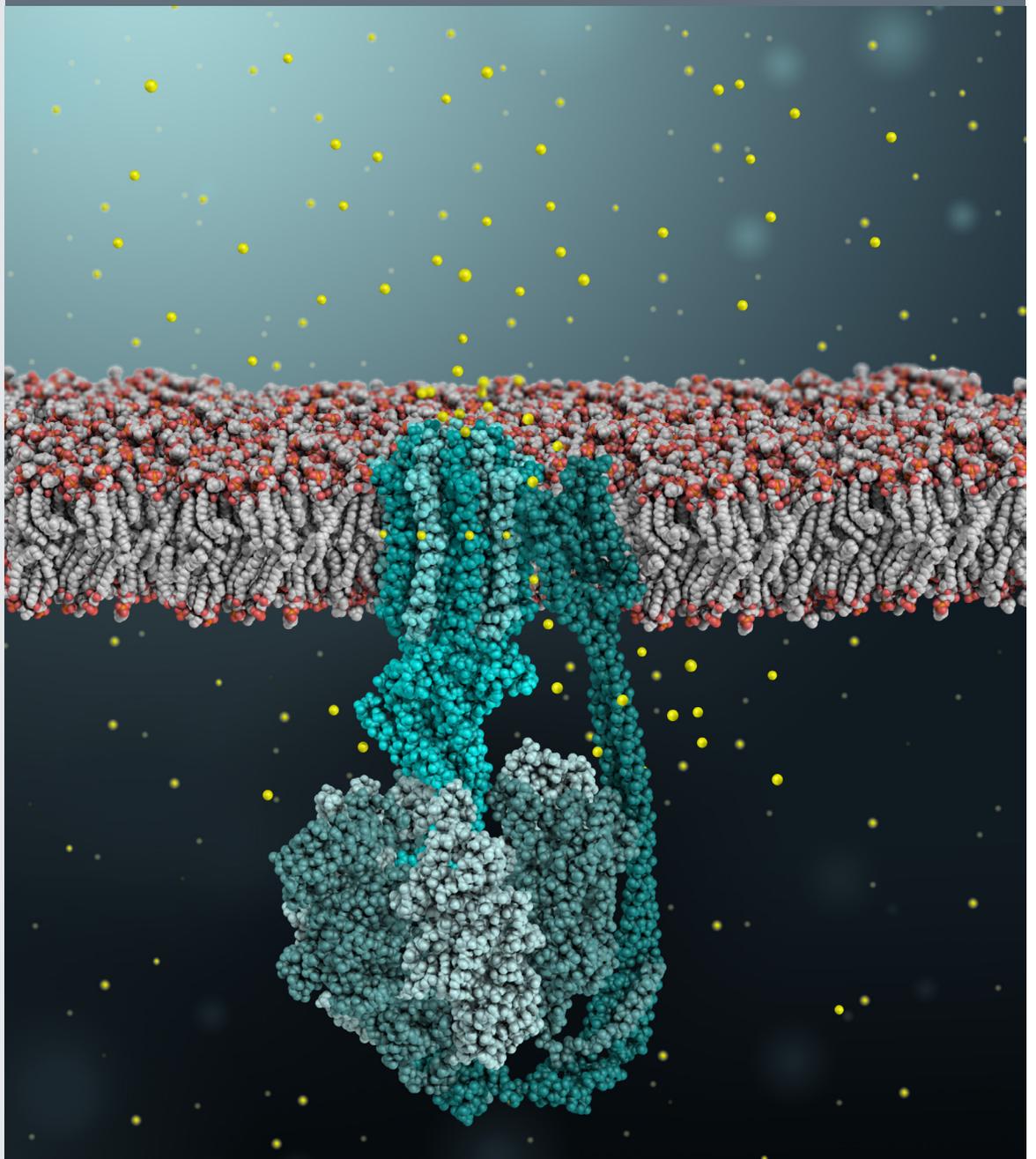


Cell-Free Synthetic Biology and Biocatalysis:

Prototyping and Conversion Platforms

Listening Day Summary Report

October 2018



About the cover:

The image was produced from 5T4Q.pdb by Max Fairlamb and Bret Freudenthal, University of Kansas Medical Center.

The image depicts the enzyme ATPsynthase in a membrane. The enzyme system could operate across a cell-free membrane system to generate the energy storage molecule adenosine triphosphate (ATP) from adenosine diphosphate (ADP). ATPsynthase works to drive the energetically unfavorable synthesis by coupling it to an electrochemical gradient created by a proton (yellow spheres) difference across the membrane.

Preface

The U.S. Department of Energy's (DOE's) Office of Energy Efficiency and Renewable Energy (EERE) invests in a diverse portfolio of technologies to ensure domestic energy security, continued economic competitiveness, environmental sustainability and the availability of cleaner fuels and power.

This report summarizes the input received from attendees of a public listening day sponsored by DOE/EERE in Denver, Colorado, on July 30, 2017. The views and opinions of the listening day attendees, as summarized in this document, do not necessarily reflect those of the United States government or any agency thereof, nor do their employees make any warranty, expressed or implied, or assume any liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product or process disclosed, or represented that its use would not infringe upon privately owned rights.

Summary Report from the July 30, 2017,
Cell-Free Synthetic Biology and Biocatalysis Listening Day in Denver, Colorado

Listening Day and Summary Report Sponsored by the U.S. Department of Energy
Office of Energy Efficiency and Renewable Energy
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Introduction

Continued advances in the design and engineering of new pathways to convert renewable biomass and waste carbon dioxide into valuable fuels, products, and materials are needed to ensure future economic prosperity and environmental sustainability in an increasingly resource constrained world. New tools and technologies are being developed, and a greater understanding for and ability to manipulate cellular biochemistries are presenting new opportunities to enhance conversion and process reaction efficiency. One potentially promising approach being developed is to use the machinery inside microbial cells to perform targeted biochemical conversions without the need for the whole cell. As will be discussed, “cell-free” biotechnologies present numerous opportunities to improve efficiency and add capabilities for pathway engineering. Thus, there is significant interest in eliciting input on how best to develop these technologies.

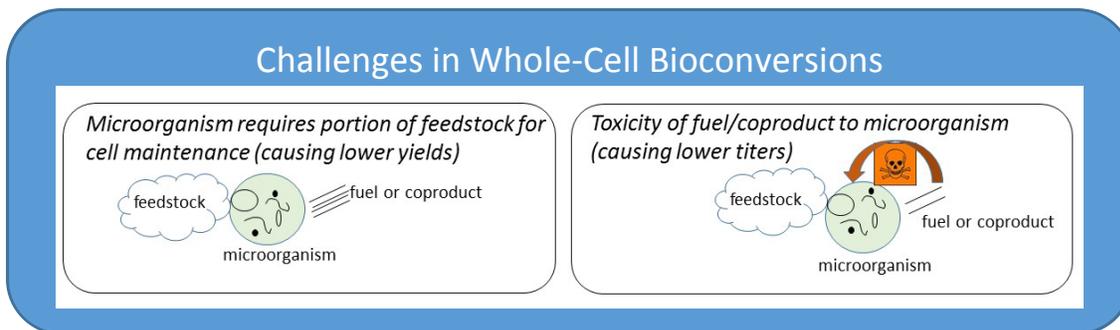


Figure 1 – Some identified challenges in whole-cell bioconversion that could be addressed with the use of cell-free technologies include feedstock diversion to microorganism metabolism and maintenance as well as microorganism poisoning due to the accumulation of toxic products.

On July 30, 2017, the US Department of Energy (DOE’s) Bioenergy Technologies Office (BETO; the Office) within the Office of Energy Efficiency and Renewable Energy hosted a listening day in Denver, CO, to discuss research and development (R&D) opportunities related to cell-free synthetic biology and biocatalysis. Cell-free systems are in vitro biochemical technologies where enzymes and/or other biological material is extracted from an organism and used outside the host organism. A major advantage for cell-free systems is increased carbon utilization compared to whole-cell biological conversions. However, beyond potential opportunities to improve system carbon conversion efficiency, cell-free synthetic biology and biocatalysts could create many new tools for conversion pathway engineering and offer numerous additional benefits. In fact, leveraging cell-free prototyping techniques to increase the rate of design-build-test-learn cycles, avoid product accumulation toxicity during conversion, and enable novel synthetic biochemistries allowing non-homologous catalytic enzymes to be combined in new pathway designs are just some of the additional opportunities identified.

The concept of cell-free biocatalytic systems dates to Buchner’s Nobel Prize in chemistry in 1907, for discovering cell-free fermentation. More recently, the tools of synthetic biology have been applied to cell-free systems. Experts in the literature refer to this novel technology area as cell-free synthetic biology. The authors of this report colloquially refer to cell-free synthetic biology as “cell-free” throughout this report. The objective of this listening day was to examine how these advanced cell-free techniques could be leveraged in the development of biofuels and bioproducts. Thirty-three attendees from academia, DOE national laboratories, other federal agencies, and industry provided information on the state of the technology; Fig. 1 shows the breakdown by organization.

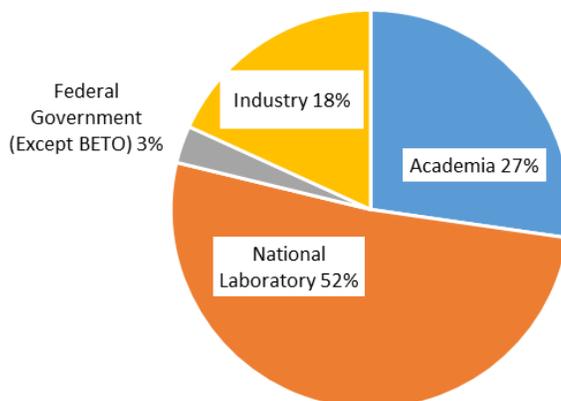


Figure 2. Listening Day Attendee Affiliations

Listening Day Concept and Process

BETO partners with technology developers to support applied R&D aimed at achieving a thriving bioeconomy that efficiently leverages non-food sustainable biomass feedstocks to produce renewable biofuels, biopower, and bioproducts. The Conversion R&D program within BETO supports applied R&D in feedstock deconstruction, intermediate upgrading, and enabling technologies (e.g., design-build-test-learn biomanufacturing; separations; reactor modeling via computational physics and chemistry). BETO investments have been used to develop dozens of cellulases and hemicellulases to convert oligomeric sugars to monomers in a cell free manner, and has adopted one use of cell-free in BETO design cases for biochemical conversion that assume enzymatic hydrolysis for feedstock deconstruction, where *T. reesei* secretes cellulase enzyme to breakdown cellulose into cellulosic sugars.¹ However, BETO has not comprehensively invested in developing cell-free conversion systems to do extensive transformations or product upgrading.

The use of a single enzyme or a cocktail mix secreted from one or more microorganism(s) and used in a hydrolysis reactor may not be the end of opportunities for cell-free to support the bioeconomy. The field of cell-free biological systems started achieving market impact several decades ago in the pharmaceutical, food and beverage, and, as mentioned, biofuel industries. Several technology trends have spurred interest in advanced applications of cell-free synthetic biology: most notably, the development of synthetic biology as a multi-disciplinary platform for optimizing organisms; additionally, materials science for bioreactor design.

BETO recognizes that emerging synthetic biology tools are increasing the potential of cell-free biological systems to advance the bioeconomy and so prioritized hearing input from stakeholders on the topic. Initially when planning the listening day, BETO considered two potential ways that cell-free could support the bioeconomy: 1) cell-free prototyping (collecting the enzymes of an organism or organisms, studying metabolic pathways over many different test conditions, identifying the optimal system, and then using the tools of metabolic engineering to promote, edit, and/or delete genes so that the organism behaves more similarly to the optimal cell-free experiment) and 2) conversion (developing stand-alone conversion platforms that utilize cell-free bioreactors). While planning the listening day, it became clear that the second option could be separated into two sub-options: 2a) cell-free free-enzyme conversion (i.e., use of cell lysates or purified enzymes) and 2b) cell-free scaffold conversion (i.e., mobile feedstock in a bioreactor with surfaces fixed with the enzymes and other biocatalytic material; somewhat analogous to bed-type heterogeneous reactors common in inorganic catalysis).

The listening day format included invited presentations, open talks from attendees, and facilitated meetings. The day began with four 25-minute overview presentations from invited technical experts. The experts focused on cell-free for prototyping, cell-free free-enzyme conversion, and cell-free scaffold conversion. Following these plenary presentations, a segment of “3x5” presentations featured 10 open-invite participants who each presented three slides for five minutes on cell-free systems.

The afternoon included a breakout session, in which the participants were separated into groups to cover the cell-free synthetic biology topics: cell-free prototyping, cell-free free-enzyme conversion, and cell-free scaffold conversion and stabilized biocatalysis. Each group was led by a facilitator that guided the breakout session and was supported by a BETO subject matter expert. Facilitators and scribes captured notes to record the stakeholder input provided. During the breakout session, attendees discussed challenges, opportunities, and resources needed to advance the state of technology related to each of the three sub-topics covered. Facilitators led standard working meeting activities such as brainstorming, sorting, and prioritizing ideas using notecards. After each of the three sessions, a pre-selected rapporteur for each group provided a 5-minute summary to all breakout session participants.

¹ <https://www.nrel.gov/docs/fy15osti/62498.pdf>

Summary of Presentations

Plenary Presentations

Purified Cell-Free Systems as a Metabolic Engineering and Biochemicals Production Platform

Dr. Joseph Rollin gave the first presentation. Dr. Rollin is currently a postdoctoral researcher at NREL, supported by a NREL Director's Fellowship. He also works as a Special Government Employee for DOE Advanced Research Projects Agency - Energy (ARPA-E). Previously, he co-founded Cell-Free Bioinnovations. Dr. Rollin provided some perspectives on the opportunity for biomass, citing that 9% of U.S. energy use is for non-light duty transportation and over 5% of U.S. energy use is for chemical production. Biomass can serve as the feedstock for these carbon-based materials, offering potential carbon-efficient and energy-efficient conversions, delivering novel platform molecules, and utilizing waste streams. While microbial (whole-cell) fermentations are the prevailing biomanufacturing platform, Dr. Rollin suggested that cell-free conversion offers a number of potential advantages. These include: faster reaction rates, broad reaction conditions (solvent tolerance; tolerance for a range of inhibitors/toxins, temperatures, and pH), and complete orthogonality (allowing for high yields at the refinery). Key challenges cited were unstable, expensive cofactors, which would require pathway design and cofactor engineering to overcome.

Dr. Rollin provided some additional context for cell-free, noting that Eduard Buchner won the Nobel Prize in Chemistry in 1907 for cell-free ethanol fermentation. Yeast cells were ground and filtered, and the filtrate, heavy with enzymes, was added to sterilized sugar cane juice. The juice and filtrate were allowed to ferment. Dr. Rollin illustrated some more recent work, including work of Sutro Biopharma and Greenlight Biosciences, two start-up firms.

Lastly, Dr. Rollin presented on recent research conducted by his group using cell-free systems conducted on a range of feedstocks, including sugars and lignin. The lignin work is focused on biological funneling of lignin derived monomers to the intermediate protocatechuic acid and the subsequent conversion to central metabolites such as pyruvate and oxaloacetate. The production of pyruvate from protocatechuic acid has been demonstrated by researchers at NREL and elsewhere in microbial fermentations using *P. putida* KT2440. As it pertains to cell-free bioconversion, the pathway has been recreated using purified enzymes responsible for this biochemistry. Through the addition of seven enzymes, Dr. Rollin's group has demonstrated the production of pyruvate. Further, by modeling the reactions using Michaelis-Menten kinetic rate equations, the production of pyruvate can be predicted with good accuracy. Ultimately, through the replication of the lignin biological funneling pathway in a cell-free system, this work can inform:

- Kinetic parameters at the reaction and system level
- Strain improvements that could be used in the whole-cell fermentation (e.g., enzyme expression levels, enzyme variants)
- Whether a cell-free bioconversion pathway can exceed performance of whole-cell fermentations.

Cell-Free Systems for Prototyping and Biodiscovery

Dr. Zachary Sun presented on "Cell-Free Systems for Prototyping and Biodiscovery." Dr. Sun is co-founder and Chief Executive Director of SynvitroBio, Inc., a start-up company working to commercialize cell-free prototyping technologies. SynvitroBio, Inc. has received funding from the Defense Advanced Research Projects Agency (DARPA), the National Science Foundation (NSF), the National Institutes of Health (NIH), and Lawrence Berkeley National Laboratory's Cyclotron Road.

Dr. Sun discussed efforts on coupled translation-translation cell-free systems as a simplified platform for engineering biological pathways and for biodiscovery of novel chemicals. The general design is to cycle through cells to determine the best carrier to produce a desired protein. Dr. Sun pointed out that the company can work with eukaryotic cells. The biggest obstacle is cofactor regeneration, Dr. Sun said.

Synthetic Biochemistry: Making Biofuels and Commodity Chemicals the Cell-free Way

Dr. James Bowie is Professor and Vice Chair, Department of Chemistry and Biochemistry, University of California, Los Angeles, and a member of the Journal of Molecular Biology editorial board. Dr. Bowie won the 2017 Biophysical Society Anatrache Membrane Protein Award, was the Protein Society President from 2013 to 2015, and founded and co-chaired the Gordon Conference of Membrane Protein Folding in 2015. Dr. Bowie presented on “Synthetic Biochemistry: Making Biofuels and Commodity Chemicals the Cell-free Way.” The main advantages cited of cell-free systems, as opposed to whole cell fermentations, were high yields, easy optimization, the ability to redesign central metabolism, reduced impact of inhibitory/toxic compounds, easier product purification, and potential for much higher productivity. Dr. Bowie cited enzyme costs, stability, and challenges with cofactor recycling and maintenance as the key barriers to adoption of these systems by the biotechnology community.

A major focus of Dr. Bowie’s presentation was on the subject of cofactor recycling and maintenance. In central metabolism, the conversion of pyruvate to Acetyl-CoA generates one reducing equivalent (either nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate hydrogen (NADPH)). Biological systems have evolved to regulate these redox cofactors through other reactions elsewhere in metabolism. Conversely, any cell-free bioconversion pathway that requires the use of cofactors would require these be supplied to the media and a means of purging any excess that might be generated over the course of the cell-free fermentation. This would render the economics completely infeasible. Dr. Bowie’s research group presented on self-regulating solutions to maintain levels of nicotinamide adenine dinucleotide phosphate (NADP⁺), NADPH, adenosine diphosphate (ADP), and adenosine triphosphate (ATP). Thus, only a small amount of these cofactors needs to be supplied in the initial media in order for the reactions to proceed.

Following the discussion of these cofactor maintenance solutions, Dr. Bowie presented several case studies that utilize these systems. A first example was presented on the production of limonene, a ten-carbon terpene. Starting from glucose, Dr. Bowie’s research team was able to produce greater than 11 g/L of limonene at a yield of 88%. Notably, limonene is toxic to cells at levels of less than 5 g/L, presenting a key barrier to the biochemical synthesis of this and related terpenes through microbial fermentations. The use of the in vitro cell-free approach circumvents these toxicity challenges.

Illustrating the Co-factor Imbalance Challenge

Attendees frequently commented on challenges related to co-factor imbalances for cell-free synthetic biology. This challenge is not found in whole-cell fermentations, where the microorganism can properly produce co-factors and leverage mechanisms to maintain co-factor balancing. See Figure 3 for a representation in the case of glycolysis.

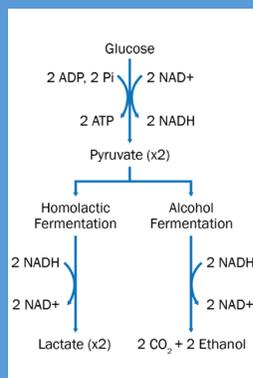


Figure 3. Representative schematic illustrating co-factor accumulation challenges. In the metabolic pathway of glycolysis, glucose is converted to pyruvate in 10 enzyme-catalyzed reactions (individual reactions not shown). The reaction steps require 2 ADP and 2 [P]_i, which are converted to ATP. The reaction steps also require 2 NAD⁺, which are converted to NADH. If pyruvate is converted via homolactic fermentation to lactate, or via alcohol fermentation to ethanol and CO₂, the NADH is required and converted to NAD⁺. This leaves an imbalance in ADP, [P]_i, and ATP. Without support, there will be unmet demand for ADP and [P]_i. Also, there will be feedback inhibition due to excess ATP.

A second example demonstrated the production of isobutanol. Isobutanol is another product that due to its toxicity mechanism (disruption of the cell wall) presents significant challenges for microbial fermentations. Typical fermentations for butanol and isobutanol encounter cell viability challenges at titers of 15-20 g/L. Through a cell-free approach, Dr. Bowie’s group was able to achieve isobutanol concentrations of 24 g/L at a yield of 91% in a 2-day fermentation. These results illustrate the potential promise of cell-free bioconversion in overcoming some of the challenges present in microbial fermentations.

Dr. Bowie noted that toxic end product can lead to reduced titers of the desired product. An example for a win in cell-free synthetic biology suggested was phase separation of isobutanol.

Bioprocess Intensification at the Intersection of Biology and Advanced Manufacturing

Dr. Sarah Baker is a Research Scientist at the Materials Science Division, Lawrence Livermore National Laboratory. Dr. Baker specializes in materials chemistry, with an emphasis on surface chemistry, nanomaterials, and the synthesis of biologically active materials. She has led projects on electrochemical and optical biosensor development, and synthesis of materials for encapsulation of enzymes for energy applications. She presented on “Bioprocess Intensification at the Intersection of Biology and Advanced Manufacturing.”

Dr. Baker explained that first generation technology is immobilization of the enzyme or enzymes involved in a given cell-free conversion strategy. This would include strategies such as adsorption or crosslinking with the main objective being continuous fermentation. Second generation technology would be the encapsulation with an aim of extending the lifetime of the enzymes. Third generation technology seeks rational design of the cell-free systems with materials science; see Fig. 2. The aim of this strategy would be to improve engineering parameters such as mass, heat, or electron transfer. At the same time, these novel reactor geometries would maintain low aeration and operation costs by significantly increasing the available surface area.

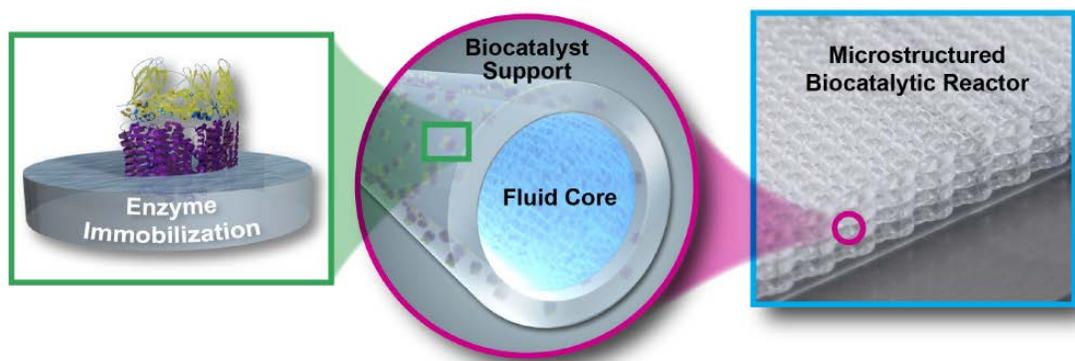


Figure 4. Potential Novel Bioreactor Designs Compared to Traditional Designs; Image courtesy Sarah Baker, Lawrence Livermore National Laboratory

Dr. Baker presented some exemplary data wherein a particulate methane monooxygenase enzyme was printed into a reactor. When the reactor was fed methane gas, the printed reactor demonstrated enzyme activity and production of methanol at levels comparable to those of whole-cell methanotrophs. While reducing equivalents had to be provided to the media in order to drive the reaction, this data shows proof of concept that reactor geometries can be optimized in concert with the enzymatic systems.

The presentation concluded with the identification of several research needs in the space. First, in regards to economics, an advanced manufacturing approach needs justification by identifying the cost and value of surface area gained. Second there is the need for highly stable enzymes that could last for months as opposed to days. Also there is the need for solutions to recycle or eliminate the use of reducing equivalents or other cofactors. And finally, comprehensive understanding of the kinetics and materials properties is necessary to optimize the enzymatic kinetics with reactor design itself.

Participant Presentations

Following the plenary presentations, listening day attendees were invited to give brief, five minute presentations to discuss their own experiences with cell-free systems and to broadcast their work more broadly. A total of ten presentations were given including:

- Sensors Caged in Droplets for Cell-free Synthetic Biology; Genome Science at Los Alamos National Laboratory; and Core Catalysis Capabilities, Dr. Amanda Barry, Los Alamos National Laboratory
- Enhanced In Vitro Biosynthesis with Nanoparticle-Enzyme Bioconjugates, Dr. Scott Walper, U.S. Naval Research Laboratory
- Systems for Advanced Biomanufacturing, Dr. Claudia Schmidt-Dannert, University of Minnesota
- The LanzaTech Process; Guidance of Strain Engineering Through Cell-Free Synthetic Biology; and Opportunities & Challenges, Dr. Michael Koepke, LanzaTech
- Advancing Cell Free Technologies for Diverse Applications; Analytical Technologies are used for Interpreting Cell Free Processes; and Microfabricated Platforms can Carry Out Cell-Free Processes at a Range of Scales, Dr. Mitch Doktycz, Oak Ridge National Laboratory
- Biofilm Integrated Nanofiber Display; Mutually Orthogonal Enzyme Conjugation Domains; and Boot-strapped Biocatalysis, Dr. Neel Joshi, Harvard University
- Synthetic Biology Special Issue: Cell-Free Expression Systems, Dr. Jean Peccoud, Colorado State University
- Relevant Capabilities, Dr. Yu-shen Cheng, National Yunlin University of Science & Technology
- National Renewable Energy Laboratory Cell Free Enzyme Capabilities: Enzyme Engineering; Tunable Biocatalysts for Biomass Conversion; and Synthetic Proteomes for the Production of Bioproducts, Dr. Roman Brunecky, National Renewable Energy Laboratory
- Advanced Biofuels and Bioproducts Demonstration Unit: A Community Resource: Broad Product and Industry Partner Base: Spanning Bioprocess, Chemical & Cell Free System Development; and JGI (Joint Genome Institute) Partnership Opportunity: Emerging Technology Opportunities Program, Dr. Todd Pray, Lawrence Berkeley National Laboratory.

Copies of released presentations can be found online linked to the event web page: <https://energy.gov/eere/bioenergy/cell-free-synthetic-biology-and-biocatalysis-listening-day>.

Listening Day Participant Responses

Challenges

Cell-Free Prototyping

Participants in the cell-free prototyping breakout session discussed several challenges impeding the development of cell-free systems for the in vitro discovery and optimization of efficient enzymatic routes for biomass-relevant transformations. Challenges identified include:

- Lack of Awareness
- In Vitro Applicability to In Vivo Systems

- Fundamental R&D, including but not limited to:
 - Cofactors
 - Pathway diversity
 - Post-translational modifications.

Lack of Awareness

Several non-technical challenges for the wider adoption of prototyping for biological pathways in vitro were addressed. There was broad agreement that there is low awareness of the potentially useful applications of cell-free prototyping beyond model organisms and kits offered by biotechnology companies. The application of cell-free prototyping in these new areas is not generally understood and there is skepticism about the broad applicability of the technology. From an industrial standpoint, it is also unclear what a successful business model might look like for a company providing broadly enabling cell-free solutions. Without successful examples beyond model organism applications, and a lack of general awareness, further commercial development in this area may be slow.

It was also identified that a public enzyme source or database analogous to the ATCC (American Type Culture Collection) for cell strains does not exist for enzymes and proteins. The lack of a public enzyme source impedes groups from creating advanced databases for cell-free prototyping systems limiting industry to capabilities it can develop in house

In Vitro Applicability to In Vivo Systems

Cell-free prototyping can be an enabling tool for metabolic engineering by vastly increasing the combinatorial space available to pathway engineers. One factor limiting the utility of this methodology is an insufficient understanding of how or if in vitro results translate to in vivo results. Without careful control of in vitro conditions, improvements in enzymatic activity found in in vitro experiments do not necessarily translate to improvements in product production in vivo. There is insufficient knowledge of how to mimic intracellular conditions for non-model organisms to the degree necessary to get meaningful results for the wide variety of organisms used in industrial biofuel and bioproduct applications. In addition, many enzymes may not be active in in vitro systems due to improper protein folding, a lack of post-translational modifications (e.g., methylation, glycosylation, acetylation), or other reasons, ultimately limiting the utility of cell-free preparations as combinatorial screening tools.

To truly realize the benefit of cell-free prototyping methods in metabolic engineering better predictive models are needed that can correlate in vitro and in vivo results. To-date, there are limited large, multi-dimensional datasets to compare performance between in vitro and in vivo systems. Collection of such data would enable a better understanding of which parameters are most important to replicate in vitro to mimic in vivo performance.

Fundamental R&D

Listening day attendees identified several areas within the broader challenge of fundamental research and development: cofactors, diversity, and post-translational modification. There were also some concerns about general limitations when using cell-free to inform in vivo conversion.

Participants also commented on the insufficient basic knowledge on the area of cofactors. For example, there needs to be development of product-specific systems such as engineering transcription factors (proteins that regulate gene expression). Similarly, there are challenges to producing enzymes that are complex with respect to cofactor (metallocenter), have complex/multiple peptide domains, or function in unique cell environments (e.g., membrane protein). Lack of fundamental scientific knowledge on the function of these types of enzymes can preclude the understanding of many cell-free systems.

Attendees also discussed the challenge posed by pathway diversity. There is such a diverse range of potential targets that more basic knowledge is needed on biochemical pathways. Coupling work with high-throughput analytics could potentially assay for intermediates and enzyme function at a reaction level as opposed to a system level. Alternative biochemical platforms (for example, anaerobic, photosynthetic) and alternative substrates (for example, CO₂, lignin), pose another diversity challenge compared to current knowledge. With each of these, the route(s) to central metabolism can vary widely.

The topic of post-translational modification was another area where attendees said more fundamental research was needed. Post-translational modification can impact enzyme folding; the area of enzyme folding was also cited where fundamental R&D was needed.

Overall, participants reported that there needs to be an improved understanding of how in vitro platforms may guide in vivo work. Possible limitations include complexes, cofactors, and ratio of redox equivalents, anaerobic reactions, and other reactions.

Other Challenges

Additionally, stakeholders suggested that the following cell-free prototyping challenges also be addressed in order to advance the potential bioeconomy implications of this technology:

- Cell-free systems could be limited to soluble proteins²
- Cell-free lysate/extracts are limited to a few organisms. If those organisms are unable to express the required genes and produce ample quantities of enzymes, certain biochemical routes might be precluded
- Transcription/translation system challenges with difficult gene templates, (e.g., high GC (guanine-cytosine) or AT (adenine-thymine) regions of DNA)
- Enzyme stability is required on the magnitude of days at a minimum in order to overcome high costs of enzyme production and purification
- Assay development and development of sensitive assays that are capable of detecting small quantities of analytes, particularly key reaction intermediates. Listening day attendees noted that there are no “out-of-the-box” strategies for these assaying challenges and that many are developed ad-hoc
- Reduce biosafety concerns (phage/virus assembly).

Cell-Free Free-Enzyme Conversion

Listening day attendees identified categories of challenges facing cell-free free-enzyme conversion, including:

- Product Separation
- Mixing / Separation / Phases
- Cofactors and Energy
- Enzyme Stability
- System Considerations (Scale, Unit Operations, Optimization)
- Feedstock – Cell-Free Interface.

Product Separation

Attendees considered that free-enzyme reactors would offer new solutions, but also some new problems, in separation and recovery of products from complex mixtures. Uncertainty surrounded the ability of membrane technology for enzyme retention, product separation, and selective product removal. Phase-transfer catalysis (PTC)³ would be needed to overcome separation challenges.

Breakout session participants questioned the actual orthogonality of cell-free free-enzyme reactors especially with respect to dealing affordably with host cells propagating into the cell-free reactor and consuming the enzymes. While some attendees thought product recovery could be easier and less expensive for cell-free, the assumption was also considered more of a theory than a statement. The ultimate complexity of a separations or purification process would be contingent on the physical properties of the product being produced (e.g., solubility in water, pKa, freezing/boiling point).

Mixing / Separation / Phases

Attendees noted that durability of enzymes in cell-free reactors is a major challenge for the advancement of cell-free systems.

² Soluble proteins are those in the cytoplasm within the cell; insoluble proteins refer to membrane proteins.

³ PTC refers to catalysts that also act as solvents to separate a reactant from one phase to another phase, where the catalytic reaction occurs.

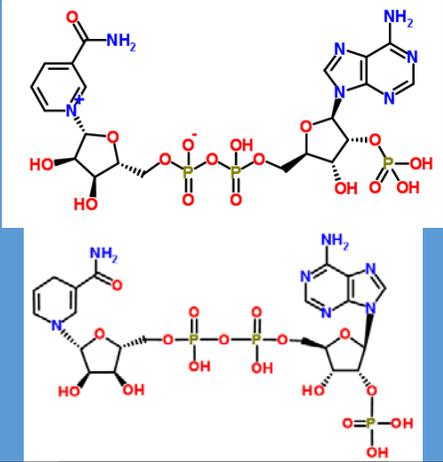
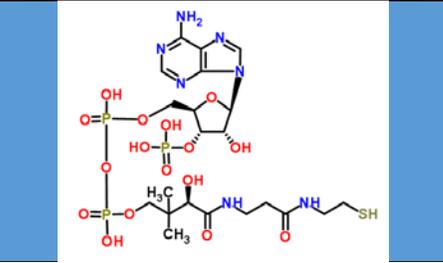
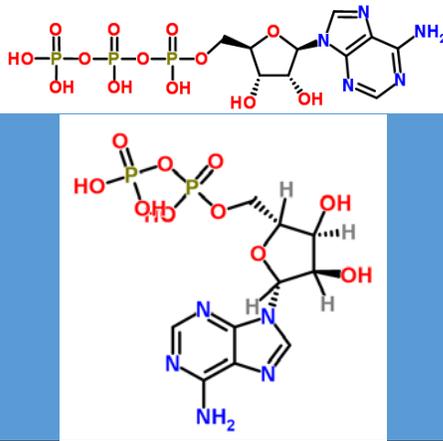
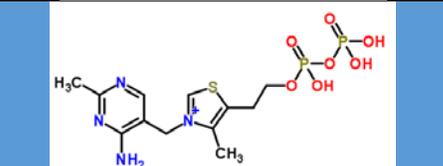
Attendees acknowledged a number of factors that would impact the ability of the enzyme to maintain activity including high pressures, shear forces associated with mixing, and the need to buffer products (in the event that an acid was being produced). Compared to the state-of-the-art for biochemical conversion, there was uncertainty about whether cell-free could be competitive given that free enzymes are far less equipped to tolerate conventional biochemical engineering conditions.

Cofactors and Energy

Breakout session participants discussed cofactors as a major challenge for the feasibility of cell-free free-enzyme reactors. Specifically mentioned were methods needed to generate the high energy cofactors ATP and NAD(P)H, and specifically electron carriers such as NAD(P)H. Metallic cofactors were also discussed as a challenge. There was consensus that exogenously supplying these cofactors in a continuous fashion would render the economics infeasible.

Priority Co-factor Maintenance

In cell-free conversions, co-factors are not readily produced and made available for enabling enzyme reactions. But co-factors serve critical functions for enzymes to act properly in biocatalytic conversion. Novel strategies are needed for maintaining balancing of several different co-factors. Attendees commented that the following co-factors are of particular importance.

<p>NAD(P)H/NAD(P) are used in cycles and pathways including the Calvin cycle (conversion of CO₂ to glyceraldehyde-3-phosphate), the non-mevalonate pathway (conversion of pyruvate and glyceraldehyde-3-phosphate to isopentenyl pyrophosphate and dimethylallyl pyrophosphate), glycolysis, and fatty acid synthesis (conversion of acetyl-CoA to fatty acids).</p>	 <p>The image shows two chemical structures. The top structure is Nicotinamide Adenine Dinucleotide (NAD), consisting of a nicotinamide ring (oxidized form) linked to a ribose sugar, which is further linked to an adenine ring and another ribose sugar. The bottom structure is Nicotinamide Adenine Dinucleotide Phosphate (NADP), which is identical to NAD but has a phosphate group attached to the second ribose sugar.</p>
<p>CoEnzyme A is used in pathways including fatty acid synthesis, fatty acid oxidation, and pyruvate decarboxylation (conversion of pyruvate to acetyl-CoA).</p>	 <p>The image shows the chemical structure of Coenzyme A (CoA). It features a thiazolium ring system (isoalloxazine) linked to a ribose sugar, which is further linked to a chain of phosphate groups, a pantoic acid derivative, and a cysteamine chain ending in a thiol (-SH) group.</p>
<p>ATP/ADP are used in pathways and cycles including glycolysis and the citric acid cycle.</p>	 <p>The image shows two chemical structures. The top structure is Adenosine Triphosphate (ATP), consisting of an adenine ring linked to a ribose sugar, which is further linked to a chain of three phosphate groups. The bottom structure is Adenosine Diphosphate (ADP), which is identical to ATP but has only two phosphate groups.</p>
<p>Thiamine pyrophosphate is used in the decarboxylation of pyruvate to acetaldehyde.</p>	 <p>The image shows the chemical structure of Thiamine pyrophosphate (TPP). It features a thiazolium ring system (thiazolopyrimidinone) with a methyl group and a hydroxyl group, linked to a methylene group, which is further linked to a pyrimidine ring with a methyl group and a hydroxyl group. The structure is also linked to a chain of two phosphate groups.</p>

Enzyme Stability

Attendees identified enzyme thermal and chemical stability as major challenges facing cell-free free-enzyme conversion reactors. The longevity of enzymes under relevant reactor conditions is an uncertainty, but stability on the orders of days to weeks is critical for economic feasibility of cell-free synthesis pathways. Methods for regeneration, such as recycling or introduction of new enzymes, were not known for commercial systems. Related to this, it was unknown whether chaperonins (e.g., heat shock 10 kDa protein 1 (GroES)) would be able to enhance the stability to the time durations required and the common energy requirements associated with these repair functions.

Attendees suggested that the solution would require implementing spatial and temporal control into biocatalytic networks. Solutions to recycle inactive enzymes as feed for living cells in a separate reactor for enzyme production were also suggested.

System Considerations (Scale, Unit Operations, Optimization)

Breakout session discussions addressed many challenges for considering systems design of cell-free free-enzyme conversion platforms. These included challenges specific to (1) scale-up of the cell-free reactor, (2) unit operations surrounding the cell-free reactor, and (3) optimizing the entire process.

Regarding (1) scale-up of the cell-free reactor, attendees said the rules for scaling cell-free processes would likely be different from traditional fermentation reactors and the ultimate size of a commercially viable reactor remains unknown. While there are analogies that can be looked upon (e.g., cellulases for cellulose deconstruction), when multiple enzymes are used in concert, the concentrations of enzymes required are largely unknown. In particular, the mixing rates for bench vs. pilot vs. commercial reactors can vary by orders of magnitude.

Regarding (2) the unit operations surrounding the cell-free reactor, attendees commented on scaling up the enzyme production system. The pinch point would be systems that can produce “cheap enzymes.” One suggestion was a protein-driven bioeconomics approach for developing fungal protein production systems based on high-yielding enzyme productivity principles. There was also a question of variables for scaling membrane proteins. More specifically, the unit operations for an enzyme production system would include production, purification/isolation, and assembly of enzymes and size/cost would need to be optimized. For assembly, multi-enzyme immobilization⁴ was suggested. In addition, the system would need enzyme stabilization for storage at scale. For cellulase enzymes, storage stability has been improved to the order of years. Drawing from these experiences, storage methods that were suggested included lyophilization⁵ and freezing. Additionally, the biocatalysis operations should be properly interfaced with chemocatalytic conversions or upgrading.

Regarding (3) optimizing the entire process, attendees wanted to see co-development of the cell-free free-enzyme reactor and the enzyme production system. The co-development would serve to minimize the time to achieving requisite robustness. Attendees suggested model-driven optimization, including metabolic redesign for optimizing carbon efficiency.

Feedstock – Cell-Free Interface

Participants commented on the challenge of a “feedstock – cell-free conversion interface” and associated challenges, including dirty feed streams and the importance to test utilization of “real-world” feedstocks (including lignin) and impact on enzyme performance and enzyme stability. For instance, it is well-established that the presence of lignin species inhibits cellulase activity. Challenges would exist in characterizing inhibitory effects associated with common hydrolysate species (e.g., acetate, furfural) and free enzymes involved in glycolysis or other product/fuel production pathways. Thus, there could be added costs for purifying or conditioning the feedstock before the cell-free free-enzyme conversion. Additionally, to follow up on the earlier discussion on Product Separation from the enzymes, there may also be challenges with separating product from the feedstock, if there are not pathway intermediates as commonly found in whole-cell fermentations. Conversely, there is potential for techno-economic upside in that some enzyme pathways may not be impacted by the presence of these inhibitory compounds.

Post-translational modifications were also discussed as a challenge (for more discussion, in Challenges: Cell-Free Prototyping, please see topics In Vitro Applicability for In Vivo and Fundamental R&D Needs).

⁴ Immobilization refers to reacting an enzyme solution with other chemicals to produce a structure (such as beads) where the enzyme is attached to an inert, insoluble material. Immobilization improves enzyme reactivity over a range of temperatures and pH, and also allows for easier product extraction.

⁵ Lyophilization, or freeze-drying, refers to freezing the material, and then reducing the pressure to sublimate frozen water to water vapor.

Cell-Free Scaffold Conversion

Stakeholders who participated in the cell-free scaffold conversion discussions identified several challenges facing scaffold/stabilized biocatalysis. These challenges can be grouped into categories including:

- Cofactors and Reactants
- Scale Up
- Enzyme Stability and Toxicity
- Material Development
- Reactor Design.

Cofactors and Reactants

Attendees were excited about the possibility of multi-enzyme constructs enabling reaction cascades, but they said cofactor recycling would be needed in order to realize the full potential of this technology. A substrate requirement would be needed in one-step reactions (this topic is discussed further in the Cell-Free Prototyping Challenges section, under Fundamental R&D Needs as well as in the Cell-Free Free-Enzyme Conversion Challenges section, under Enzyme Stability). As noted in Sarah Baker's presentation, gas substrates such as methane and CO₂ may have poor mass transfer and low volumetric productivity in stirred-tank reactors, and could require innovative printed reactors.

Attendees noted that providing the proper reducing equivalents needed for energy supplementation would be difficult to accomplish. Scaffolds could be designed to interface with new energy sources (e.g., electric) to provide the required reductants. Cell-free pathways might need to utilize such non-natural reducing equivalents in the absence of an in situ regeneration scheme. Alternatively, feeds would need to be chemically reduced prior to cell-free conversion (e.g., chemically reducing CO₂ to CO and then feeding CO for the cell-free conversion).

Scale Up

Breakout session participants also mentioned the challenges related to scaling up cell-free scaffold conversion for industrial biomanufacturing. There would need to be a way to cheaply produce the enzyme or use crude extract from cell lysate and then selectively bind/assemble the enzyme(s) to a scaffold. Low-cost production of the scaffold itself would also be necessary for this technology to be cost-effective.

Enzyme Stability and Toxicity

The stability of enzymes in cell-free scaffold conversion was another challenge identified by listening day attendees. Breakout session discussions related to this topic reiterated that stability refers not just to the enzyme, but to the enzyme-substrate complex. Also, there are several types of stability, including the structural stability of the enzymes as well as the enzyme activity. Long lifetimes and recycling of enzymes would be needed to address stability. These lifetimes would need to be longer than non-scaffolded systems, given the additional costs associated with scaffold production and the binding of these enzymes. Thus, the enzymes need to be stable on the order of months in order to justify the costs. Additionally, to address toxicity, it was stated that the microenvironment would need to be controlled for stabilizing enzymes. Attendees suggested that there would need to be optimization of enzymes for pH, temperature, and buffer factors.

Material Development

Listening day attendees recommended that new materials would need to be developed for cell-free scaffold conversion. There is considerable excitement around developing material scaffolds from renewable sources as well as developing new biocompatible materials. Nonetheless, scientists in this field expect concurrent challenges in both enzymology and materials science. Funding and programs that cross-cut materials science and synthetic biology will be needed to overcome these challenges. To design these new materials, attendees said there is a need for theoretical methods for joint scaffold/enzyme optimization as well as for robust and reliable predictive tools for scaffolding improvement.

Reactor Design

Reactor design is another area that was identified by breakout session discussions as a challenge to advancing cell-free scaffold conversion. Overcoming this barrier would allow for enhancing catalysis by substrate channeling reactor design. Listening day participants suggested that this would be possible if cell-free reactor systems were tailored to gaseous feedstocks. Challenges for cell-free scaffold conversion include both mass transfer limitations and reaction kinetic limits, with researchers finding that scaffold/membrane thickness is one variable to manipulate.⁶

Successful scaffolding is expected to increase enzyme productivity rates by positioning relevant enzymes in close proximity. However, the effective enzyme concentration level required is not yet known. As discussed previously, these challenges would vary depending upon reactor scale due to non-linear mixing rates that are observed as the volumes of reactors increase. Another reactor design challenge is developing methods to maximize mass transfer without large mixing and operational expense costs.

Opportunities

Cell-Free Prototyping

Listening day attendees discussed both near- and long-term opportunities for cell-free synthetic biology prototyping.

Near-Term

Attendees said there were many opportunities for cell-free synthetic biology prototyping. They are addressed in this section under the following categories:

- High-throughput screening
- Predictive models
- Target products
- Pathway development.

High-throughput Screening

Breakout session participants expect that unlocking the diversity of information available for use in cell-free synthetic biology prototyping will prove the value of high-throughput screening. This includes exploring metagenomics diversity and other vast genomic information that is currently unexplored. The potential implications include discovering new biochemical reactions.

High-throughput screening would also be valuable for protein engineering and rapid evaluation of reaction pathways. Due to costs for automation and the status of tools, high-throughput pathway engineering is currently limited to a small number of research groups generally exploring prominent organisms and biochemical pathways. The immediate opportunity is for developing high-throughput characterization of single enzymes or matrix combination screening to test activities of enzyme combinations. Other high-throughput screenings would be for structural analysis, including utilization of cryo-electron microscopy, nuclear magnetic resonance, and x-ray crystallography.

Predictive Models

Listening day attendees also discussed opportunities for developing and utilizing predictive models to evaluate biochemical pathways and enzymes. To develop predictive models for pathway engineering, there needs to be an understanding of enzyme kinetics, pathways, efficiencies, and stoichiometry, all of which need to be informed and validated with experiments. Breakout session participants referenced Dr. Rollin's plenary presentation, which mentioned his investigation of the kinetic model for protocatechuate to pyruvate using cell-free experiments⁷.

If correlations between cell-free systems and in vivo systems can be established, then the time to develop novel biochemical production pathways can potentially be reduced. Moreover, it would help inform optimal expression levels for particular enzymes

⁶ C.D. Blanchette, J.M. Knipe, J.K. Stolaroff, J. R. DeOtte, J. S. Oakdale, A. Maiti, J. N. Lenhardt, S. Sirajuddin, A. C. Rosenzweig, S. E. Baker. *Nature Communications* (2016) 7:1190. <https://www.nature.com/articles/ncomms11900>

⁷ For more information, refer to Dr. Rollin's plenary presentation: https://www.energy.gov/sites/prod/files/2017/12/f46/rollin_cell-free_synbio_listening_day.pdf.

or biochemical pathways.

Target Products

Listening day attendees identified several target products and end-uses for valorizing cell-free synthetic biology prototyping. For example, in the near-term, cell-free systems could address challenges and opportunities related to small-quantity/high-value markets (such as pharmaceuticals, materials, and chemicals). Some stakeholders questioned whether completely cell-free could be relevant for commodity-scale markets in the near-term.

Attendees also mentioned potential high-value, specialized products including non-natural bioproducts as well as non-natural pathways. Additionally, participants said there were opportunities for antibody optimization/production in eukaryotic cell-free systems. Other discoveries could be made for new enzymes, natural variants of known enzymes, and novel functions of enzymes. These high-value products could help de-risk some of these technologies, making them more relevant for future commodity scale applications.

Pathway Development

Breakout session participants also discussed some of the specific opportunities for developing pathways utilizing cell-free synthetic biology prototyping. In order to reach that point, though, R&D investment is needed to establish accurate characterizations of solubility-structural studies. Pathways could utilize an accelerated design-build-test-learn cycle that could have advantages in that cell-free protein synthesis and transcription/translation would be much faster than in vivo approaches. Throughput could increase dramatically allowing for thousands of mutated templates and by extension inform ways to improve productivity and yields for analogous in vivo systems.

Additionally, attendees described multiple catalytic domains and linked enzymes. They envisioned multi-enzyme reactions in one pot. For instance, if in a whole cell fermentation system multiple organisms were needed in series, the requisite enzymes could be integrated from both organisms to complete the synthesis in a single reaction vessel.

Long-Term

Breakout session participants also described a variety of long-term opportunities related to cell-free prototyping. Many of the suggestions are predicated on cell-free systems being developed to a significant extent within the biotech community, including:

- A comprehensive biochemical model to inform intelligent cell-free systems: this model would integrate enzymes and reaction kinetics across a wide range of organisms to inform the most efficient and productive pathways. Cell-free systems could pick and choose the most effective enzymes in developing existing and novel in vitro pathways. The model would include information to develop the most efficient systems in regards to yield, cofactor requirements, avoidance of competing/promiscuous reactions, and other cost-parameters.
- Hybridization of cell-free systems with chemical catalysis: multiple conversion steps could be combined into a single reactor as a means of process intensification. For example, one could use cell-free systems to funnel cellulosic sugars to a biochemical intermediate (e.g., acetate) and then within the same reactor, chemical catalysis could convert the intermediate to a higher value product or biofuel.
- Cell-free systems for the development of de novo and retrosynthetic pathways: existing and engineered enzymes from multiple organisms could be combined into systems to screen and develop new-to-science chemical syntheses.

Cell-free Synthesis Opportunities: Cell-free technologies can fundamentally alter biochemical reactor design and system operation

Traditional biochemical and fermentation reactor engineering strategies optimize system parameters around the growth kinetics and limitations of the platform microorganism. In fact, the inherent growth rate of the organism and its particular sensitivities to accumulated products and co-products may not permit reaction engineering that optimizes the kinetics of the target biochemical pathway. Cell-free technologies could liberate biochemical reaction engineering from the limitations of the cellular growth rate and sensitivities of a host organism. Beyond providing bioprocess engineers with new options to maximize conversion rates and product titers, cell-free systems could offer additional benefits by avoiding some mass transfer and product secretion limitations seen in whole cell systems. Overall biochemical kinetics and reactor engineering could be transformed through the use of cell-free technologies perhaps opening up the viable production routes for new biochemicals that are too complicated or expensive to produce using traditional fermentation.

Some of the opportunities discussed by participants are highlighted here below. See Figure 4 for one schematic of a cell-free free-enzyme production setup that exploits the advantages of cell-free.

- Enabling production of products (e.g., butanol, fatty aldehydes) that are toxic/inhibitory to host microorganisms
- Acidic products
- Non-secreted products (e.g., PHB)
- Processes utilizing toxic/inhibitory feedstocks (e.g., acetate, lignin monomers)
- Products separable by solvents incompatible with cells
- Traditionally batch fermentations

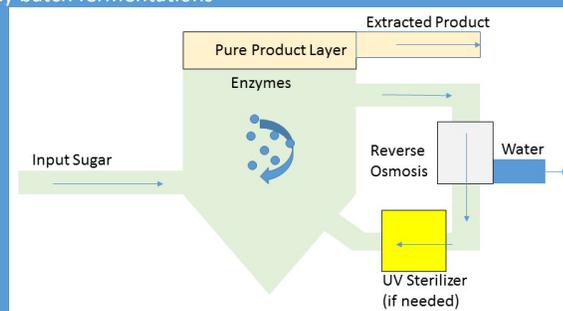


Figure 5: Example schematic of cell-free free-enzyme production, that capitalizes on the opportunities listed above. Image courtesy James Bowie. Source https://www.energy.gov/sites/prod/files/2017/12/f46/bowie_cell-free_synbio_listening_day.pdf

Other items discussed with respect to long-term solutions included non-protein-based biocatalysts (e.g., ribozymes), miniaturization of cell-free systems, and discovery of secondary metabolite production pathways.

Cell-Free Free-Enzyme Conversion

Listening day attendees discussed several opportunities for cell-free free-enzyme conversion, including:

- New Chemicals and Products
- Mixing / Separation / Phases
- Cofactors and Energy
- Unit Process Optimization
- Tolerance of Toxicity
- Conversion Efficiency
- New Engineering Toolkit.

New Chemicals and Products

Attendees were enthusiastic that cell-free free-enzyme conversion would enable more versatility so that innovators could produce new molecules. Key product opportunity spaces identified for cell-free systems included chiral molecules that can leverage the stereoselectivity of enzymes. Recent advances in in vivo protein engineering can be leveraged in these efforts. More generally, they could access new chemical spaces, such as producing functional groups, particularly heteroatoms, which petroleum chemicals cannot make efficiently. This could open up spaces to novel fuel molecules, polymers, surfactants, and other large market chemicals. In general, attendees also were excited about the opportunity for completely orthogonal systems.⁸

⁸ An orthogonal interaction occurs when there are two pairs of substances and each substance can interact with their respective partner, but does not interact with either substance of the other pair

Cell free Synthesis Opportunities: Novel biochemical pathways can be conceived of and engineered using cell-free technology

The possible conversion biochemistries that can be realized by traditional whole-cell pathways, even among pathways engineered using synthetic biology, are constrained by the platform organism. In fact, specific enzyme suites and metabolic pathways are often unique to a particular platform microorganism, and may not be viably expressed heterologously. Individual enzymes or enzyme sequences coupled together from multiple disparate microbial hosts may not be efficiently expressed in all cases and there may not be a single non-native host for such complex engineered pathways. However, cell-free pathway engineering could offer unique opportunities to more efficiently realize complex and novel biochemical pathways by allowing targeted enzymes and pathway sequences to be put together from a variety of diverse hosts without metabolic limitations from the chosen platform microorganism. Such cell-free pathways could offer entirely new conversion possibilities and yield new product chemistries currently impossible to synthesize biochemically.

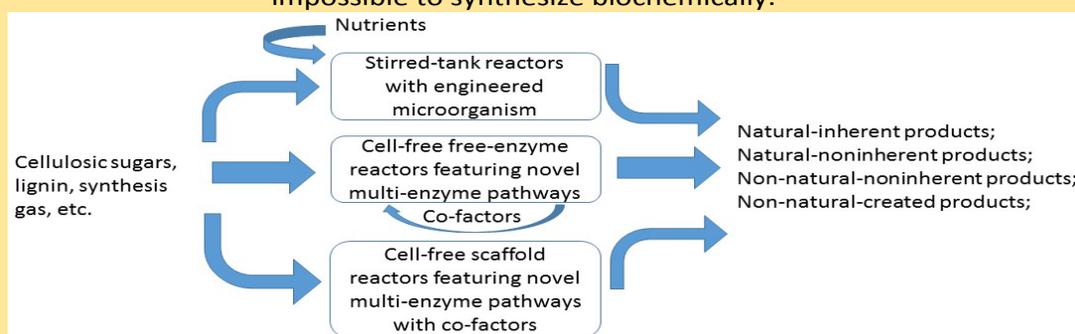


Figure 6: Novel multi-enzyme pathways in new reactor setups would enable new products from biomass.

Mixing / Separation / Phases

Attendees commented on several opportunities for cell-free free-enzyme conversion to leverage in regards to reactor phenomena such as mixing and phase separation. One opportunity would be enzymes in microchannel reactors, which stakeholders suggested could take advantage of continuous flow, which would modify the concentration profile as opposed to conventional batch reactors. Potentially for some reaction mechanisms, efficient mixing could improve the reaction rates.

Opportunities exist for the engineering of organisms being used to produce the extracts used in cell-free systems. Whether using cell lysates or purified enzymes, it is important for the organism being used to produce said enzymes to maximize growth and productivity of the targeted enzymes. This opportunity space can potentially leverage some of the learnings from the cellulase production industry wherein highly productive hosts (e.g., *T. reesei*) were engineered to produce large quantities of the cellulase enzymes. In these organism development efforts, the ultimate goal is to eliminate unwanted enzymes or pathways so as to increase the specific activity of the lysate or to improve the economics of enzyme purification.

Cofactors and Energy

Attendees suggested there may be new opportunities for leveraging cofactors, for example, the electrochemical method of cofactor regeneration. An example is using the electrical grid as a source of electrons or H₂ to provide reducing power to cell-free systems.

Another opportunity identified for overcoming barriers associated with cofactors and energy requirements of cell-free systems was biomimetics. Biomimetics is the imitation of natural systems, and in this context may refer to emerging concepts such as artificial

enzymes, such as nanozymes, or nanomaterials designed to behave like enzymes. Attendees suggested there could be methods for cofactor synthesis or recycling, by developing artificial cofactors.

In general, attendees reiterated that building orthogonal multi-enzyme catalyst networks was an opportunity, with enzyme engineering and smart use of cofactors involved.

Unit Process Optimization and System Simplification

Breakout session participants discussed several process optimization opportunities for cell-free free-enzyme conversion. In general, process flows may be significantly different than those for traditional in vivo biochemical conversion technologies. Attendees said there could be economic advantages for a conversion reactor to have the attributes of biochemical conversion (for example, high yield, titer, and productivity) but without a living organism in the conversion reactor. The traditional biochemical restraints that would no longer present themselves include mixing and aeration issues of active fermentations.

Additionally, attendees also suggested that cell-free free-enzyme conversion would exploit some opportunities for downstream activities. Product separation could be improved, and by-products could be eliminated without the use of a living organism in the conversion reactor. This is particularly true for biofuels and bioproducts that are difficult to secrete/transport from the cell or that typically require a means of cell-lysis. New opportunities for continuous production may also arise which can manifest in high productivity rates and yields. For example, processes for bioproducts and biofuels that are insoluble or phase separate in aqueous streams.

There was also discussion of how cell-free conversion strategies could simplify the processes. One example that was discussed was the ability to operate at higher temperature and pressure than in vivo biochemical conversion technologies. This would enable higher reaction rates and system throughputs while at the same time perhaps reducing or eliminating contamination issues. This would obviate the need for complex clean-in-place systems and reduce system downtime. Use of cell-free systems may also create opportunities to use more efficient solvents for extractions and separations that would otherwise be toxic to whole-cell systems.

Tolerance of Toxicity

Utilization of cell-free technologies could lead to new conversion processes that can tolerate levels of toxic molecules lethal to production microorganisms (here, the use of the word “toxicity” refers specifically to the microorganisms used in the bioreactor, as opposed to toxicity of a product to consumers or the environment).

Attendees commented that cell-free conversion would enable the production of molecules toxic to cell membranes and other cellular components, making them difficult or not possible to produce from in vivo processes. In addition to the ability to produce toxic end-products, intermediate toxicity can also be avoided in cell free systems. This discussion mirrored many of the points made in the plenary presentation given by Dr. James Bowie on the subject of butanol and terpenes production. For example, microbial production of limonene and other terpenes is limited due to toxicity, due to causes including lipophilicity of terpenes leading to build-up in membranes causing functional disruptions.⁹

Conversion Efficiency

Attendees said that cell-free free-enzyme conversion offered opportunities for greater conversion efficiencies. Carbon conversion efficiency was specifically cited. There were also comments that conversion efficiency would be improved through better defined, “bare bones” reaction pathways that eliminate side reactions. Over long-duration cell-free fermentations, yields could be enhanced by avoiding the loss of carbon and other atoms to cell growth and maintenance by having defined stoichiometry and synthesis steps.

Another impact would be through assembling mixed lysates from multiple organisms. Attendees commented on opportunities for assembling novel conversion pathways by mixing enzymes/lysates from different organisms. New conversion pathways may also be assembled from gene bioprospecting of un-culturable organisms. Reasons for unculturability include missing a nutrient in the

⁹ Jongedijk, E., Cankar, K., Buchhaupt, M. et al. Biotechnological production of limonene in microorganisms. *Applied Microbiology and Biotechnology* (2016) 100: 2927-2938. <https://doi.org/10.1007/s00253-016-7337-7>

culture medium, or toxicity of the culture medium to the organism, or inhibitory effects from other bacteria in the medium.¹⁰ There was also a suggestion to “swap in different unit enzymes for a family of products.” This would allow a process to toggle between multiple products in order to react to varying market conditions and prices.

New Engineering Toolkit

Breakout session participants discussed several opportunities derived from new tools that could each be developed into a new engineering toolkit for cell-free free-enzyme conversion technologies. These included:

- 1) The engineering of enzymes that enable direct use of cheap reducing power: sources of reducing agents included hydrogen, carbon monoxide, carboxylic acids, or electrons
- 2) Pathway optimization
- 3) Engineered allostery: specifically, allosteric regulation is enzyme regulation by binding an inhibitory molecule at a site on an enzyme other than the enzyme’s active site
- 4) Biomimetic cofactors (and enzymes that can use them)
- 5) “Modular” “switchable” systems with flexibility for different feedstocks and/or different products
- 6) Computational toolkits that could inform de novo enzyme design and/or protein-protein interactions predictions with high confidence.

Cell-Free Scaffold Conversion

Listening day participants discussed several opportunities for cell-free scaffold conversion including:

- Scale and Separations
- Specificity and Yield
- Mixed Platforms
- Tool Development.

Scale and Separations

Attendees were excited about the opportunity for cell-free scaffold conversion to be a biomanufacturing platform at reduced cost, due to requiring less biomass, less purification, and providing higher yields. Purification requirements could be lowered by simpler separations processes and may be combined with catalytic separation, such as reactive distillation. Such improvements can lower costs through process intensification. Additionally, scaffold approaches could be compatible with novel solvents that are otherwise incompatible with whole-cell systems.

As with other breakouts, attendees were enthusiastic about prospects of creating pathways not possible in living cells. On a related note, attendees said it could be possible to provide different microenvironments that optimize certain reaction steps.

Specificity and Yield

Attendees also discussed how cell-free scaffold conversion could improve specificity and yield for conversion technologies in the bioeconomy. There would be an opportunity for high throughput screening of enzyme cascades on scaffolds. One vision described was a one-pot, self-sufficient, self-organizing protein scaffolding/enzyme orchestration system. Advantages for scaffold conversion include the absence of competitive pathways and optimal proximity of enzymes to improve reaction rates and productivity.

The outcome envisioned is a system that achieves higher substrate/energy efficiency by creating systems of optimal enzyme

¹⁰ Wade W. *Unculturable bacteria—the uncharacterized organisms that cause oral infections.* *Journal of the Royal Society of Medicine* (2002) 95:81-83. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1279316/>

concentration (i.e., by controlling the number of scaffolding sites, you can optimize the concentration of enzymes in concert with the kinetic parameters).

Mixed Platforms

Mixed platforms were another opportunity for cell-free scaffold conversion. Opportunities included mixing biocatalysis with chemical catalysis, and improving efficiency for electro-biocatalysis platforms. A mix of combining enzymes with electro-chemical synthesis was also suggested.

Tool Development

Attendees said there could be opportunities for tool development to help the designers of such scaffold technologies. Genetic tools for optimizing scaffold design were recommended. This would provide key design rules for scaffold design of enzyme complexes and pathways. The outcome would be facilitating better understanding of enzyme/pathways function to guide optimization for further engineering. Currently there are not good public examples for biomanufacturing, but work has been explored in optimizing pathways, especially turnover of RNA messaging, for cell-free genetic circuits.¹¹

Resources Needed

Breakout session participants discussed the tangible actions to take advantage of opportunities and overcome barriers related to cell-free prototyping, free-enzyme conversion, and scaffold conversion. The resources needed were broadly categorized into those available through investments and collaboration compared to those requiring stakeholder contributions. This section provides an overview of the ideas discussed.

Investments and Collaboration Strategies

Attendees wanted to see longer funding cycles to allow researchers to explore concepts thoroughly. If the issue is more money per investment or more time per investment, they would like to receive more time: “do less with more, but do it well...longer funding cycles.” Attendees want to see investments in funding enzymes / pathways / process development. Attendees cited DARPA (Defense Advanced Research Projects Agency) as a model for innovation funding.

Attendees identified that cell-free free-enzyme conversion researchers could partner with several DOE programs. These programs included the Agile BioFoundry¹² managed by BETO, the Small Business Vouchers Pilot¹³ managed by EERE, and the Industrial Seedlings¹⁴ program managed by BETO and commonly operated at Lawrence Berkeley National Laboratory’s Advanced Biofuels and Bioproducts Demonstration Unit. Attendees mentioned the CRADA¹⁵ model of collaboration, a common legal framework for the national laboratories to work with external partners such as companies and universities.

Attendees were clear on a number of collaborations needed to advance cell-free free-enzyme conversion. From the perspective of the researcher, these include collaborations with government and industry. Cell-free researchers need an opportunity for collaboration with the petroleum, biofuels, and bioproducts industry to understand if there are platform chemicals that may be easier to produce through cell-free synthetic biology. Further, this could help identify if there are molecules that could be useful but cannot be made through traditional means. Cell-free researchers also expressed a desire to collaborate with companies doing traditional fermentations at scale in order to inform additional ways in which cell-free researchers can help solve industry problems.

Specifically, collaboration activities discussed or identified were:

- FOAs that emphasize Technology-to-Market plans with commercialization partner

¹¹ Vincent Noireaux, Roy Bar-Ziv, Albert Libchaber. *Proceedings of the National Academy of Sciences* Oct 2003, 100 (22) 12672-12677; DOI: 10.1073/pnas.2135496100

¹² <http://agilebiofoundry.org/>

¹³ <https://www.sbv.org/>

¹⁴ <http://abpdu.lbl.gov/wp-content/uploads/sites/4/2016/06/BETO-Lab-Call-FY16-Seedlings-30March2016-FINAL.pdf>

¹⁵ <https://energy.gov/gc/downloads/doe-cooperative-research-and-development-agreements>

- Leverage existing synthetic biology and chemical catalysis centers and consortia
 - CRADA (between national laboratory and external partner such as a company)
- Small Business Innovation Research (SBIRs), especially for technology developers without resources
- Process scale-up (at facilities like the ABPDU or the Integrated Biorefinery Research Facility (IBRF))
- Biofabrication design pipelines
- Develop centers of excellence and/or public-private partnerships to drive R&D
- Collaborations to gain knowledge/understanding from pharmaceutical industry
- Development of data repositories for enzymes and material/scaffold synthesis.

Attendees identified several resources needed for advancing cell-free synthetic biology prototyping and categorized them by the stakeholder category which they believed to be best suited for the role. The stakeholder categories identified include:

- DOE-BETO
- DOE – Office of Science
- DOE – ARPA-E
- DOE national laboratories
- Other federal agencies
- DOE national labs, industry, and academia.

DOE - BETO

Attendees wanted to see longer funding cycles to allow researchers to explore concepts thoroughly. If the issue is more money per investment or more time per investment, they would like to receive more time: “do less with more, but do it well...longer funding cycles.” Attendees want to see investments in funding enzymes / pathways / process development. Attendees cited DARPA as a model for innovation funding.

- Development of guidelines for technoeconomic analysis (TEA) including TEA benchmarks (e.g. product titers, yields, productivity rates)
- Near term focuses for BETO should be:
 - Near term: cell-free systems that can be applied to multiple pathways (e.g., sugars to pyruvate)
 - Longer term: Once a pathway is chosen, cell-free fermentations that target commodity-scale products from the platform intermediates above
- Develop predictive models and devices in the context of fuel/chemical production
 - Develop feedback loops between enzyme kinetic experiments and models to identify optimal pathways
 - Translate knowledge obtained from cell-free systems to whole-cell fermentations

- Identify priority/candidate molecules in the areas of
 - Fuels
 - Fuel or bioproduct intermediates
 - Materials
- Strategy to work in parallel with the Agile BioFoundry
 - Use of cell-free systems for high-throughput screening of specific enzymes
 - Enzyme overexpression strategies (specifically for enzyme production hosts)
 - Partnerships between gene synthesis and cell-free transcription/translation systems
- Raise awareness of cell-free systems
 - Facilitate discussions with technology providers, feedstock providers, enzyme producers, product manufacturers
 - Convene a large workshop or plan a session at a major conference on the topic
- Developing rules for process scaling
 - Identification of scaling heuristics that are/are not consistent with in vivo biochemical conversion.

DOE - Office of Science (including Biological and Environmental Research)

- Bioprospecting
 - Enzyme production hosts
 - Novel enzyme discovery
 - Gene discovery
- Promoter/transcription factor development and data dissemination
- General enzymology
 - Fundamental science on protein structure/activity
 - Standardized data on enzyme kinetics (Km, Kcat, etc.)
- Develop generalized enzyme scaffolding strategies.

DOE - ARPA-E

- Methane metabolism
- Plant/Agriculture prototyping

DOE National Laboratories

Attendees suggested several roles that the DOE national laboratories could aid cell-free synthetic biology:

- Genome discovery (promoters/terminators, transcription factors)
- Development kit Cooperative Research and Development Agreement (partnership between laboratories and industry)
- Cofactor recycling and regeneration R&D
- Standardized data on enzyme kinetics
- Standardized TEA methodologies for assessing cell-free systems
- Development of models/correlations to translate cell-free system performance into whole-cell fermentation performance.

Other Federal Agencies

Attendees recommended the involvement of other federal agencies, in as much they can aid in supporting the discovery of enzymes that are relevant to biofuel and renewable chemical production with BETO. Specific activities suggested included support of retrosynthesis (for biofuel), pathway mapping, and bioprospecting. Some offices cited and their possible points of collaboration:

- U.S. Department of Defense - Defense Advanced Research Projects Agency
 - More technology development funding
 - Safe genes (biosecurity)
- U.S. Department of Defense - Army
 - Computational sensors from cell-free
- U.S. Department of Health and Human Services - National Institutes of Health NIH
 - Novel therapeutic development
 - Cell-free for systems biology
 - Identification of new molecules (pharmaceutical/medical)
- U.S. Department of Agriculture
 - Plant/Agriculture prototyping
- National Science Foundation
 - Educational outreach kit for K-12 and academia.

Additionally, attendees said that international cooperation in the form of bilateral projects could be helpful.

Industry

Attendees commented that, in the near-term, it would be a challenge to get industry involved in cell-free systems and thus there would need to be an effort to stimulate industry interest. Initial work would be needed in academia and at the national laboratories. The goals of the work would be cell-free tools development and devices development. This work would promote industry interest. Participants said industry would respond to those goals. In response to tools development, industry could engage in large datasets/relevant hosts. Work in devices development from academia and national laboratories would enable industry activities in scale up.

Academia

Attendees said there would be demand from academia to use cell-free systems as a tool. It was recommended to host an awareness challenge to increase interest.

Listening Day Conclusions

The listening day provided attendees and BETO with a clearer perspective on cell-free systems including opportunities and resources needed. A number of key messages received included: potential for new products that cannot be produced from petroleum or current biotechnology; novel setups for separations; new toolboxes for synthetic biology; new tools to investigate converting novel substrates such as lignin and waste carbon gases.

Appendix 1: Attendees

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Appendix 2: Listening Day Agenda

Cell-Free Synthetic Biology and Biocatalysis Listening Day Agenda <i>U.S. Department of Energy, Bioenergy Technologies Office (BETO)</i> <i>Sunday, July 30, 2017</i> <i>Brown Palace Hotel</i> <i>Denver, Colorado</i>		
<i>Time</i>	<i>Agenda Item</i>	<i>Speaker</i>
8:00 a.m. – 8:30 a.m.	<i>Coffee and Registration</i>	
8:30 a.m. – 8:45 a.m.	Introductory Remarks	David Babson, BETO
Cell-Free Synthetic Biology as an Enabling Tool for the Bioeconomy		
8:45 a.m. – 9:10 a.m.	<i>Purified Cell-Free Systems as a Biofuels and Biochemicals Production Platform</i>	Joe Rollin, National Renewable Energy Laboratory
9:10 a.m. – 9:35 a.m.	<i>Cell-Free Systems for Prototyping and Biodiscovery</i>	Zachary Sun, Synvitrobio, Inc.
Cell-Free Synthetic Biology as a New Conversion Platform for the Bioeconomy		
9:35 a.m. – 10:00 a.m.	<i>Synthetic Biochemistry: Making Biofuels and Commodity Chemicals the Cell-Free Way</i>	James Bowie, University of California, Los Angeles
10:00 a.m. – 10:25 a.m.	<i>Bioprocess Intensification at the Intersection of Biology and Advanced Manufacturing</i>	Sarah Baker, Lawrence Livermore National Laboratory
10:25 a.m. – 10:30 a.m.	Debrief and Breakout Instructions	
10:30 a.m. – 10:50 a.m.	Break	
10:50 a.m. – 11:50 a.m.	Participant Presentations: 3 x 5 Session	
11:50 a.m. – 12:00 p.m.	Breakout Session Overview	
12:00 p.m. – 1:00 p.m.	Lunch	
1:00 p.m. – 1:40 p.m.	Breakout Question #1: Identifying Challenges	
	A. Prototyping B. Conversion Platform 1) Free Enzyme Biocatalysis 2) Scaffold/Stabilized Biocatalysis	
1:40 p.m. – 2:10 p.m.	Breakout Question # 2: Opportunities	
	A. Prototyping B. Conversion Platform 1) Free Enzyme Biocatalysis 2) Scaffold/Stabilized Biocatalysis	
2:10 p.m. – 2:40 p.m.	Breakout Question # 3: Resources / Partnerships Needed	
	A. Prototyping B. Conversion Platform 1) Free Enzyme Biocatalysis 2) Scaffold/Stabilized Biocatalysis	
2:40 p.m. – 2:55 p.m.	Synthesize Findings and Prepare Report-Out Slides	
2:55 p.m. – 3:05 p.m.	Report-Out from Group A	
3:05 p.m. – 3:15 p.m.	Report-Out from Group B-1	
3:15 p.m. – 3:25 p.m.	Report-Out from Group B-2	
3:25 p.m. – 3:30 p.m.	Closing Remarks	David Babson, BETO

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For more information, visit: to <https://www.energy.gov/eere/bioenergy>

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