assessed for implementation in the Southeast region of the United States, utilizing regionally abundant forestry residues as feedstocks.

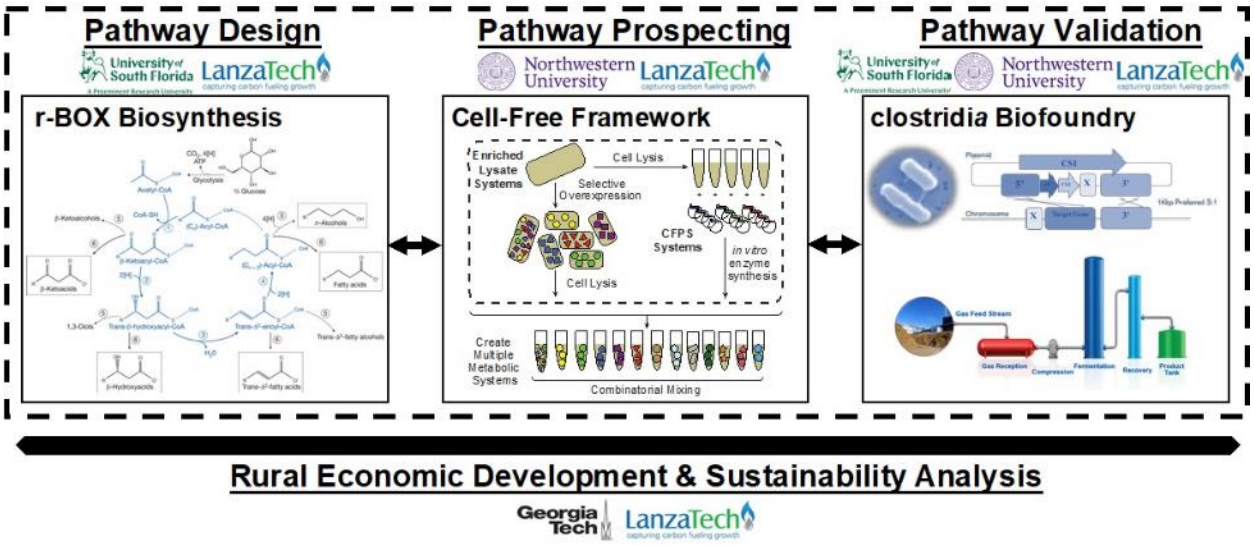


Photo courtesy of Northwestern University

OVERALL IMPRESSIONS

- The goal of this new project is to produce a wide range of value-added chemicals from syngas using a fermentation process. The key step involves reversing the beta-oxidation cycle, which has previously been shown to produce a wide range of valuable chemicals in a sugar-based process. Overall, the project has a strong technical plan. Clear milestones are provided. The work is compelling, though high-risk, as it is not clear how easy it will be to reverse the beta-oxidation cycle in an anaerobic bacterium. That said, this is the best team imaginable to accomplish such a task.

- The team assembled has a good past record of accomplishment. The approach taken is valid.

- This is a relevant project with well-defined project plan and targets. Regardless of having a positive outcome, the approach the PIs are taking is great.

- This project is a collaboration among three academic labs with LanzaTech to convert syngas to a range of advanced bioproducts by *Clostridium* fermentation. This is a highly capable team composed of leaders in their respective fields. Distinct tasks are well outlined, and quantitative goals defined. Most challenges are well understood, and the team has a good plan to address them. However, even in model organisms like *E. coli*, productivity and yield of compounds with more than four carbons via the reverse beta-oxidation pathway has been low to date. Use of a syngas substrate in a non-model organism poses an even greater challenge. There is no specific product selected to achieve milestones. Although this project could address a range of products derived from the TCA cycle, a particular model product should be

chosen to demonstrate that chain-length-specific enzymes can be found. A challenge that is not yet addressed is the need to balance enzyme rates to prevent buildup of intermediates.

RECIPIENT RESPONSE TO REVIEWER COMMENTS

- We thank the reviewer for this critical evaluation. The specific products we have in mind for the initial testing are not yet public information.

ENZYME ENGINEERING AND OPTIMIZATION

National Renewable Energy Laboratory

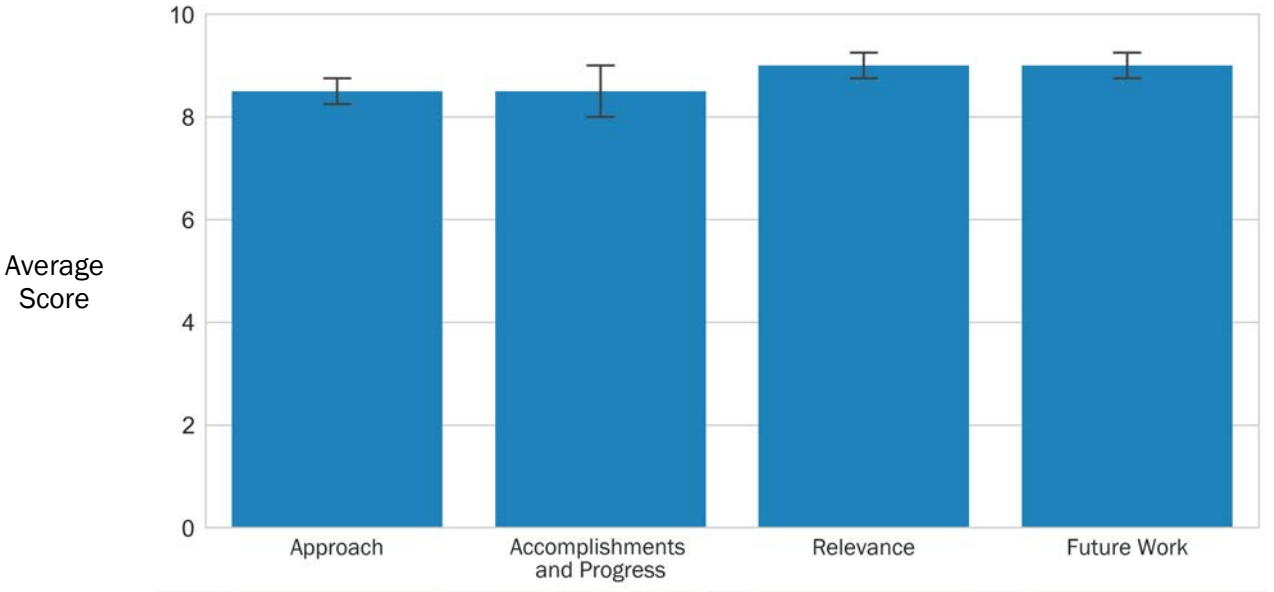
PROJECT DESCRIPTION

WBS:	2.5.4.100
CID:	NL0026686
Principal Investigator:	Dr. Mike Himmel
Period of Performance:	10/1/2015–9/30/2021
Total DOE Funding:	$5,846,452
DOE Funding FY16:	$1,600,000
DOE Funding FY17:	$1,600,000
DOE Funding FY18:	$1,746,452
DOE Funding FY19:	$900,000
Project Status:	Ongoing

This project continues to be one of the leading research efforts worldwide in understanding and improving cellulases for biomass conversion to fuels, especially in the context of the industrial production host *Trichoderma reesei*. Pivoting from deacetylated dilute acid to DMR pretreatment of corn stover will dramatically improve the lignin quality and pretreatment CAPEX aspects of the biofuels process. However, DMR solids require an improved and differently formulated enzyme blend. In the context of the biochemical platform, cellulase hemicellulose use-related costs account for approximately 10% of the cost to produce advanced fuels and intermediates. As an example, our approach will target achieving a cost savings of about $1.20/GGE relative to the 2018 SOT case for 2,3-BDO production. This will be accomplished by meeting very specific enzyme loading and sugar yield (final glucan and xylan conversion) goals. In collaboration with the Low Temperature Advanced Deconstruction and Biochemical Process Modeling and Simulation projects, we propose a two-task project for FY 2019–2021. In Task 1, Cellulase Improvement (CI), we will identify the best cellobiohydrolase (CBH) I enzymes from natural diversity for enhanced digestion of DMR solids (Megatron I). In several Enzyme Engineering and Optimization milestone reports last FY, we noted the discovery of new CBH I enzymes from preliminary diversity studies that have improved kinetics compared to the *T. reesei* enzyme. Note that the co-development of Megatron II, which will

Weighted Project Score: 8.8

Weighting for Ongoing Projects: Approach - 25%; Accomplishments and Progress - 25%; Relevance - 25%; Future Work - 25%



I One standard deviation of reviewers' scores

deliver improved CBH II enzymes, is based on a separately DOE-funded proposal. In Task 2, Lignin Modifying Enzymes and Xylanases, we will deliver enzymes found to enhance the digestion of DMR feedstocks in the presence of the improved cellobiohydrolases from Task 1. Task 2 will interrogate enzymes from diversity, as well as commercial formulations. To ensure success, we have built the special constitutive and native CBH I delete strains of *T. reesei* needed to quickly produce and test new enzyme candidates. Finally, collaboration with Novozymes will help us achieve our enzyme performance goals by ensuring process relevance and utility into new enzyme formulations.

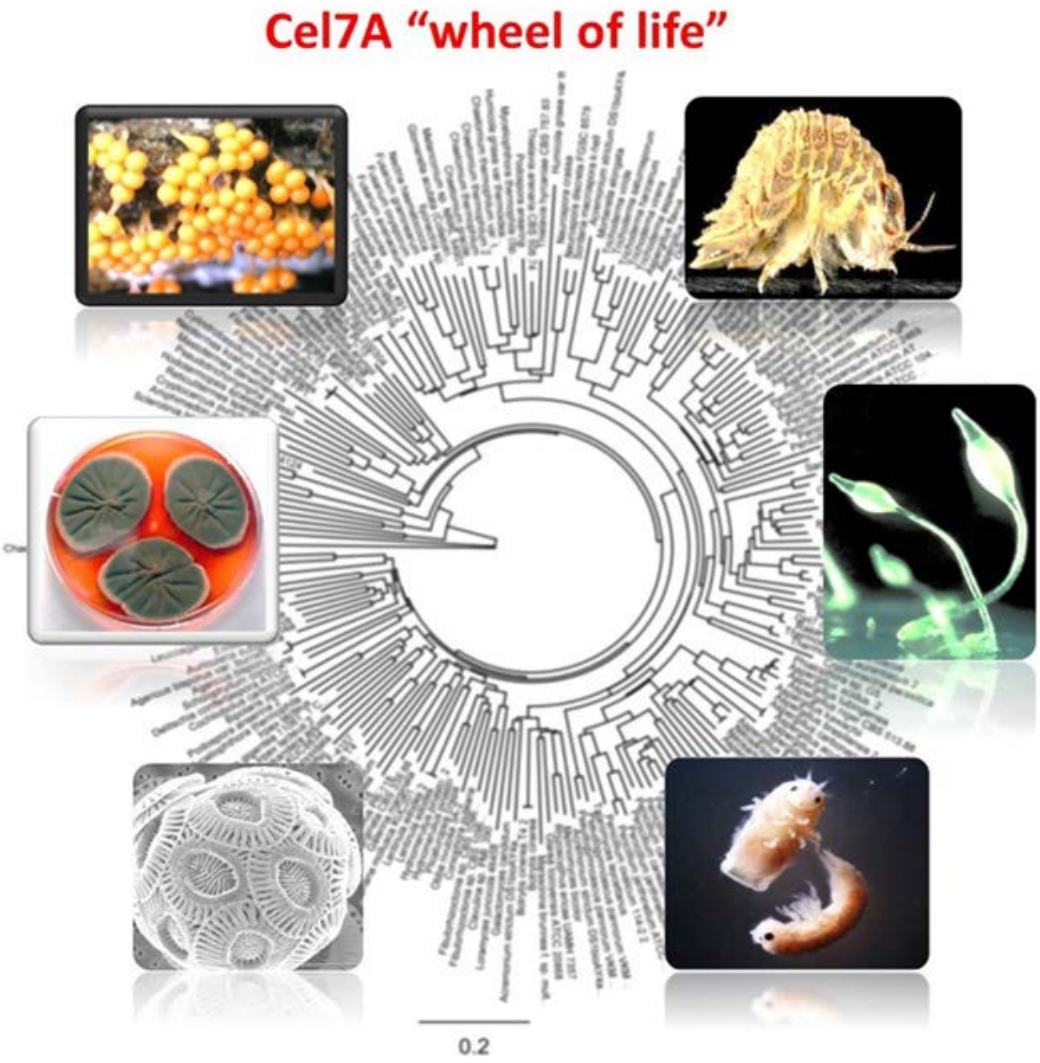


Photo courtesy of National Renewable Energy Laboratory

OVERALL IMPRESSIONS

- The goal of this project is to develop enzyme cocktails for DMR-treated biomass (which is the focus of other BETO-funded projects). The team provides a compelling argument for improving these enzyme cocktails—namely, that these commercial cocktails do not work well on DMR-treated biomass. In addition, this work is central to all sugar-based conversion strategies. Overall, the team is making excellent progress. The raptor-enzyme concept is especially appealing, as is the collaboration with

Novozymes, which highlights the commercial viability of the work. That said, addressing feedstock variability would strengthen this project.

- This team has combined enzymology, enzyme design, genome mining, and strain engineering to produce a comprehensive platform for optimizing enzymatic treatment and saccharification of DMR solids. Key results include identifying critical protein domains, establishing a cell-based production and secretion system, engineering an enzyme with increased activity, and preparing an enzyme library for screening. Although the final results of this project have yet to be fully realized, the enzyme-optimization platform itself is an achievement that can likely be applied to other challenges in the future.

- This an impressive tour de force that combines enzyme discovery and engineering with the development and testing of new cellulase/other hemicellulase/lignin-modifying blends. It brings a credible team of internal and external collaborators together with a key enzyme supplier. Achieving realistic enzyme cost reduction is critical to the success of an industry that aims to commercialize or use these enzymes. A parallel development can be envisioned for algal/oilseed/high-protein feedstocks. BETO should consider broadening the scope of this project and to look for other enzyme conversion opportunities beyond lignocellulosics.

- This team is developing an enzyme formulation that works well on DMR solids, while most traditional formulations are more suited for deacetylated dilute acid solids. They are also developing an improved enzyme expression system. There is clear commercial interest in the outcome of this work, and industrial partners were engaged early in the project. The approach involves a combination of enzyme discovery in nature and enzyme engineering via domain shuffling. The team is encouraged to try additional directed evolution methods in the coming year, as their work transitions from basic R&D to achieving performance targets. In addition, this team could be of use in engineering pathway enzymes that may be bottlenecks in microbial bioconversion processes, so a broadening of scope is encouraged.

RECIPIENT RESPONSE TO REVIEWER COMMENTS

- Thank you. The raptor concepts are indeed intriguing and follow an interesting principle from nature—you get the best biomass deconstruction performance by bringing all tools to the table. It is true that feedstock variability will impact enzyme formulation. The extent of this impact is not known at this time; however, the Feedstock-Conversion Interface Consortium platform should begin to address these issues and we will certainly assist in any way possible.

- Thank you for your comments. This work is indeed the culmination of years of technical preparation and partnering with industry. Also, knowing the performance targets in the context of a more mature process was very important. Evolutionary methods regarding cellulase performance improvement (specific activity) have always been challenging. One historical problem has been the sometimes-poor connection between activity on surrogate substrates and actual biomass. For this reason, we take a reductionist approach aimed at reducing numbers of candidates quickly and then employing more relevant assays. Of course, when outstandingly superior or inferior enzymes are found, we examine them closely using structural biology. We are involved as a team in supporting some of the pathway projects in the program, including Cell Free Technology (Bomble), Carbonic Anhydrase for Flu Gas Cleanup (Zhang), and Targeted Microbial Development (Zhang).

CELL-FREE AND IMMOBILIZATION TECHNOLOGIES

National Renewable Energy Laboratory

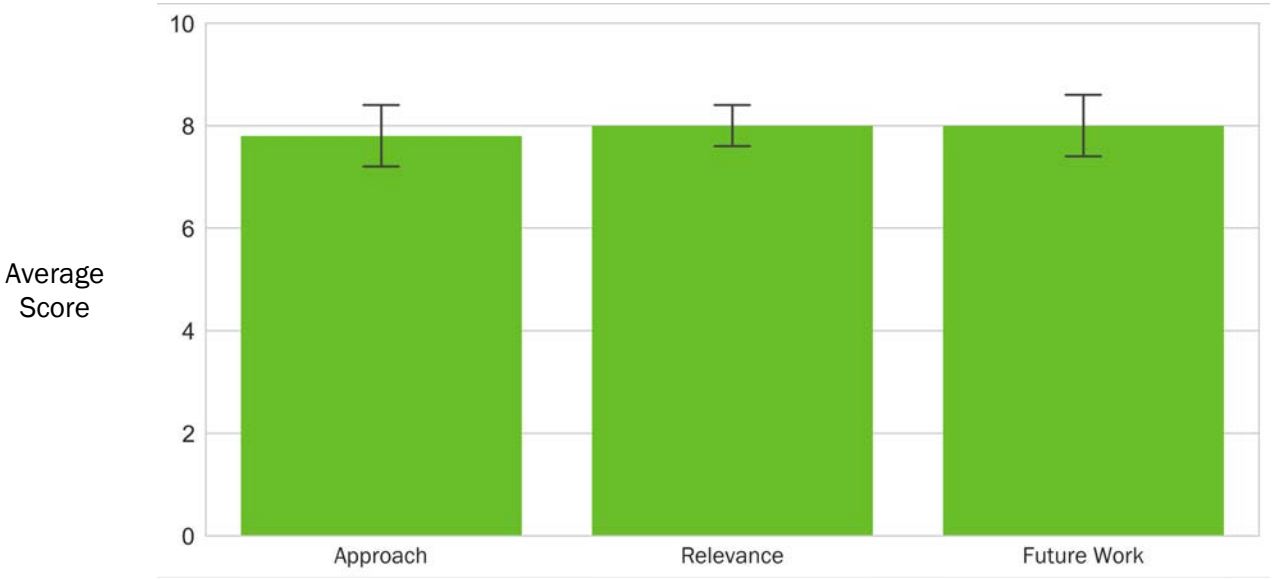
PROJECT DESCRIPTION

WBS:	2.5.4.101
CID:	NL0034445
Principal Investigator:	Dr. Yannick Bomble
Period of Performance:	10/1/2018–9/30/2021
Total DOE Funding:	$900,000
DOE Funding FY16:	$0
DOE Funding FY17:	$0
DOE Funding FY18:	$0
DOE Funding FY19:	$900,000
Project Status:	New

Today, several key factors negatively impact the production of fuels and chemicals from renewable sources. Common hindrances in the biological production of biochemicals are: (1) end-product or intermediate toxicity to the microbial biocatalyst, (2) the diversion of carbon to biomass formation, and (3) coproduction of undesired byproducts. A particularly attractive alternative is to eliminate the biocatalyst entirely and instead operate the desired metabolic pathways in isolation, thus circumventing the roadblocks of biological toxicity, lower yields, and lack of specificity. However, cell-free enzyme systems still suffer from low productivities owing in part to the effects of free diffusion of intermediates, lack of long-term enzyme stability, cofactor cost or inefficient recycling rates, and, finally, the cost of enzyme production purification. This project represents a new effort to propose innovative and cost-competitive routes to producing biochemicals from a variety of feedstocks using cell-free approaches. These routes will help reduce the current risk and cost associated with classical cell-free production. Cell-free technologies show promise for application to the production of toxic inhibitory products or products difficult to separate from microbial growth media and can help reduce the production barriers in multiple areas of biological conversion of feedstocks to biochemicals.

Weighted Project Score: 8.0

Weighting for New Projects: Approach - 25%; Relevance - 25%; Future Work - 50%



I One standard deviation of reviewers' scores

More specifically, we will develop new technologies and routes that could be used to produce high-value biochemicals such as 1,3-propanediol, 3-hydroxypropionic acid, 2,3-BDO, or polyhydroxybutyrate from biomass-derived C5/C6 sugars or lignin, but also from waste byproducts such as glycerol. This project will lead to significant innovation and also lead to new concepts and rational design of pathways and enzymes. Within this project, we will develop new metabolic enzyme cascades that will represent natural or artificial combinations of enzymes to produce the desired biochemicals from a variety of feedstocks. We will also develop basic design principals for constructing synthetic metabolons, using fusion proteins and synthetic protein scaffolds, to promote substrate channeling and stability while conserving peak activity. Additionally, our efforts will include a TEA of cell-free approaches to provide the sensitivities of the process to enzyme loading, activity, pH, reactor volumes, and cofactor recycling rates. Finally, we will focus on further increasing stability, operating lifetime, and efficiency of the pathway enzymes by immobilization on support surfaces. We will focus on immobilizing pathway enzymes or combinations of enzymes on several different conducting polymers and evaluate the effect on stability and operating lifetime. As more combinations become available, we will conduct a more systematic study of the means of immobilizing these enzymes. This preliminary work will enable the in-depth study of cofactor recycling at these interfaces using mediators for electron transfer.

Taken together, these approaches will enable process intensification, continuous operation, lower capital and separations costs, and end-product flexibility, and thus has the potential to contribute significantly to BETO's goals of cost-competitive biofuels and bioproducts.

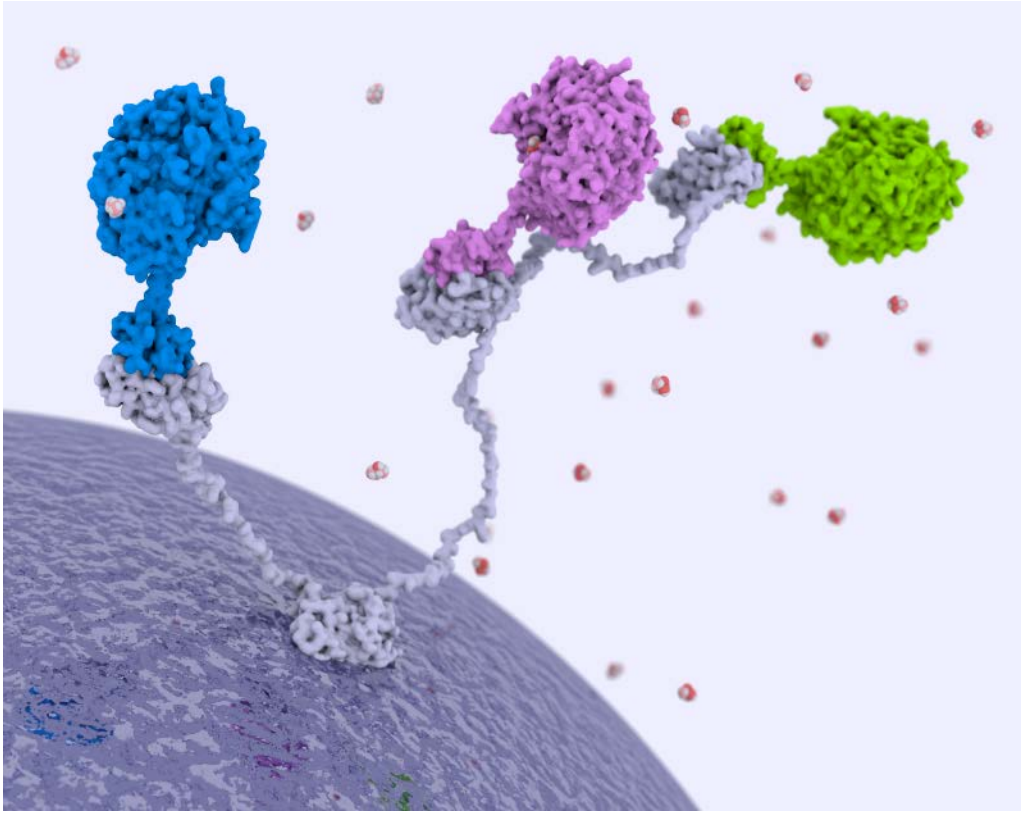


Photo courtesy of National Renewable Energy Laboratory

OVERALL IMPRESSIONS

- The project has a clear focus. The PI is looking into TEA and used that for prioritizing tasks and selecting the main focus for the project, which is a great approach. Manufacturability—especially in

regard to achieving needed stability of the enzyme and the cost associated with making such stable proteins—needs to be addressed.

- This team seeks to demonstrate the potential for bioproduction by applying immobilized cell-free biocatalysts to industrially relevant hydrolysates. If successful, this project could provide an interesting and relevant alternative for products that are toxic or that could benefit from higher reaction densities. Even though the project was only recently initiated, they have already shown cell-free production of 1,3-propanediol.

- The goal of this project is to develop a cell-free process for 2,3-BDO production. While cell-free technologies are promising, it is not clear whether these technologies can be scaled for the production of commodity chemicals. One key challenge will be developing efficient strategies for recycling cofactors. While the team addresses this challenge, the details regarding how they will solve this problem are vague. In addition, the work associated with encapsulation has many potential problems; key among them are the costs associated with using such approaches. Again, the key question is whether these technologies can be applied at scale for the production of a commodity chemical.

- Cell-free manufacturing of a chemical such as 2,3-BDO represents an intriguing possibility with many challenges. Proof of concept and cost effectiveness from continuous operation need to be established for the commercial viability of this approach.

- The project is aimed at developing cell-free processes to enable biochemical and fuel production, which would reduce downstream separation costs. This could facilitate implementation of non-natural pathways with toxic intermediates and allow higher-density reactions. This work is intended to address the major barriers to commercialization of cell-free processes, including enzyme stability and cofactor regeneration. The first task is to engineer enzymes to construct an *in vitro* bioconversion pathway, and the second task focuses on immobilization technology. Initial success has been demonstrated in tethering, immobilization, and cofactor recycling, and about half of a long metabolic pathway has been demonstrated. The team has a good understanding of the risks, and the milestones are oriented around de-risking the approach; however, there is no clear technical plan on how the risks of enzyme production and stability will be addressed. Also, issues with scale-up are not considered. Overall, this is a high-risk project as no cell-free process for a long metabolic pathway has been commercialized. Furthermore, the economics of this process compared to a conventional fermentation are not clear. This will be addressed in the TEA.

RECIPIENT RESPONSE TO REVIEWER COMMENTS

- We would first like to thank the reviewers for their effort to carefully evaluate this project. Their feedback will help us redirect some of our efforts and consider details we had overlooked.

- There are indeed challenges in scaling up the production of biochemicals using cell-free biocatalysis: (1) enzyme production costs and operating lifetime and (2) cofactor management. However, we believe these can be overcome using either tools that have already been demonstrated for enzymatically driven processes, or increased R&D.

- Enzyme production costs is a risk that can be remediated, as enzyme production can be dramatically improved using dedicated production strains, as is the case for cellulases or other industrial enzymes. We are currently investigating the cost of producing these enzymes at large scale.

- Enhanced enzyme stability can be achieved using several approaches such as immobilization, enzyme engineering, or prospecting. All of these approaches have been successfully used in industry to reduce operating costs of enzymatically driven processes. Additionally, cell-free biocatalysis offers more process flexibility, as they allow for the replacement of less-stable enzymes in the pathway while others

can keep operating for many more production cycles. Reviewers mention issues with encapsulation, which can indeed lead to problems such as enzyme inactivation, limited diffusion, or additional costs. We aim to test several encapsulation and immobilization technologies and assess the most promising for stability and production while also minimizing costs.

- Finally, proper cofactor maintenance can be achieved using balanced pathways for efficient recycling; in this case, cofactor costs only reside in priming the system, allowing easier scale-up. Cofactor costs at scale can also be reduced by creating a market for these cofactors to be mass produced, isolating cofactor mixes from spent cells from other processes, or developing the use of biomimetic cofactors.