

Systems Biology of Bioenergy-Relevant Microbes to Enable Production of Next-Generation Biofuels and Bioproducts

Summary of Projects Awarded in 2021 under Funding Opportunity Announcement DE-FOA-0002448

Genomic Science Program

genomicscience.energy.gov

The ability to manipulate microbial biosynthetic pathways and metabolism using synthetic biology provides unprecedented opportunities to advance U.S. Department of Energy (DOE) missions in sustainable bioenergy development. Among these opportunities is research to (1) enhance the production of advanced biofuels and bioproducts and (2) convert and upcycle synthetic polymers. Within DOE's Office of Biological and Environmental Research, the Genomic Science program (GSP) is well-positioned to address these opportunities through its support of basic research that investigates the foundational principles driving biological systems. GSP research seeks to provide the necessary fundamental science to understand, predict, manipulate, and design plant and microbial systems and processes that underpin energy innovations. As part of this objective, GSP issued a funding opportunity announcement (FOA) in fiscal year 2021 to solicit research proposals aimed at enabling a future where biological systems can be designed and modified for desired specific outcomes while delivering positive impacts on the environment and bioeconomy. GSP awarded 21 projects in the FOA's two subtopic areas: sustainable bioenergy and polymer upcycling. Descriptions of the subtopics and awarded projects follow.

Subtopic A: Sustainable Bioenergy

The immense diversity and versatility of microbial metabolism offer the potential to sustainably produce bioenergy and bioproducts that are not dependent on fossil fuels. Realizing this potential requires fundamentally understanding how biological systems behave. To advance this understanding, this subtopic focuses on systems biology-driven basic research to design and control the functional capabilities within living systems, specifically targeting the production of bioproducts and "advanced" biofuels, or biologically synthesized compounds that can potentially serve as energy-dense transportation fuels, such as gasoline, diesel, and aviation fuel, and that are compatible with existing engines and fuel distribution infrastructure. GSP sought sustainable bioenergy proposals in two areas:

- Developing emerging model microbes or microbial communities with unique or enhanced capabilities to produce advanced biofuels and bioproducts.
- Understanding novel microbial functional capabilities and biosynthetic pathways relevant to the production of advanced biofuels and bioproducts.

Subtopic B: Polymer Upcycling

Globally, more than 350 million metric tons of plastic polymers are produced annually, and their production is anticipated to quadruple by 2050. Though synthetic polymers are typically highly resistant to biological depolymerization or breakdown, evidence indicates that some plastics [e.g., polyethylene terephthalate (PET) and ester-based polyurethanes] can be enzymatically deconstructed. However, the enzymatic pathways for depolymerizing many other polymers, including polystyrene, polyamides, or ether-based substrates, remain unknown. To meet this challenge, the polymer upcycling subtopic targets research that builds on rapid advances in genomic science, biosystems design, and computational biology to develop enhanced capabilities for biologically converting and reusing synthetic polymers. In coordination with the DOE-wide Plastics Innovation Challenge, GSP sought polymer upcycling proposals in two areas:

- Identifying and developing novel biological mechanisms, enzymes, and pathways for polymer deconstruction and conversion in both model and environmental microbes.
- Designing new biosynthetic pathways for converting polymers into new products or their precursors.

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Websites and Links

BER Genomic Science program
genomicscience.energy.gov

DOE Office of Biological and Environmental Research
science.osti.gov/ber

DOE Office of Science
energy.gov/science

Biological Systems Science Division Strategic Plan
genomicscience.energy.gov/2021bssdstrategicplan/

FOA description
science.osti.gov/-/media/grants/pdf/foas/2021/SC_FOA_0002448.pdf



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Energy and Carbon-Optimized Conversion of Lignocellulose to Biobased Chemicals by Extreme Thermophiles

- **Principal Investigator:** Michael Adams (University of Georgia)
- **Collaborators:** Robert M. Kelly (North Carolina State University), Dmitry Rodionov (Sanford Burnham Prebys Medical Discovery Institute), Ying Zhang (University of Rhode Island)

Project Goal and Summary

This project aims to use systems biology-guided approaches to develop nonmodel microbial metabolic engineering platforms based on *Caldicellulosiruptor bescii* and *Pyrococcus furiosus*. *C. bescii* grows optimally at 78°C and is the most thermophilic lignocellulose-degrading organism known; *P. furiosus* grows optimally at 100°C and is the most thermophilic fermentative organism known. This work leverages recent breakthroughs in the development of molecular genetic tools for these organisms, complemented by a deep understanding of their metabolism and physiology gained over the past decade of study in the research team's laboratories. Using switchgrass and poplar as model plants, the project will apply the latest metabolic reconstruction and

modeling approaches to optimize biomass conversion to industrially relevant chemicals.

Bioprocessing above 70°C can have important advantages over near-ambient operations. Highly genetically modified microorganisms usually have a fitness disadvantage and can be overtaken easily in culture when contaminating microbes are present. The high growth temperature of extreme thermophiles precludes growth or survival of virtually any contaminating organism or phage, thus reducing operating costs associated with reactor sterilization and sterile facility maintenance. In addition, at industrial scales, heat production from microbial metabolic activity vastly outweighs heat loss through bioreactor walls that would require cooling.

Using carbon dioxide supplementation as an additional carbon source and hydrogen gas supplementation as an additional energy source, the team will engineer *C. bescii* and *P. furiosus* to produce several key industrial chemicals and enhance their production. The overarching goal is to demonstrate that nonmodel microorganisms, specifically extreme thermophiles, can be strategic metabolic engineering platforms for industrial biotechnology.

Optogenetic Control of Microbial Consortia for Biofuel and Chemical Production

- **Principal Investigator:** Jose Avalos (Princeton University)

Project Goal and Summary

Microbial fermentations using co-cultures, as opposed to monocultures, have been proposed to improve fuel and chemical production. Dividing biosynthetic pathways across two or more microbial strains can improve productivity by (1) reducing the metabolic burden on any one strain, (2) exploiting advantageous attributes of different microbial species, (3) optimizing metabolic modules separately in each strain, and (4) avoiding metabolic crosstalk or regulatory feedback. However, stabilizing consortial populations to avoid member loss and optimizing population compositions are formidable challenges that have stifled their application. Researchers have used strategies such as controlling inoculation ratios, utilizing exclusive nutrients for different members, or engineering co-dependencies between species to stabilize consortia. However, these strategies are limited in their ability to dynamically adjust populations and thus optimize them for chemical production.

The goal of this project is to develop optogenetic controls of microbial consortia for biofuel and chemical

production. Optogenetics uses light-responsive proteins to control biological processes, a technique only recently applied to control metabolic activity in microbial fermentations. The research team has developed new optogenetic systems to control growth rates in several microbial species, including yeast and bacteria. This capability could enable not only the stabilization of microbial consortia with light, but also their optimization for chemical production. The team will develop mono- and polychromatic optogenetic controls of microbial consortia and scale them up to lab bioreactors. They then will develop light-controlled co-culture fermentations, in which engineered biosynthetic pathways are fragmented into modules and optimized in separate strains, as well as consolidated bioprocessed optimized with light for cellulose or PET utilization. Mathematical models and feedback controls will help advance basic understanding of these biological systems and optimize them for chemical production. These technologies constitute a new paradigm to engineer and control microbial consortia, which could help realize their promise for biofuel and chemical production.

Quantitative Analysis of Metabolic Segregation of Lignin Deconstruction and Catabolism in Outer Membrane Vesicles of Soil *Pseudomonas* Species

- **Principal Investigator:** Ludmilla Aristilde (Northwestern University)
- **Collaborators:** Neha Kamat (Northwestern University), Gregg Beckham (National Renewable Energy Laboratory)

Project Goal and Summary

This research seeks to address a long-standing metabolic shortcoming for a biocatalytic platform that can efficiently couple lignin depolymerization and the carbon flux of breakdown derivatives to important product biosynthesis. Outer membrane vesicles (OMVs) in pseudomonads have recently been implicated in lignin depolymerization and aromatic carbon catabolism, thus offering a means to develop improved biocatalysts for these two key processes. These OMVs could be combined with the cellular metabolic versatility of pseudomonads, which are well-known for processing both lignin and cellulose derivatives toward biosynthetic pathways of target products. A major knowledge gap that prevents harnessing the functional capabilities of OMVs is a quantitative understanding of their metabolic networks and regulation in relation to the intracellular metabolic network.

The overarching research goal is to elucidate the reaction network responsible for OMV metabolic functionalities in soil *Pseudomonas* species with demonstrated accelerated lignin catabolism and subsequently evaluate the metabolic relationship of OMVs in fueling lignin-derived carbon fluxes toward intracellular biosynthetic pathways. This fundamental understanding is required to leverage encapsulated OMV catabolic reactions to enhance the intracellular metabolic network for concerted lignin catabolism and valorization. The research team will use a unique interdisciplinary approach that combines stable isotope–assisted metabolomics, proteomics profiling, genome-scale quantitative flux modeling, biomimetic tools for vesicle design, and metabolic engineering. Research outcomes are aimed at achieving significant advances in exploiting the biosynthetic processing power of microbial systems for converting lignocellulose into valuable biofuels and bioproducts. Beyond advancing sustainable energy objectives, these research outcomes will provide metabolic roadmaps to guide biotechnological applications of OMV production, a widespread attribute in several bacterial species.

Novel Systems Approach for Rational Engineering of Robust Microbial Metabolic Pathways

- **Principal Investigator:** Laura Jarboe (Iowa State University)
- **Collaborators:** Robert Jernigan (Iowa State University), Peter St. John (National Renewable Energy Laboratory)

Project Goal and Summary

The goal of this project is to develop and implement a new process for improving bioproduction under harsh conditions. This new process, which will have significant economic advantages for industrial processes, uses an approach that addresses the failure of reactions required for bioproduction, due to inhibition, denaturation, misfolding, or disorder of individual enzymes. The research team will develop and implement a framework that identifies first these enzymes and then their robust replacement enzymes from sets of extremophiles. Additionally, the team will apply a systems genomics approach to improve bioproduction under two distinct stressors—high temperatures and low intracellular pH—using *Escherichia coli* as the model organism. This approach is complementary to evolution-based improvement of microbial robustness because it incorporates existing knowledge.

The tasks span three length scales: (Aim 1) performance of metabolic pathways as enzyme networks, (Aim 2) enzyme sequence, and (Aim 3) functional output of metabolic pathways in the form of organism activity. Aim 1, metabolic systems modeling, will use flux balance analysis to model changes in system temperature and intracellular pH through enzyme inhibition and changes to reaction thermodynamics. To identify potential replacements for rate-limiting enzymes, Aim 2 will computationally assess enzymes from thermophilic organisms known to be robust under high temperature and low pH conditions. This approach utilizes the body of knowledge of protein sequence, structure, and evolution. Structures of sequence variants will incorporate entropy evaluations to assess protein stabilities. Aim 3 will build on previously developed organism engineering strategies by assessing the identified enzymes and replacements. Success will be judged by the sensitivity of product titer, rate, and yield to temperature or acidification. This approach represents a new path for building on existing knowledge by complementing existing organisms with enzyme replacements; it is also generic in terms of production organism and product identity.

Developing, Understanding, and Harnessing Modular Carbon/Nitrogen-Fixing Tripartite Microbial Consortia for Versatile Production of Biofuel and Platform Chemicals

- **Principal Investigator:** Xiaoxia Nina Lin (University of Michigan)
- **Collaborators:** Maciek Antoniewicz, Andrew Allman (University of Michigan), Sujit Datta (Princeton University), Jagroop Pandhal (University of Sheffield, United Kingdom)

Project Goal and Summary

Microbial communities are ubiquitous in nature, exhibiting incredibly versatile metabolic capabilities and remarkable robustness. Inspired by these synergistic microbial ecosystems, the rational design of synthetic microbial consortia is emerging as a new paradigm for bioprocessing and offers tremendous potential for solving some of the biggest societal challenges. This project focuses on a tripartite consortium consisting of a photosynthetic specialist that fixes carbon dioxide (CO₂), a second specialist that fixes nitrogen (N₂), and a third specialist that can convert the organic carbon and nitrogen generated by the first two specialists into a desired product. In addition to CO₂ fixation, a noteworthy

feature of this design is the elimination of the requirement for nitrogen fertilizer, whose production through ammonia synthesis using the Haber-Bosch process accounts for an estimated 2% of global energy expenditure.

The project's overall goals are to (1) develop a modular and flexible model system capable of producing diverse biomolecules (varying the C:N ratio) as advanced biofuel or platform chemicals, (2) dissect this complex ecosystem using a spectrum of cutting-edge systems approaches, and (3) ultimately derive scalable and broadly applicable design principles for maximizing system performance. Specifically, by integrating complementary expertise from multiple research labs at three institutions, the research team will pursue three specific objectives: (1) develop tripartite microbial consortia for carbon and nitrogen fixation and production of biomolecules with various C:N ratios, (2) investigate molecular and cellular mechanisms governing the tripartite consortia via omics studies and predictive modeling, and (3) explore alternative spatial configurations and develop scalable design principles.

Converting Methoxy Groups on Lignin-Derived Aromatics from a Toxic Hurdle to a Useful Resource: A Systems-Driven Approach

- **Principal Investigator:** Christopher Mark (University of Idaho)
- **Collaborators:** Jeremy Draghi (Virginia Polytechnic Institute and State University), N. Cecilia Martinez-Gomez (University of California, Berkeley), Andreas E. Vasdekis (University of Idaho)

Project Goal and Summary

Lignin-derived compounds are an abundant resource for bioproduction but are difficult to convert due to toxicity, including formaldehyde released from abundant methoxy groups. The research team discovered that *Methylorubrum extorquens*—a model system well known for dealing with formaldehyde during growth on methanol—grows very well on aromatics and does not release formaldehyde into the medium while doing so. Besides the well-characterized formaldehyde oxidation systems in this organism, the team recently discovered two additional functions: a complex formaldehyde stress response involving novel regulators and the unexpected involvement of lanthanide-dependent dehydrogenases. These functions are critical for effective vanillic acid conversion.

The goal of this project is to take advantage of the novel pathway the team discovered in *M. extorquens*. This pathway converts acetyl-CoA and β -ketoacid into acetoacetate and succinyl-CoA, thereby allowing carbon to flow efficiently into the species' high-flux glyoxylate-regeneration pathway. Whereas the team's target molecule is butanol, the same glyoxylate-regeneration pathway is naturally used to generate an internally accumulated compound, poly- β -hydroxybutyrate (PHB), that will be leveraged as a visualizable single-cell proxy for production capacity to guide the design process. In particular, the team developed a new optical method to assay PHB in single, live cells to examine the correlations between growth, stress response, and production in single cells. This capability is critical because these traits can vary tremendously, suggesting that stability, and not just catalytic capacity, may be critical to develop effective growth and production from these difficult feedstocks. As such, this project combines genome-scale approaches that will broadly identify the physiological hurdles that cells face in growth and production from vanillic acid, with a suite of single-cell approaches to examine phenotypic heterogeneity. In the process, this project aims to demonstrate a novel approach that embraces heterogeneity as a source of innovation in biosystems design.

Synthetic Metabolic Pathways and Biosensors to Expand Lignin-Based Bioconversion

- **Principal Investigator:** Ellen Neidle (University of Georgia)
- **Collaborator:** Ramesh Jha (Los Alamos National Laboratory)

Project Goal and Summary

Although extremely abundant and energy rich, the lignin portion of lignocellulosic biomass is underutilized. Therefore, the ability to convert this renewable resource to valuable compounds is an important environmental goal, as it will enable biorefineries to reach their full commercial potential and be competitive with petroleum-based industries. This project focuses on new methods and approaches to help accomplish this goal by harnessing the power of the soil bacterium *Acinetobacter baylyi* ADP1.

The research team will generate new ADP1 strains and enzymes to support lignin valorization efforts. Specifically, they will expand this bacterium's ability to degrade syringol,

a lignin-derived aromatic substrate. The premise of this approach is that natural microbial capabilities responsible for slow lignin degradation in nature can be engineered for increased metabolism in the laboratory. Expansion of syringol metabolism will also facilitate the metabolism of other aromatic compounds and enable the synthesis of wax esters, or bacterial storage compounds that have important commercial and industry uses. The team will construct a synthetic biological pathway and optimize it using novel biosensors and protein engineering methods.

ADP1 will be developed as a platform organism and as a generator of portable resources (enzymes and pathways) for use in other organisms. The team will also develop tools that will be broadly useful for metabolically engineering aromatic compounds, which are significant in efforts both to reduce fossil fuel consumption and to synthesize and degrade plastic polymers. Knowledge and resources from this project will be important not only for lignin biodegradation, but also for the bioeconomy in general.

Metabolic Modeling and Genetic Engineering of Enhanced Anaerobic Microbial Ethylene Synthesis

- **Principal Investigator:** Justin A. North (The Ohio State University)
- **Collaborators:** William R. Cannon (Pacific Northwest National Laboratory), Kelly C. Wrighton (Colorado State University)

Project Goal and Summary

Ethylene is the most synthesized industrial organic platform chemical. It is the foundation of several multibillion-dollar industries, particularly plastics. Annually, nearly 200 million metric tons of ethylene are produced worldwide, with demand steadily increasing. Ethylene is derived almost exclusively from fossil fuels by energy-intensive processes, resulting in substantial carbon emissions. Therefore, biological production of ethylene is necessary for a sustainable bioeconomy.

This project's long-term goal is to develop robust and efficient pathways for the microbial conversion of renewable lignocellulose and CO₂ feedstocks into impactful levels of ethylene. Many organisms use pathways requiring oxygen to synthesize ethylene, leading to combustion hazards. These pathways are circumvented by the newly discovered

anaerobic ethylene cycle, which enables oxygen-free conversion of CO₂ and lignocellulose to ethylene. Genes for a complete or partial anaerobic ethylene cycle are found throughout photosynthetic proteobacteria and lignocellulosic *Clostridia* species. Current metabolic models of the native ethylene cycle from the photosynthetic proteobacterium *Rhodospirillum rubrum* reveal a key flux constraint in all organisms: production of ethylene precursor compounds from methionine by tightly regulated secondary metabolic processes.

This project—based on the research team's discovery of a genetically regulated ethylene cycle and the key metabolic bottleneck that limits ethylene yields—will address three specific aims: (1) overcome the known flux and regulation constraints of the anaerobic ethylene cycle, (2) construct and employ systems-level predictive metabolic models of photosynthetic and lignocellulosic bacteria to identify points of regulation that support or compete with ethylene production, and (3) engineer photosynthetic and cellulolytic bacteria for high-yield ethylene production from CO₂ and lignocellulose. Ultimately, this project will result in engineered bacterial systems that produce robust ethylene yields from CO₂ and lignocellulose.

Engineering Synthetic Anaerobic Consortia Inspired by the Rumen for Biomass Breakdown and Conversion

- **Principal Investigator:** Michelle O'Malley (University of California, Santa Barbara)
- **Collaborators:** Christopher E. Lawson (University of Toronto), Scott Baker (Pacific Northwest National Laboratory)

Project Goal and Summary

Plant biomass (lignocellulose) is a widely abundant renewable resource that can be harnessed for value-added production of fuels and chemicals. While microorganisms have been engineered to break down plant waste and turn released sugars into products, this process remains energy-intensive and requires expensive pretreatment and separation steps. Furthermore, engineering into a single organism all desirable traits for breakdown and conversion is difficult.

This project will develop a new strategy to break down plant biomass inspired by the microbial partnerships found in the digestive tract of grazing herbivores (e.g., cows, goats, sheep). A diverse team of microbes in the rumen of large herbivores work together to liberate sugars from crude

plant biomass and convert that sugar to nutrients for the animal. These microbial consortia consist of fungi, bacteria, and archaea that form tight associations, which divide and conquer the difficult tasks of lignocellulose conversion.

The research team will leverage a “synthetic rumen” consortium composed of anaerobic fungi and chain-elongating bacteria to study which chemical metabolites are shared and exchanged between microorganisms and identify strategies to bolster lignocellulose conversion to value-added products. The project’s approach will develop high-throughput systems and synthetic biology tools to realize stable synthetic consortia that route plant waste into short- and medium-chain fatty acids (SCFAs/MCFAs) rather than methane.

Key research objectives are to (1) design and predict anaerobic fungal and bacterial consortia that efficiently convert plant biomass into MCFAs, (2) understand how cultivation parameters and microbe-microbe interactions regulate and drive microbiome metabolic fluxes, and (3) use genomic editing to alter the fermentation byproducts of anaerobic fungi and bolster MCFA titers and yields.

Cell-Free Systems Biology of an Atypical Glycolytic Pathway

- **Principal Investigator:** Daniel Olson (Dartmouth College)
- **Collaborators:** Costas Maranas (The Pennsylvania State University), Yannick Bomble (National Renewable Energy Laboratory), Daniel Amador-Noguez (University of Wisconsin, Madison)

Project Goal and Summary

Using lignocellulosic biomass to produce fuels and chemicals requires developing organisms that can effectively utilize it as a substrate. Cellulose-fermenting, anaerobic bacteria show great promise for this task. However, engineering them for high titer product formation is challenging due to several distinctive aspects of their metabolism. First, their glycolytic pathway differs from canonical glycolysis at several key steps, including phosphofructokinase and pyruvate kinase, suggesting substantial differences in regulation that interfere with both engineering and modeling efforts. Second, datasets with diverse metabolic fluxes are needed to parameterize kinetic models. In cellulolytic anaerobes, their limited substrate range (generally restricted to cellulose and

its hydrolysis products) and reliance on EMP glycolysis prevent the generation of these datasets by either gene deletions or feeding with different substrates, as is commonly done in model organisms.

To address these obstacles, this project will study *Clostridium thermocellum* metabolism using individual enzyme assays as well as cell lysates to characterize metabolic modules. Results from these experiments will be incorporated into a new multiscale kinetic model. Improved understanding will be demonstrated by *in vivo* production of 2,3-butanediol from cellulose.

The primary impact of this work will be an improved systems-level understanding of central metabolism in *C. thermocellum*, a representative of the cellulolytic anaerobes. This understanding will inform the use of this organism group as a platform for fuel and chemical production from lignocellulose. Demonstrating the utility of lysate-based cell-free systems for studying metabolism will lay the foundation for a new systems biology workflow responsive to the unique features of emerging model organisms.

Improving Bioprocess Robustness by Cellular Noise Engineering

- **Principal Investigator:** Gregory Stephanopoulos (Massachusetts Institute of Technology)
- **Collaborator:** Andreas E. Vasdekis (University of Idaho)

Project Goal and Summary

When observed with cellular or subcellular resolution, a clonal population exhibits cell-to-cell differences that range from morphological features to protein content, and, eventually, function and metabolism. This phenomenon, termed cellular noise, may seem insignificant at the global scale. However, its importance is ascertained when considering that most cell populations, natural communities, and infections start from a single or just a few cells. Research has established that cellular noise can confer robustness to a biological system, namely the ability to maintain function in the presence of environmental fluctuations. To this end, cellular noise imposes a nongenetic form of division of labor, and, as such, some cells of the population can express pathways that enable them to continue functioning in the new environment. While the effects of cellular noise have been extensively investigated in the context of microbial physiology and disease, knowledge of how to engineer cellular noise

and design robust biosystems in a synthetic biology context remains at its infancy.

To address this knowledge gap, this project is developing an integrative workflow that combines genome-wide editing methods, multiomic and single-cell analyses, and computational models. The project then applies this workflow to bestow robust cellulosic oil and alkane production in *Yarrowia lipolytica* under genuine industrial conditions that exhibit varying concentrations of toxic lignocellulosic hydrolysate inhibitors and temperatures. This approach involves two steps: (1) evolving *Y. lipolytica* by both rational and combinatorial means to obtain tolerant and overproducing variants; then (2) conducting multiomics analyses and constructing genome-wide metabolic kinetic models. These models subsequently guide noise-engineering efforts followed by robustness validation in programmable microfluidics and large-scale bioreactors. Overall, the proposed noise-engineering project is anticipated to not only yield robust biosystems, but also generate novel design principles that enable efficient bioproduction beyond standard laboratory settings.

Harnessing the Robust Metabolism of *Bacillus coagulans* for Efficient Conversion of Lignocellulosic Biomass Hydrolysates to Designer Bioesters

- **Principal Investigator:** Cong Trinh (University of Tennessee)
- **Collaborators:** Bruce Dien (U.S. Department of Agriculture, Agricultural Research Service), Richard Giannone (Oak Ridge National Laboratory)

Project Goal and Summary

The overarching goal of this project is to fundamentally understand and redirect the metabolism and regulation of thermophilic *Bacillus coagulans* for efficient conversion of undetoxified lignocellulosic biomass (i.e., switchgrass) hydrolysate sugars into designer bioesters, which have broad utility for fuels and chemicals. This project will address technical barriers and fundamental knowledge gaps to develop new technologies for domesticating a promising nonconventional microorganism for industrial biotechnology. Specifically, the research team will elucidate the underlying mechanisms behind *B. coagulans*' robustness and further optimize it for co-utilizing mixed sugars in inhibitory biomass hydrolysates to facilitate downstream targeted strain engineering. To achieve this end, adaptive laboratory evolution and systems analysis of wildtype and evolved strains

will be used in combination with metabolic and regulatory engineering strategies.

Furthermore, capitalizing on the endogenous overproduction of lactate in *B. coagulans*, this project will design a new class of ester-producing pathways derived from the pyruvate metabolic node for the synthesis of acetate and lactate-based esters. The team will use model-guided protein engineering to create novel thermostable enzymes and *de novo* metabolic pathways that function as exchangeable metabolic modules for effective biosynthesis of designer esters. Finally, the research team will use biosystems design based on a modular cell-engineering framework to construct *B. coagulans* strains that are highly compatible with mixed sugar consumption and rapid implementation of exchangeable ester production modules. Overall, this research is directed toward solving critical challenges in transitioning to sustainable fuels and chemicals. It will introduce the use of the promising thermophilic *B. coagulans* as a biomanufacturing platform for effective conversion of bioenergy biomass feedstocks (switchgrass) into designer esters for use in fuels and chemicals.

Engineering Bacterial Microcompartments in *Clostridium autoethanogenum* to Overcome Bottlenecks in Sustainable Production of Synthetic Rubber

- **Principal Investigator:** Danielle Tullman-Ereck (Northwestern University)
- **Collaborator:** Michael Köpke (LanzaTech)

Project Goal and Summary

One promising route to sustainable bioproduction of fuels and chemicals is the engineering of organisms, such as acetogens, to efficiently convert abundant and low-cost carbon monoxide or carbon dioxide and hydrogen-containing gases to desirable products at high efficiency and low cost. This approach not only provides an avenue for repurposing greenhouse gases, but also minimizes the necessity for harsh chemicals and hazardous byproducts common in petroleum-based processes. However, many biochemicals are not yet produced biologically due to roadblocks in the cellular biosynthesis process. These roadblocks can include toxicity of intermediates, redox imbalances, and/or product loss to off-pathway reactions. Nature uses spatial organization strategies, such as sequestration in organelles, to alleviate these issues. In bacteria, organization occurs

in protein organelle-like structures known as bacterial microcompartments.

This project has three objectives that explore the native regulation, assembly, and function of microcompartments in the industrially relevant nonmodel host *Clostridium autoethanogenum*. The objectives include the sequestration of key biosynthesis enzymes from two distinct metabolic pathways to make compounds involved in rubber production, showcasing the strategy's power for reducing toxicity and product losses due to side reactions. Finally, this work couples modeling with experiments to understand the native system and identify the most promising targets for compartmentalization. If successful, this work will (1) provide insight into the native function of these structures in this organism, (2) be the first direct demonstration of this feature of a bacterial microcompartment in a nonmodel organism, and (3) provide a detailed method for repeating this success in other organisms and with other pathways. Ultimately, this research will lead to cost-efficient production of petroleum-derived chemicals.

Systems Biology to Enable Modular Metabolic Engineering of Fatty Acid Production in Cyanobacteria

- **Principal Investigator:** Jamey Young (Vanderbilt University)
- **Collaborators:** Doug Allen (U.S. Department of Agriculture, Agricultural Research Service, and Danforth Plant Science Center), Bradley S. Evans (Danforth Plant Science Center), Carl Johnson, John McLean (Vanderbilt University), Brian Pflieger (University of Wisconsin, Madison)

Project Goal and Summary

Cyanobacteria are attractive hosts for biomanufacturing because of their ability to rapidly fix carbon dioxide (CO₂), grow in nutrient-poor environments, and produce renewable chemicals directly from photosynthesis. Unlike triacylglycerol production in green algae, production of free fatty acids (FFAs) using genetically engineered cyanobacteria results in secretion of the product into the culture medium where it can be efficiently recovered. However, there is a major gap in the understanding of how lipid metabolism is regulated in cyanobacteria that limits the ability to rationally engineer high-titer FFA production in cyanobacterial hosts.

The overall objective of this project is to use systems biology approaches to identify metabolic control points and

bottlenecks that regulate flux to FFAs in cyanobacteria. The project's central hypothesis is that cyanobacterial lipid metabolism can be modularized into pathways that are "upstream" and "downstream" of the nodal metabolite acetyl-CoA, which can be separately studied and optimized to enhance overall FFA production. The research team will apply a suite of systems biology approaches, including ¹³C flux analysis, metabolomics, lipidomics, proteomics, and CRISPRi screens, to rigorously define flux regulation within each module. As proof of principle, this modular approach will be applied to optimize cyanobacterial production of FFAs in the fast-growing, halotolerant strain *Synechococcus* sp. strain PCC 7002.

The rationale for the proposed research is that a deeper understanding of how fatty acid flux is regulated upstream and downstream of acetyl-CoA will enable integrated "push-pull" metabolic engineering strategies to produce lipid products directly from photosynthetic CO₂ fixation in cyanobacteria. This research will directly contribute to DOE's mission by advancing efforts in biological production of renewable fuels that do not compete with agriculture.

The Whole is Greater than the Sum of Its Parts: Multiscale Modeling and Engineering of Microbial Communities for Next-Generation Bioproduction

- **Principal Investigator:** Karsten Zengler (University of California, San Diego)
- **Collaborator:** Michael Guarnieri (National Renewable Energy Laboratory)

Project Goal and Summary

Interspecies metabolic exchanges directly influence the functional organization of microbial communities, where each member fulfills specific functions according to its niche. This metabolic collaboration thereby allows the community to achieve functions that are unreachable by axenic community members, thus directly affecting the ecosystem. Effective and robust conversion of complex biomass in nature is always performed by multifaceted communities. While evolution has clearly favored communities to generate short chain fatty acids from complex biomass, the vast majority of bioproduction processes have been focused on single-organism systems. Through both top-down and bottom-up approaches, this project seeks to vastly increase scientific understanding and design capabilities for complex multiorganism systems to enable robust biomass deconstruction and conversion.

By unraveling the underlying principles and contributions of individual organisms to optimized functionality in complex communities, this project will develop a knowledgebase and working pipeline that enables microbial community design for producing advanced biofuels and bioproducts from biomass. At the foundational level, the research team will leverage a mutualistic co-culture consisting of *Clostridium thermocellum* and *Clostridium thermobutyricum* for the highly effective conversion of lignocellulosic biomass to butyric acid. The team will unravel the metabolic interactions of this co-culture and augment it with additional microbes to further increase the versatility of variable biomass conversion, stability against perturbation, and improvements in bioproductivity. Moreover, the team will design engineering strategies for increased performance of this community, resulting in a complete conversion of biomass including deconstruction and metabolic assimilation of sugars to generate butyric acid. The primary objective of this project is to elucidate the fundamental mechanisms driving commensal biomass conversion and to define community design principles. Lessons learned will be crucial for designing stable microbial communities for various biotechnology applications in the future.

A Gene-Editing System for Large-Scale Fungal Phenotyping in a Model Wood Decomposer

- **Principal Investigator:** Jiwei Zhang (University of Minnesota)
- **Collaborators:** Jonathan Schilling (University of Minnesota), Daniel Cullen (University of Wisconsin, Madison), Igor Grigoriev (Lawrence Berkeley National Laboratory), Allison Thompson (Pacific Northwest National Laboratory)

Project Goal and Summary

This project aims to combine system biology approaches and gene-editing to develop a high-throughput genetic platform for large-scale phenotyping in a model wood-decomposer fungus relevant to DOE mission areas. Wood decay fungi offer industrially relevant pathways to extract carbohydrates from lignocellulose, and their mechanisms have broad relevance to global carbon cycling. Among these organisms, brown rot fungi evolved “upgraded” degradative pathways compared to others. These fungi use a two-step mechanism (oxidation then hydrolysis) to degrade wood to quickly and selectively release soluble sugars, but they leave lignin relatively intact as a byproduct. Although the scientific community has made genomically informed advances in the last decades in brown rot fungi, progress is limited by an inability to manipulate genes in any brown rot fungal

strain. Targeted gaps remain for understanding the brown rot genetic mechanism.

First, multiomics approaches have advanced the knowledge of the two-step mechanism, but the gene functions involved remain unverified and ambiguous. Second, brown rot fungi have adapted distinct gene regulatory mechanisms to precisely control and consolidate the two steps during wood decay, but little is known about this process. This knowledge gap limits scientists’ ability to refer to brown rot gene functions from other fungi with known decay pathways. Third, most genes identified by multiomics are of hypothetical or unknown function, leaving major gaps for discovering novel functional genes.

This project will integrate systems biology, genome-editing, and network modeling to address these key gaps. The goal is to provide stand-alone tools and resources for discovering novel fungal genetic features that can also be used in combination to advance relevant brown rot research in the post-genomic era. This will allow scientists to rapidly link genotype to the phenotypes that enable brown rot efficacy. The work will aid in the development of high-throughput genetic tools to elucidate fundamental microbial processes, advancing new engineering designs for lignocellulose bioconversion.

Optimizing Enzymes for Plastic Upcycling Using Machine Learning Design and High-Throughput Experiments

- **Principal Investigator:** Nicholas Gauthier (Dana-Farber Cancer Institute)
- **Collaborators:** Chris Bahl (Institute for Protein Innovation), Gregg Beckham (National Renewable Energy Laboratory), Debora Marks (Harvard Medical School), Chris Sander (Dana-Farber Cancer Institute), David Weitz (Harvard University)

Project Goal and Summary

Plastic use is ubiquitous in the modern world. In particular, polyethylene terephthalate (PET), which is used in plastic bottles, is one of the most abundantly produced plastics, with ~65 million metric tons manufactured annually. As with many plastics, mechanical and chemical PET deconstruction and upcycling are costly and inefficient. Recently, biological enzymes capable of breaking down PET into its basic building blocks have garnered significant interest as a sustainable solution to the plastic challenge. These enzymes are undergoing pilot studies for implementation in enzyme-based recycling. However, there are significant limitations

to current enzymes, including the need to perform costly preprocessing of plastic waste before the enzymes can work. Further optimization of these enzymes is necessary to make the process profitable, thereby incentivizing commercialization of this biology-based recycling technology.

This project aims to apply machine learning to design new enzymes capable of breaking down PET. Based on preliminary experiments, the research team intends to create a diverse set of enzymes with exceptional properties useful for industrial recycling. Testing these enzymes is typically labor intensive, but the use of a robotically-enabled platform will allow the team to experimentally characterize key enzymatic properties of thousands of designed enzymes. Moreover, the team will be able to optimize existing enzymes known to break down plastic by using a novel method it developed to test many small changes in enzyme structure. Ultimately, knowledge gained from this project will result in highly optimized enzymes capable of breaking down PET plastics in an industrial recycling setting, enabling a green solution to the plastic problem.

SynThetic BiolOgy Driven Approach to Repurpose PolyAmides (STORM)

- **Principal Investigator:** Kate Kucharzyk (Battelle Memorial Institute)
- **Collaborators:** Jacob Lilly, Morgan Evans (Battelle Memorial Institute), Joshua Britton (Debut Biotechnology), Jaydeep Bardhan (Pacific Northwest National Laboratory)

Project Goal and Summary

The environmental and economic footprint of Nylon 6 (PA6), a synthetic polymer recalcitrant to degradation, could be reduced through polymer degradation and upcycling. Little is known about PA6 biological degradation, and no direct enzymatic degradation pathway has been identified. The STORM project will generate a degradation pathway for PA6, for which there is currently no effective disposal technology beyond incineration or removal to landfills. The research team will use advancements in systems biology approaches, computational modeling, sequence-based homologue searching, and molecular docking to elucidate the basis for PA6 biological degradation using several target enzymes classes. By identifying and characterizing enzymes capable of degrading PA6, the project aims to advance the science of polymer upcycling. Because the technology used will be enzymatically and biologically produced, the associated cost and environmental footprint will be minimal.

The team will investigate a direct relationship between PA6 material morphology, topology, and biodegradation efficiency using modeling approaches and experimental testing. The intercept between polymer morphology and enzyme kinetics will enable the research team to propose a model pathway for PA6 degradation previously unattainable by biological systems.

STORM contains both polymer degradation and polymer morphology modeling approaches. For degradation, the research team will: (1) integrate computational modeling, (2) elucidate the biological catalyst for degradation via homologue searches and evolutionary analyses, (3) conduct testing of enzyme efficacy, and (4) optimize enzyme expression in model organisms. Enzymes of interest will be fed into enzyme-substrate models to down-select effective enzymes. The team will use homology searches and alignments to identify target enzymes in currently available organisms. Laboratory testing will be performed on the down-selected enzymes to determine degradation efficacy. These enzymes will then be engineered into a model organism for overexpression capable of degrading PA6 at higher rates. This work will provide the first known technology for degradation and upcycling of recalcitrant PA6 polymers.

Discovery of Distributed Pathways for Plastic Conversion in the Yellow Mealworm Microbiome

- **Principal Investigator:** Kevin Solomon (University of Delaware)
- **Collaborators:** Mark Blenner (University of Delaware), Aaron Wright (Pacific Northwest National Laboratory), and Andrea Steiger (Pacific Northwest National Laboratory)

Project Goal and Summary

This project aims to develop enhanced minimal consortia and microbial platforms for the upcycling of plastics derived from the digestive tract of the yellow mealworm (larvae of *Tenebrio molitor*). Microbial communities living in the digestive tracts of insect larvae can rapidly process a wide array of dirty plastics without pretreatment. The yellow mealworm microbiome is particularly versatile, degrading recalcitrant polyethylene post-consumer wastes in addition to other plastics. However, little is known about the molecular mechanisms used by these microbial communities to degrade wastes, the role of various microbes in metabolism,

or the importance of relationships between bacteria, fungi, and archaea within these communities.

This project uses top-down and bottom-up approaches to probe the function of these complex communities and develop efficient systems for the processing of various plastics. The research team will enrich for microbes and proteins involved in plastics degradation through growth selective pressure in the mealworm and molecular “fishing” strategies with engineered chemical “bait” that resembles plastics. Enriched populations will be profiled with integrated omics and biochemical analyses to identify novel enzymes for plastics degradation. In parallel, the team will pursue bottom-up reconstruction of plastic-degrading communities to tease out design rules for plastics degradation. Finally, identified enzymes and plastic degradation pathways will be tested and optimized for efficient plastic waste processing in engineered microbes and cultivated microbiomes. Ultimately, research results will lead to sustainable biological methods for the degradation of untreated, mixed plastic wastes and enable their upcycling to new products.

Novel Enzymes and Synthetic Metabolic Pathways for Complete Degradation and Upcycling of Recalcitrant Polyamides

- **Principal Investigator:** Alexandre Zanghellini (Arzeda Corporation)
- **Collaborators:** Chris Voigt (Massachusetts Institute of Technology), Jacob Bale (Arzeda Corporation)

Project Goal and Summary

As of 2015, 6.3 billion tons of plastic waste have been generated globally. According to estimates, only 9% of this total has been recycled, while 12% has been incinerated to recover energy values. The rest entered landfills or the natural environment. New technologies are needed to address this ever-growing problem. While closed-loop recycling methods offer new potential routes for dealing with plastic waste, competition with cheap, fossil fuel-derived precursors is likely to inhibit progress on this front for the foreseeable future. An alternative approach involves harnessing the power of biology to not only depolymerize plastics back to their monomer precursors, but also convert them into higher-value products. This approach offers stronger economic incentives and, in turn, would be expected to drive more rapid and widespread adoption.

This project proposes to combine computational protein design and synthetic biology to address the problem of biodegradation and upcycling of Nylon 6 and Nylon 66. Although natural enzymes can degrade amorphous portions of polyamides such as Nylon 6 and Nylon 66, complete enzymatic degradation has not been demonstrated. The inability to achieve complete degradation is likely due to a lack of natural amide hydrolases with active sites “open” enough to bind the crystalline portion of the polymer. This project aims to computationally design hydrolase enzymes to alleviate this limitation by introducing polyamide hydrolysis activity into scaffolds with open active sites.

In parallel, the research team will screen and engineer bacterial strains that can metabolize Nylon 6 and Nylon 66 degradation byproducts directly into central metabolism. Once achieved, such platform strains can be used to produce a wide variety of fermentation products from central metabolites. Integrating this project’s designer Nylon 6 and Nylon 66 depolymerizing enzymes into these engineered hosts will provide a novel, elegant, and cost-effective consolidated fermentation process for nylon upcycling to higher-value sustainable materials.

Developing a Consolidated Biological Process to Upcycle Plastics

- **Principal Investigator:** Tae Seok Moon (Washington University)

Project Goal and Summary

The goal of this project is to solve the plastic waste crisis by re-designing the global plastic economy to upcycle plastic wastes into high-value compounds rather than discarding or simply recycling them. To this end, the research team aims to provide an innovative strategy to valorize waste polyethylene terephthalate (PET). Specifically, they will develop the foundational knowledge for effectively depolymerizing PET by discovering and engineering novel PET-degrading enzymes (PETases). The team will also develop a transformative consolidated process that does not require costly PETase purification by engineering a bacterial strain to both secrete PETase and use the depolymerized monomers. Finally, they will integrate the engineered strain's PET utilization ability with its high-value compound production ability.

This project is motivated by the team's discovery that a salt-tolerant *Rhodococcus* strain (called RPET) can grow well on the alkaline hydrolysis products of PET as the sole carbon source without any purification step. Research indicates that some microbes can use either terephthalic acid (TPA) or ethylene glycol (EG), but microbes that can use both PET monomers have rarely been reported. Although the team's novel strain enables a two-step process consisting of PET alkaline or enzymatic hydrolysis and biological conversion of the hydrolysis products by RPET, a consolidated biological process will minimize the use of sodium hydroxide (NaOH) and purified PETase. However, engineering

efficient mesophilic PETases is challenging despite some recent advances in PETase engineering that generated highly active thermophilic PETases. To the team's knowledge, there is no thermophilic TPA/EG-utilizing microbe for which efficient genetic engineering tools are available to develop a commercially viable PET upcycling process.

To address these gaps, this project will (1) discover and create both mesophilic and thermophilic PETases by leveraging the team's demonstrated approaches, including synthetic metagenomics and protein modeling-assisted directed evolution, and (2) develop a protein secretion system in mesophilic RPET. To better understand and engineer this novel chassis organism, the team is examining the complexity of TPA degradation pathways. Their preliminary analysis revealed redundant TPA pathways in RPET, explaining RPET's robust growth at extremely high TPA concentrations. To further enhance understanding of RPET's complex metabolic and regulatory networks, the team will provide a pathway map, starting from TPA, along with relevant regulators. Based on this detailed understanding, the project will culminate with demonstrating the consolidated process that converts PET into value-added products. As a proof-of-concept demonstration, the team will produce muconate and polyhydroxyalkanoate from PET as the sole carbon source. These two products were chosen to demonstrate the generalizability of the team's PET-based process by diversifying the product range instead of providing a commercially viable PET valorization process. Such a commercially relevant process will help drive the U.S. economy and reverse negative environmental impacts.