

# BIOCHEMICAL CONVERSION AND LIGNIN UTILIZATION

TECHNOLOGY AREA

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# INTRODUCTION

The Biochemical Conversion and Lignin Utilization Technology Area is one of 11 technology areas that were reviewed during the 2023 Bioenergy Technologies Office (BETO) Project Peer Review, which took place April 3–7, 2023, in Denver, Colorado. A total of 18 presentations were reviewed in the Biochemical Conversion and Lignin Utilization session by five external experts from industry, academia, and nonprofit sectors. For information about the structure, strategy, and implementation of the technology area and its relation to BETO's overall mission, please refer to the corresponding Program and Technology Area Overview presentation slide decks (https://www.energy.gov/eere/bioenergy/biochemical-conversion-and-lignin-utilization).

This review addressed a total U.S. Department of Energy (DOE) investment value of approximately $42,369,587.00, which represents approximately 8% of the BETO portfolio reviewed during the 2023 Project Peer Review. During the Project Peer Review meeting, the presenter for each project was given 20 minutes to deliver a presentation and respond to questions from the review panel.

Projects were evaluated and scored for their approach, impact, and progress and outcomes. This section of the report contains the Review Panel Summary Report, the Technology Area Programmatic Response, and the full results of the Project Peer Review, including the scoring information for each project, comments from each reviewer, and the response provided by the project team.

BETO designated Beau Hoffman and Lisa Guay as the Biochemical Conversion and Lignin Utilization Technology Area review leads, with contractor support from Ryan Lawrence (Boston Government Services). In this capacity, Beau Hoffman and Lisa Guay were responsible for all aspects of review planning and implementation.

# BIOCHEMICAL CONVERSION AND LIGNIN UTILIZATION REVIEW PANEL

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# BIOCHEMICAL CONVERSION AND LIGNIN UTILIZATION REVIEW PANEL SUMMARY REPORT

*Prepared by the Biochemical Conversion and Lignin Utilization Review Panel*

## INTRODUCTION

BETO focuses on developing technologies that convert domestic lignocellulosic biomass (e.g., agricultural residues, forestry residues, dedicated energy crops) and waste resources (e.g., municipal solid wastes, animal manure, biosolids) into affordable low-carbon biofuels and bioproducts that significantly reduce carbon emissions on a life cycle basis compared to equivalent petroleum-based products. These bioenergy technologies can enable a transition to a clean energy economy, create high-quality jobs, support rural economies, and spur innovation in renewable energy and chemicals production—the bioeconomy.

The program supports diverse projects under two primary focus areas: low-cost sustainable aviation fuel (SAF) and lignin valorization. The biochemical track supports projects for biomass pretreatment, sugar production, and the fermentation of sugar and syngas to fuel chemicals and polymer precursors. The lignin valorization track supports projects that utilize lignin for chemicals, fuels, and polymer synthesis. Scale-up and demonstration projects focus on identifying and addressing commercial-scale challenges and reducing production costs. Novel technology-based projects focus on developing the knowledge base and overcoming barriers early in technology development. Industry engagement is strong in most of these projects. Several projects show great commercial potential and hit their milestones. To gauge whether these metrics are meaningful, it would have helped to see more failure to gauge how much risk BETO is taking. It was unclear whether only successful projects were presented or whether every project was successful, which seems somewhat questionable, or, at very least, that the program is too conservative.

The program is funding fantastic science and technology projects that are well aligned with BETO's near-term (2030) goals of supporting the development of SAF and renewable chemicals and recyclable plastics with low-carbon emissions. The reviewed projects demonstrate alignment with the strategic goals for this technology area. The new (2023) Multi-Year Strategic Plan presents a valuable opportunity to consider place-based and regional approaches to this technology area (as well as others), which is for the most part absent in the existing suite of funded projects. It appears that BETO is appropriately managing project progress, as evidenced by "go/no-go" decisions that have curtailed unproductive lines of inquiry as well as the redirection of certain research focus during the course of the merit review process. Several principal investigators (PIs) noted constructive communications with BETO staff in providing active guidance on research projects and direction.

Overall, the general strategy makes sense with multiple complementary projects, and most projects are on track. The focus on techno-economic analysis (TEA) and scale-up are key strengths of this program. Overall, the technology area is successful. It has a defined strategy, including goals and technical targets that complement BETO's overall mission.

## STRATEGY

The program focuses on developing new approaches and technologies to support the BETO 2030 goals of low-cost biofuels and lignin valorization. The program's goal is well reflected in the diverse projects it supports. From biomass fractionation, sugar production, and sugar/syngas fermentation to thermochemical and biological conversion of lignin and lignocellulose-derived sugars, all projects' goals directly or indirectly support low-cost SAF. The portfolio is weighted toward projects linked to the development or utilization of lignin and lignin-derived sugars resulting from deacetylation and mechanical refining (DMR) pretreated biomass.

The program supports technology development and demonstration at multiple scales. BETO has also funded a range of commercial projects, thus diversifying the portfolio to incorporate projects at higher technology readiness levels (TRLs). Due to the proprietary nature of work and the lack of technical details, it is more difficult to accurately evaluate these projects, and it is recommended that steps be taken to increase visibility into technology development status and the associated performance metrics. While developing commercially viable technology is the program's strong focus, the portfolio also supports fundamental studies at multiple scales.

In addition to supporting mature technologies in their scale-up and commercially relevant process development, the portfolio explores novel technologies and innovative concepts. The addition of cell-free systems seems appropriate, but the focus on products rather than technical de-risking and scale-up appears to be a missed opportunity. The expansion of the BETO product portfolio to include more biochemicals and bio-based material projects is viewed as a strength. These products are typically of greater value than fuels, and thus, generally speaking, have a higher probability of commercial success.

As the field of biofuels and biochemicals continues to evolve, we can anticipate that artificial intelligence and machine learning technologies are expected to play increasingly important roles in bioprocess development. Proactive investment in developing competencies in these areas may help ensure that BETO projects can enhance their ability to analyze large datasets, optimize processes, and guide decision making, ultimately leading to more efficient and effective research and development (R&D) efforts.

The strength of the portfolio is the use of TEA as a guiding principle to direct the science. While this is easy to propose, it is often difficult to implement in practice. The BETO projects, specifically at the National Renewable Energy Laboratory (NREL), have done an outstanding job of using TEA to guide scientific decisions. In addition, the various support activities in analytics, modeling, etc., provide real utility not only to the BETO portfolio but also the field at large. BETO has made good progress toward developing a range of foundational technologies (e.g., TEA and life cycle analysis [LCA], modeling, analytical services) that can help support projects across the BETO portfolio. An expansion of effort and/or investment in these areas is recommended because these technologies appear to be contributing to increased R&D efficiency, shorter R&D timelines, and good project management. For example, TEA and LCA are crucial tools in evaluating the economic feasibility and sustainability of biofuel and biochemical production processes, and every project should undergo frequent and in-depth TEAs/LCAs to ensure that R&D are being efficiently directed toward achieving BETO programmatic goals.

Additionally, investing in analytical support, including advanced analytical techniques and instrumentation, can greatly aid in the characterization and analysis of complex biomass feedstocks, intermediates, and final products. Accurate and comprehensive analytical data are critical for understanding the chemical and physical properties of biomass components, evaluating process performance, and ensuring product quality. Thus, increased investment in analytical and computational support would enable BETO to obtain more robust and reliable data, leading to better-informed research outcomes.

The program portfolio stimulates multidisciplinary collaborations among academics, national labs, and industries to leverage diverse expertise, which is highly beneficial in developing commercially viable technologies backed by strong science; however, there seems to be a tension in funding projects at national labs via lab calls versus projects led by industry and academic researchers via funding opportunity announcements. Of the 18 projects we reviewed, a university researcher was the lead on only one (and the presentation was made by an industry partner). National labs serve DOE goals, cultivate extraordinary expertise, and can provide continuity over time. Overall, the quality of the research delivered is high; however, BETO may consider whether it is missing opportunities to diversify lead and supporting PIs through emphasis on lab calls. It is appropriate to fund crosscutting and analytical projects via lab calls. These projects are providing important services to ongoing research at NREL and—to an extent—other national labs and groups.

These projects have developed and made publicly available several useful tools, protocols, and analyses. To what extent can BETO expand these services to provide broader reach beyond NREL?

It is laudable that BETO began to include a requirement for diversity, equity, and inclusion (DEI) goals in proposals and funded projects as of 2021. The inconsistency of implementing this requirement for some (more recently funded) and not all funded projects in the portfolio is a challenge. While some projects funded prior to the DEI requirement acknowledged DEI goals, others did not. Many of the projects that did include DEI goals focused on intentions to develop partnerships with minority-serving institutions (MSIs) or intentions to recruit interns and graduate students from underrepresented minorities. It may be constructive for BETO to provide more guidance, case studies, or support services to advance meaningful DEI engagements in funding proposals and funded projects.

## STRATEGY IMPLEMENTATION AND PROGRESS

We commend the BETO teams for exploring different technologies, often orthogonal to the dominant approaches in the field. We need new ideas and technologies, and the work on DMR and lignin are big steps in this direction. The diversity of technical approaches being explored—particularly around lignin usage and valorization—is, at this point in time, a strength because it increases the overall impact and probability of the approach to realizing BETO's environmental (i.e., greenhouse gas [GHG] emissions reduction) and product (i.e., sugar, biofuel, and biochemical) cost targets. In short, this approach helps maximize the likelihood that the funded projects will contribute toward achieving the program's long-term biofuel and biochemical targets. Another strength is a mix of projects de-risking more established technologies while simultaneously pursuing more risky ones, like cell-free systems. The suite of funded projects represents leading-edge research as well as research that can develop solid progress or refine the state of the art (SOA). The funded projects span the process chain and are well divided between upstream processing (pretreatment and deconstruction) and downstream bioconversions (lignin valorization and fermentative biochemical production).

The use and application of TEA for guiding technology development is a key strength. This is a unique aspect of the program and one that could be a model for other biofuel-related research and technology development. Key project milestones should be, across the board, TEA and/or LCA informed, whenever possible. The program has made large strides in the development of TEA and LCA and increasing the analyses into technology development plans and tracking; however, an area where the program may fall short is in the consistent application of TEA and LCA in project milestone setting across all projects. More consistent application will help ensure that all portfolio projects are pointed toward high TRLs, improved commercial metrics, and/or environmental benefits. Many projects had go/no-go and end-of-project milestones based on process outputs that are technically relevant but not the most TEA- or LCA-relevant metrics. For example, many bioconversion processes use titer as the goal, when yield and productivity are typically more relevant for determining commercial potential. Shifting focus toward these primary performance parameters would help ensure that the technologies being developed are driving toward beneficial outcomes for the performer, the government, and, more broadly, the biofuels and biochemicals field.

DMR process: The program is funding projects that are at the leading edge of work in the field. Following the scale-up challenges associated with dilute acid biomass pretreatment, the program responded by heavily investing in DMR. BETO, via NREL, has put the focus on this process versus the conventional dilute acid process, which has a track record at the commercial scale, with the valid argument that the lignin from the DMR or chemical-recovery-free DMR (CRF-DMR) process is less condensed that than of the dilute acid process, and therefore offers a wide range of applications compared to the sulfur lignin. As the readiness level of DMR has advanced, the program is doing a good job layering in downstream projects that aim to address downstream technical challenges. BETO, via NREL, is also pursuing continuous enzymatic hydrolysis (CEH) in conjunction with the DMR processes, but it is not clear if either DMR process (DMR or CRF-DMR) is scalable.

Cell-free system: The program supports three cell-free system projects where terpenes, isobutanol, butanol, and hexanol are the target products. The program's investment in cell-free technologies is a strength; this field is inherently risky (i.e., TRLs are low), but the approach has the potential to both expand the range of accessible products and realize large step changes in process rates compared to microbial bioconversion technologies. The advantages of a cell-free system in overcoming cell toxicity, enabling diverse products, and achieving high titer and production rates are well justified in these projects. Most of these projects have made great progress by meeting or exceeding the project milestones. Robust computation-aided identification of pathways, enzyme engineering, and low-cost cofactor synthesis demonstrated in these projects greatly improves the current SOA in cell-free technologies and advances science; however, it should be noted that the barrier due to the extremely high production costs is unlikely to be overcome in the short term. For example, TEA estimates the cell-free-based isobutanol cost to be $3.57/kg even after reducing the cofactor cost by 98% (from $9,000 to $200), which is significantly higher than its current market price ($440/metric tonne). While the current projects produce fuel molecules, targeting higher-value products (e.g., bio-based pharmaceutical chemicals) will help accelerate cell-free system technology adoption and commercialization. Although higher-value products have niche markets, they are commercially more viable than cost-competitive fuels, and delivering early "wins" will help de-risk the deployment of the technology toward lower-value product targets (e.g., fuels and commodity chemicals).

Lignin valorization: The program supports several projects that produce lignin-based chemicals and SAF molecules. The approaches taken in these projects, such as obtaining reactive lignin based on lignin-first reductive catalytic fractionation (RCF), catalytic oxidation for bioavailable monomers, and biological funneling to single molecules, are scientifically meritorious and novel. These projects led by NREL nicely complement each other in their project efforts. Although the advantages of a lignin-first biorefinery in producing higher-quality lignin products are well demonstrated in the current projects, carbohydrate streams derived from the RCF process and their fermentability should also be evaluated in future projects to better justify the viability of lignin-first biorefineries. It is also noted that in all lignin valorization projects, lignin is depolymerized first and then upgraded to chemicals or for polymer synthesis. Although the projects made great progress, the challenges of this valorization pathway should not be overlooked and must be clarified. For example, the dependency of the conversion and product on lignin source, the challenges in product separation and purification, and the high capital and operating costs for catalytically converting lignin should be better informed in future projects. Other lignin valorization pathways that do not require depolymerizing lignin should also be supported. For example, utilizing lignin's high carbon content and aromatic structures to develop property-advanced materials is attractive in this context.

## RECOMMENDATIONS

### Recommendation 1: Cell-free projects should focus more on technology de-risking and scale-up and less on producing new chemicals.

Because scale-up is the key question concerning the potential of cell-free systems, the panel recommends directly investigating this issue.

### Recommendation 2: The viability of most of the commercial projects is questionable, unlike NREL's projects.

The panel suggests that the NREL team performs TEAs for these projects in order to provide a consistent baseline for evaluating feasibility.

### Recommendation 3: There appears to be an unmet opportunity to expand into single-cell protein.

Feedstock to food/feed is a gap in the BETO portfolio because alternative proteins fall into the scope of decarbonization, where there is a growing consumer demand/interest and where there is opportunity to provide substantial GHG and environmental benefits relative to the status quo. The panel recommends that BETO

incorporates this opportunity into its downstream bioconversion strategy using TEA and LCA to gauge impact relative to incumbent technologies.

### Recommendation 4: Academic researchers are less well represented in leading projects.

The program may benefit from an increased variety of research approaches, and the research community may benefit from an expanded network of BETO-funded research. The panel encourages BETO to expand its outreach and program emphasis to reach new potential PIs and participating entities (be they industry or academic institutions). The panel also encourages BETO to expand these services and/or increase accessibility to provide broader reach to diverse potential customers. The increased academic representation could take the form of independent projects but also increased collaboration/partnering with academic labs.

### Recommendation 5: The program has invested in supporting analytical and modeling technologies; however, this may be an area where additional investment is warranted since these tools have the ability to accelerate timelines and improve overall R&D efficiency.

One specific area that may warrant increased investment is the development of LCA tools as well as the application of LCA alongside TEA so that a complete picture of both financial and environmental benefits can be assessed, which is of particular note given programmatic emphasis on reducing GHG emissions.

### Recommendation 6: BETO began to include a requirement for DEI goals in proposals and funded projects as of 2021.

The inconsistency of implementing this requirement for some (more recently funded) and not all funded projects in the portfolio is a challenge. The panel encourages BETO to provide more guidance, case studies, and support services to advance meaningful DEI engagements in funding proposals and funded projects.

# BIOCHEMICAL CONVERSION AND LIGNIN UTILIZATION PROGRAMMATIC RESPONSE

## INTRODUCTION

The Biochemical Conversion and Lignin Utilization Program is grateful to the reviewers for their contributions, recommendations, questions, and feedback during the 2023 Biochemical Conversion and Lignin Utilization Project Peer Review session. The program recognizes that this is a significant investment of time to review the projects, travel, and prepare the reviews following the in-person meeting. The program appreciates that each reviewer was willing to contribute their time and expertise to this meeting.

The program is proud of the research that was showcased and appreciates that the reviewers see merit and have optimism about the commercial potential/impact for the projects. Numerous technologies funded by the Biochemical Conversion and Lignin Utilization Program have been commercialized in recent years and/or raised significant amounts of private equity funding toward scale-up and commercialization. Additionally, several of the technologies originally funded by this program have been funded at the pilot and demonstration scales by the Systems Development and Integration Program.

Reviewers noted that they were unsure if there were projects in the portfolio that have failed that would allow them to understand the amount of risk the program is taking. BETO does discontinue and has discontinued projects for lack of technical progress in the past, and the projects presented in this session represented all the actively funded projects by BETO in the space. As discussed here, the program has utilized third-party

technical verifications for more than 10 years now with these projects to assess process. The use of these experts identifies both red flags warranting discontinuation and, more frequently, ways that projects' progress can be enhanced. Projects that have been discontinued are exempt from presenting. Given the nature of R&D, BETO does not expect every project to succeed but employs rigorous merit review and project management practices to improve the likelihood of success.

The program appreciates that TEA and LCA are recognized as "guiding principles" of the program and decisions about research investments and promise. The TEA and LCA efforts at the national laboratories and that are associated with many of these projects have long been recognized for publicizing their design reports, process models, and economic assumptions. In recent years, and as decarbonization has grown in priority, BETO has encouraged projects to move away from firm goals in terms of "dollars per gasoline gallon equivalent (GGE)" and focus on the current economic viability of the processes/technologies. Some of these firm cost targets were quite ingrained as metrics by the program and researchers, and work remains to make this philosophical change from "What does it take to achieve $3/GGE?" to "What is the current cost and state of technology?" The move away from hard costs has also been influenced by non-R&D factors, such as policies and tax credits, that will have major impacts on feasibility.

New to this Project Peer Review cycle was the inclusion of a DEI criteria as part of the review. BETO recognizes that this is a new component to the program and that this can be challenging for the reviewers to assess, particularly when only some projects have specific DEI or environmental justice components. In addition to being a new focus area for the research being funded, BETO and other DOE offices are striving to provide more meaningful guidance for reviewers about how to incorporate these elements into lower-TRL projects.

Once more, the program would like to thank the reviewers for their service to the office and for their suggestions on how to continue to improve.

### Recommendation 1: Cell-free projects should focus on de-risking as opposed to the production of new chemicals.

The cell-free portfolio is relatively limited within the program; however, significant strides have been made to date, including demonstrating full cofactor recycling, the potential for biomimetics, immobilization and scaffolding, and other technologies that benefit any cell-free molecule. Many of these initial "deal-breaking" barriers were identified in a prior workshop held by BETO, and some of the current challenges being discussed were either not practical to address at that time or not yet identified. The program recognizes that there are many remaining challenges, and these include those of scale-up. BETO's approach toward cell-free R&D has been to ensure that the barriers are addressed sequentially for this technology to be viable. In the case of the initial cell-free work at NREL, 2,3-butanediol (2,3-BDO) was chosen as an exemplary molecule to demonstrate proof of principle. Since then, BETO has shifted to molecules that are much more likely to be made via cell-free pathways, including butanol and isoprenoids. BETO sees cell-free as a mid- and long-term technology and will continue to invest in this as appropriate.

### Recommendation 2: Ensure consistent TEA for industrially led projects.

The program recognizes this point and has heard this concern before. In part, this is a consequence of the meeting format (a public presentation). BETO instructs projects to *not* include any information that would be considered proprietary or confidential. The willingness of certain projects to share TEA or other commercialization data can vary depending on the entity. BETO recognizes that this can be frustrating to the reviewers and can make these projects difficult to review. National lab projects often are willing to divulge their TEA assumptions, but often industrially led projects do not include this information.

As part of the verification process, BETO does contract with subject matter experts from NREL and elsewhere to evaluate the TEA, LCA, and associated assumptions for the industrially led projects. These results are

confidential and business sensitive and thus protected under nondisclosure agreements. BETO uses the recommendations of these verifications to inform project management decisions to ensure that TEAs and LCAs have credibility.

### Recommendation 3: Study the relative impact/value of single-cell protein.

BETO concurs with the recommendation that single-cell protein and alternative proteins present significant potential for decarbonization. BETO is exploring the market and LCA analysis of single-cell protein. BETO recently ran a Small Business Innovation Research solicitation specifically targeted at sustainable protein. BETO will collaborate with other agencies, namely the U.S. Department of Agriculture (USDA), to ensure that efforts in this space are complementary. At present, BETO defers much of the focus in this area to the USDA but will continue to collaborate and explore ways that its work can enable this industry.

### Recommendations 4 and 6: Academic researchers are largely unrepresented, and overall guidance on DEI is lacking.

As noted, BETO recognizes that DEI and environmental justice elements were only included as elements of projects in 2021 and later. Since the time of the Project Peer Review, BETO has distributed updated DEI guidance to the DOE national laboratories. The guidance includes considerations and examples that researchers could consider across multiple areas: integrating into existing DEI program participation, opportunities for the research team, and considerations for the research process and outcomes. In addition, the DOE Office of Energy Efficiency and Renewable Energy as a whole has updated its funding opportunity announcement template to include more specifics and expectations on the community benefit plans for future solicitations. With that said, BETO also has considerable work to do to incorporate these principles into its culture and associated funded projects.

With regard to the inclusion of academic researchers in BETO-funded work, the program notes that more than half of the projects that presented in this session have one or more funded university partners. As recent solicitations have targeted higher-TRL approaches and higher quantities of product (such as the clean cellulosic sugars funding opportunity announcement), this has naturally resulted in more industry partnerships; however, even in the case of that solicitation, university partners play key roles in those projects.

### Recommendation 5: Increase investment in economic/environmental modeling efforts.

Funding resources for these projects have been limited in recent years, and this is reflected in a shift away from the end-to-end design cases that can take significant time from the economic analysts. To ensure that the various R&D projects in the portfolio still have access to these analysis capabilities, we have encouraged the creation of TEAs and LCAs that might explore smaller system boundaries—for example, the cost and carbon intensity of producing a clean sugar intermediate as opposed to going to a final finished fuel or product. Additionally, many of BETO's economic and environmental models, such as the Greenhouse Gases, Regulated Emissions, and Energy Use in Technologies (GREET) model, are funded under the Data Modeling and Analysis Program. That program has been working to improve the collaboration and frequency of handoffs between the experimental, process/economic modeling, and supply chain and sustainability analysis teams.

# BIOCHEMICAL PLATFORM ANALYSIS

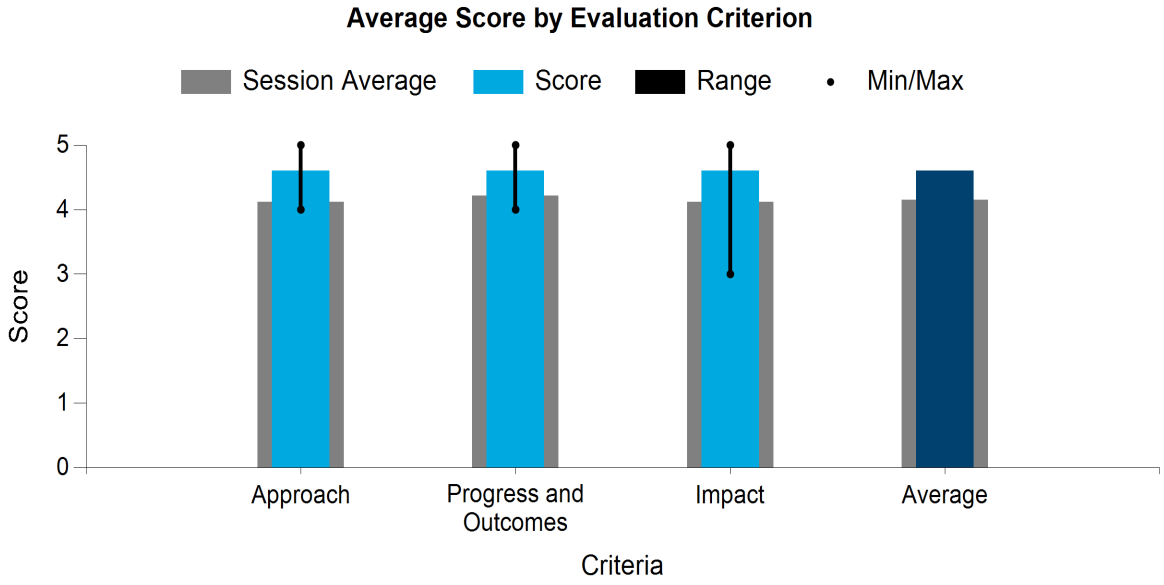
## National Renewable Energy Laboratory

### PROJECT DESCRIPTION

| | |
|---|---|
| WBS: | 2.1.0.100 |
| Presenter(s): | Ryan Davis |
| Project Start Date: | 10/01/2022 |
| Planned Project End Date: | 09/30/2025 |
| Total Funding: | $1,500,000 |

The Biochemical Platform Analysis project conducts process modeling, TEA, and support for LCA spanning a range of biomass conversion pathways based on biochemical processing concepts making use of sugars, lignin, and other biomass components. This is a high-impact project providing both direct support to DOE-BETO in establishing TEA/LCA metrics for integrated conceptual processes (which are utilized by BETO to guide program goals and planning documents) as well as support to other NREL and partner projects by evaluating specific steps within such an integrated model framework to identify key technology barriers that must be addressed through research prioritization.

This is a well-established project with a long history of delivering value to researchers and BETO management. The analysis conducted in this project serves an important function to provide a process context for the R&D activities funded by the program (i.e., to provide the "so what" answers to key questions on economics and the sustainability of conversion technology concepts), in support of ultimately meeting the latest program goals to maximize decarbonization potential at 70% or more GHG reduction from conventional fuel benchmarks while maintaining economic viability. Key achievements since the 2021 Project Peer Review include demonstrating continued improvements to state-of-technology cost benchmarks while expanding analysis to additional promising pathway options.




### COMMENTS

- The goal of this project is to provide process design and economic analysis for the BETO program. Detailed TEA is a critical aspect of the BETO program and one of its overall strengths, especially how it is used to guide the overlying science and engineering. The key applications are 2,3-BDO and butyric

acid. Both targets are well motivated based on TEA, as is the motivation for a lignin-first biorefinery concept. The project also provides some key costs for valorizing lignin, which, in turn, provides targets for the technical projects. The team is making excellent progress, providing key targets for other projects.

- This project is central to the BETO portfolio in lignin utilization and biochemical conversion. It is what makes the BETO program unique among the various federal initiatives supporting biofuel development. The impact is clearly articulated, benefiting not only the BETO program but also the entire biofuels community as it provides a clear benchmark and framework for analysis.
- This project is central to BETO's portfolio and should continue to receive support.
- The project serves a valuable purpose within BETO's biochemical conversion portfolio: providing process design and analytical support to guide research toward achieving economic and sustainability goals. It maintains consistent focus on BETO GHG and cost reduction goals. The overarching approach is reasonably aligned with the needs of the impressive number of research efforts it interfaces with, predominantly research conducted at NREL. The team seems nimble in responding to challenges and in periodically reassessing and resetting its course forward. The project's findings are available to and well received by the broader community.
- DEI goals are relatively new to BETO. The project has an interest in pursuing DEI goals, and it has initiated outreach to an MSI. Additional guidance or resourcing from BETO or NREL could strengthen these efforts.
- Considering future directions, I applaud the project's plans to expand research from a focus on traditional biomass feedstocks to include waste feedstocks. These alternative sources of biomass will be increasingly important to achieving sustainability goals.
- The project has made significant progress toward integrating TEA across the BETO portfolio, particularly at NREL. The team has effectively used TEA to support decision making in early-stage R&D planning, ensuring that biorefinery economics are considered from the outset of the projects. Further, the use of sensitivity analyses to reassess priorities based on new R&D results reflects a proactive approach to project management. In short, this project provides invaluable support for other BETO projects that may lack TEA/LCA expertise and budget and helps improve overall partner project research efficiency and direction.
- Historically, coordination between TEA and LCA has been limited; however, the project is taking steps to better align these activities and incorporate aspects of LCA, such as leveraging the GREET model to estimate carbon intensity. Given the growing importance of reducing GHG emissions as a project success criterion, this coordination between TEA and LCA is critical for robust decision making, and it is recommended that this objective is even further emphasized.
- TEA has been effectively used to support diverse technologies across the BETO portfolio. One notable example is the successful switch from adipic acid to beta-ketoadipic acid for lignin valorization, resulting in a four-times increase in productivity and addressing a key cost driver. The project has also demonstrated the value of TEA in optimizing process parameters, improving yield and productivity in projects such as the dioxolane-based technology and optimizing C5 sugar utilization.
- The project has made the TEA models and tools available to the public, with significant downloads of the TEA models in the past 3 years; however, it is noted that the tools are still perceived as black-boxed, and efforts to increase their accessibility, such as through publications or simplified tool sets (e.g., an expansion of the Excel-sheet-based models that were recently published), would further accelerate

adoption and align with the project's stated objectives of increasing industry engagement, especially among small businesses.

- Regarding DEI, the project has worked to build out TEA/LCA capabilities at MSIs, providing students with training and working toward a joint manuscript.

- It is not clear what mechanisms have been put in place to accurately screen processes knowing that TEA from a lower TRL does not usually translate into the target economic viability at higher TRL. Could the PI clarify what has been put in place?

- This project is one of the most important projects in the BETO portfolio. The project works closely with other BETO-relevant projects to bridge lab research and commercial realization of the technologies. The risk identification and mitigation strategies are adequate. The project analyzed various BETO-relevant research, such as 2,3-BDO or organic acids to fuel pathways and lignin coproduct inclusion. It is great to see how this project has updated its model to reflect the technological advances in its TEA. The most important contribution of TEA/LCA to most of the BETO projects is perhaps to inform them of the critical factors affecting product costs and GHG emissions while technology is still under development. This information will help researchers adjust the processes and identify promising pathways to move forward, de-risking technology at the early stage of technology development. The 2,3-BDO pathway optimization performed in this project is an excellent example. The high impact of this project is quantifiable, evidenced by the high downloads of TEA reports, high-impact papers the team published, and the project results that affected the industry decision. The team's approach to improving the credibility of the modeling is commendable. The team works closely with external consultants and stakeholders to receive input and feedback. A DEI plan is in place. Overall, this is an important project, and the team did a great job.

## PI RESPONSE TO REVIEWER COMMENTS

- We thank the reviewers for their positive comments and recognition of the importance of this project. We agree it will be critical to further increase coordination between TEA and LCA efforts moving forward in light of the increased priorities around decarbonization, and we plan to continue working with BETO and other laboratory partners to proactively identify ways to structure modeling efforts to co-optimize cost and sustainability metrics in concert. On the question about screening low-TRL processes, we often begin our TEA considerations for early-TRL concepts based on fundamental questions such as defining maximum theoretical yields or minimum-severity operating conditions. This can help quickly screen out concepts that cannot meet minimum thresholds for economic viability as constrained by untenably low yields and/or high processing costs compared to other established process benchmarks. From that point, concepts that pass this initial screen may be evaluated through "feasibility-level" TEA (not necessarily warranting the rigor of more comprehensive "design case" pathways) with an emphasis on sensitivity scans to highlight key drivers or uncertainties that must be addressed as the concept moves toward higher TRLs, and accordingly more in-depth TEA modeling, also often soliciting engineering subcontractor assistance to validate economic viability for more mature technology concepts. Regarding the comment on increasing public accessibility to simplified TEA tools, we note that the models that we do publish (https://www.nrel.gov/extranet/biorefinery/aspen-models/) are not necessarily "black box" because they are complete Aspen Plus model files with accompanying TEA spreadsheets, although we acknowledge that being based in Aspen Plus, they are not readily accessible to a wide audience. Although many of the fully integrated biorefinery processes that we model require the thermodynamic rigor of a process simulation package such as Aspen Plus in order to provide reliable mass and energy balance information on which to base TEA/LCA calculations, moving forward, we will also seek to prioritize the publication of more simplified cases, such as the NREL sugar model, using user-friendly Excel tools for broader accessibility.

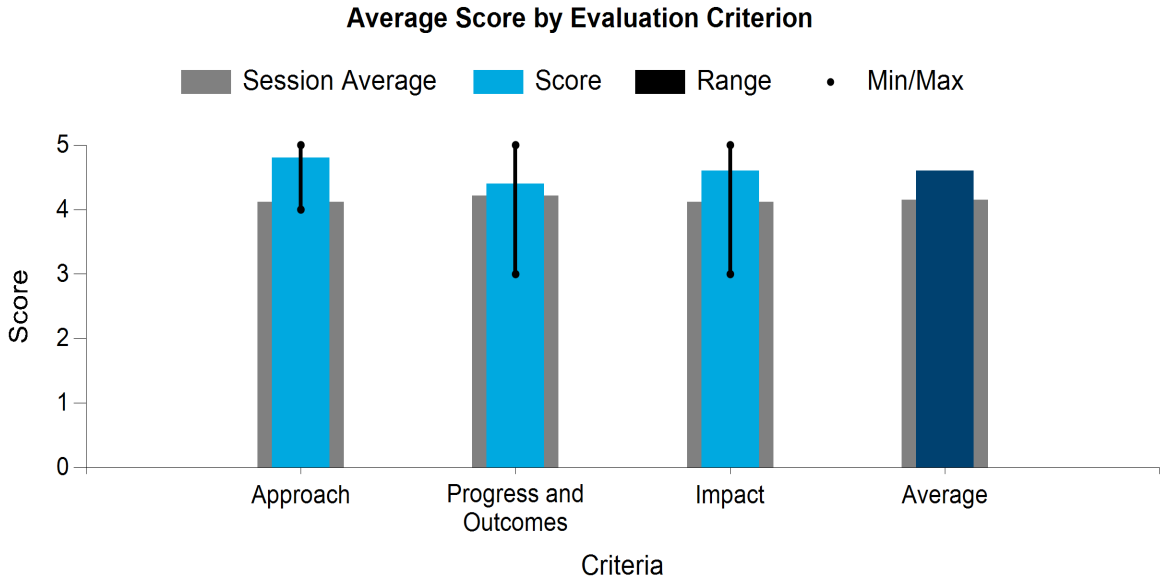
# LIGNIN-FIRST BIOREFINERY DEVELOPMENT

## National Renewable Energy Laboratory

### PROJECT DESCRIPTION

| | |
|---|---|
| WBS: | 2.2.3.106 |
| Presenter(s): | Gregg Beckham; Laura Hollingsworth; Megan Krysiak |
| Project Start Date: | 10/20/2017 |
| Planned Project End Date: | 09/30/2023 |
| Total Funding: | $2,040,000 |

The Lignin-First Biorefinery Development project aims to develop a cost-effective and scalable biomass fractionation strategy based on RCF. The RCF process uses a protic solvent, hydrogen gas or a hydrogen donor, and a metal catalyst in the presence of intact biomass to produce a stable, depolymerized lignin oil and a polysaccharide pulp, which can both be converted to value-added products in parallel processes. RCF is a promising strategy to enable the use of woody feedstocks in biochemical conversion processes and is also promising as a means to valorize lignin with equal emphasis to biomass carbohydrates. Guided by TEA and LCA and in collaboration with industry partners, we are actively developing RCF methods to (1) avoid the need for exogenous hydrogen gas, (2) substantially reduce reactor pressure via the use of low-vapor-pressure solvents, (3) separate the lignin solvolysis and catalytic processes through reaction engineering solutions, (4) reduce solvent loading below what can be achieved in typical batch reactors, and (5) avoid or minimize the use of organic solvents. The project also collaborates closely with the Lignin Utilization and Lignin Conversion to SAF projects for critical substrate handoffs and analytics. Overall, the project is enabling new approaches that ultimately can enable RCF to be a feedstock-agnostic method to valorize both polysaccharides and lignin.




### COMMENTS

- The goal of this project is to develop a bioprocess centered on valorizing lignin through reductive catalytic fractionization. The key strength of this approach is that it provides a unique solution to biofuel production and can handle woody biomass. In other words, the process is feedstock agnostic. On the technical side, the key strength is the development of analytical methods for resolving different linkages and structures. Another strength is the adaptation of an existing industrial process for cracking C–C

lignin bonds. In particular, the team is focused on developing viable and practical solutions for depolymerizing lignin.

- Overall, this is an exciting project that is making excellent technical progress, especially with reducing the key drivers in cost. The project is well managed with a clear risk management plan. The technical work is impressive and impactful, as illustrated with multiple collaborations with companies.
- This project is one of a suite of lignin projects supported by BETO and led by NREL. This portfolio of projects fits together well in direct support of BETO's goals for lignin valorization. This project, early in the process of achieving lignin utilization, pushes the process of removing lignin from the plant cell wall in the directions of greater efficiency and increased sustainability. The project appropriately uses TEA and LCA to guide the research toward the greatest impact. The project has demonstrated the success of RCF in processing a variety of feedstocks. It would be interesting to see if these successes can be achieved with additional heterogeneous or blended feedstocks.
- The research addresses challenging problems. The project's industry collaborations validate the relevance of this work to real-world interests and objectives.
- A lignin-first biorefining strategy, which involves removing lignin from the plant cell wall, has shown promise in accessing stable lignin oil from woody feedstocks. This approach is aligned with BETO's lignin valorization goals for 2030 and has broad applicability because the outputs (i.e., sugars and lignin-derived phenolics) can serve as inputs for a range of downstream conversion processes.
- One key strength of this approach is the focus on process viability, development, and optimization, rather than complete process integration to end products. By leaving downstream conversion work to industry and national lab collaborators with specialized expertise, the team can concentrate on advancing the TRL of RCF and increasing its impact as a foundational technology that can be combined with diverse bioconversion technologies.
- The extensive use of TEAs and LCAs has provided actionable recommendations for process improvements and optimization targets, guiding R&D directives such as catalyst selection, solvent type and loading, and process optimization. The progress made in the approach is significant, as evidenced by advancements across nearly all major cost centers in the TEA model, such as reducing reactor pressure and solvent loading. This progress has laid a solid foundation for planned integrated process development. While an updated TEA has not been performed to estimate the net TEA and LCA benefits of process improvements to date, it is likely that these benefits are substantial. In summary, the RCF strategy shows tremendous promise and, with further development, is anticipated to make a significant contribution toward achieving SAF and lignin valorization goals.
- Regarding DEI, as presented, there remains room for additional effort and attention to be paid here.
- The project made some satisfactory progress while investigating various parameters (single versus mixed solvents, presence/absence of hydrogen, and solvent recycling loop).
- One recommendation moving forward would be to focus on exploring the potential observed with RCF water-based solvent and find ways to improve the process efficiency when water is used as the sole solvent. One key question that would need to be answered is the rate of condensation in the RCF water-based solvent.
- The project aims to develop economic RCF of biomass by performing an experimental study guided by TEA. This project is one of the lignin valorization projects led by NREL, and the lignin oil produced in this project has been upgraded by several other projects; thus, the success of this project also benefits

other relevant projects. The team has made excellent progress toward the project goal. The team has carefully assessed the project risks and developed mitigation plans. The project approach is commendable. The RCF process is studied in greater detail in this project. From biomass type, reactor configuration, scale, use (or not) of $H_2$, solvent selection, solvent loading, and catalyst type to product separation, the team considered both the economic aspect and scalability of the process. Industry engagement is also strong. TEA sensitivity analysis to inform factors affecting the minimum selling price (MSP) is a great way to optimize the technology. It shows that RCF capital cost, delignification, and reactor pressure are the top three factors affecting the MSP. Solvent recovery is another key factor. The project evaluates a water-only RCF to reduce reactor pressure and lower solvent costs. Overall, the project made great progress and the team conducted excellent research.

- A few recommendations to consider: (1) Although the lignin-first approach can improve extracted lignin, lignin is still a supplemental source, and cellulose pulp is the main driver of the biorefinery. It would be helpful if the correlations between different RCF approaches and the resulting pulp quality (for cellulose/xylan retention, lignin content, and inhibitory compounds) were also discussed. (2) Solvent consumption can significantly alter TEA results. It is recommended to carefully evaluate solvent consumption when alcohols are used in the system. (3) Was wastewater treatment considered when water is the solvent? (4) The results with different biomass types could be elaborated to evaluate if the different RCF processes are truly agnostic to the feedstock.

## PI RESPONSE TO REVIEWER COMMENTS

- We thank the reviewers for their positive feedback on the Lignin-First Biorefinery Development project. We agree with the reviewer's comment regarding the need to extend this work to blended or heterogeneous feedstocks. Another reviewer's comment remarked on the need to update the TEA and LCA models, which we are doing as the work progresses, and the reviewer is correct that all the process advancements obtained thus far have contributed substantially to reducing estimated costs, energy consumption, and GHG emissions. In terms of DEI activities, we agree with the comment that "there remains room for additional effort and attention to be paid here," and we plan on formally incorporating DEI milestones in the proposal for future project work (fiscal years [FY] 2024–2026); however, we have many DEI activities that were not mentioned on slide 5, including mentoring of interns for workforce development, allocating funds for early-career staff to present at conferences (promotes networking and scientific outreach), and emphasizing proper data citation in meetings to ensure recognition of all staff, specifically interns and technicians. In terms of the use of water as a cosolvent or a sole solvent, that is a major focus of the project going forward, and we have used our flow-through reactor system to estimate the kinetics of condensation, which will inform the at-scale reactor design work that we are conducting with industry partners, as briefly described during the presentation. Regarding questions of pulp quality, we have previously shown that RCF pulp is readily digestible by cellulase enzymes, and work in the Feedstock-Conversion Interface Consortium has shown that the resulting sugars from RCF pulp-derived hydrolysate can be converted microbially with little performance differences relative to clean sugars. We will follow up on this again in FY 2024–2026 if the project is renewed for another 3-year project cycle. In terms of solvent consumption, this is a long-standing question in the literature, and as discussed during the Q&A, we have intentions to conduct isotope labeling experiments to this end in the near future; the use of water would mitigate this concern. Wastewater treatment is modeled explicitly in our TEA. Regarding the feedstock-agnostic comment, we only had time to cover a single slide on this work, but this work is detailed in https://doi.org/10.1039/D2GC04464A. We very much appreciate the positive feedback from the reviewers, and we look forward to sharing our future progress on this exciting fractionation approach!

# PRODUCTION OF LOW-COST AND HIGHLY FERMENTABLE SUGAR FROM CORN STOVER VIA THE CHEMICAL-RECOVERY-FREE DEACETYLATION AND MECHANICAL REFINING PROCESS

## National Renewable Energy Laboratory

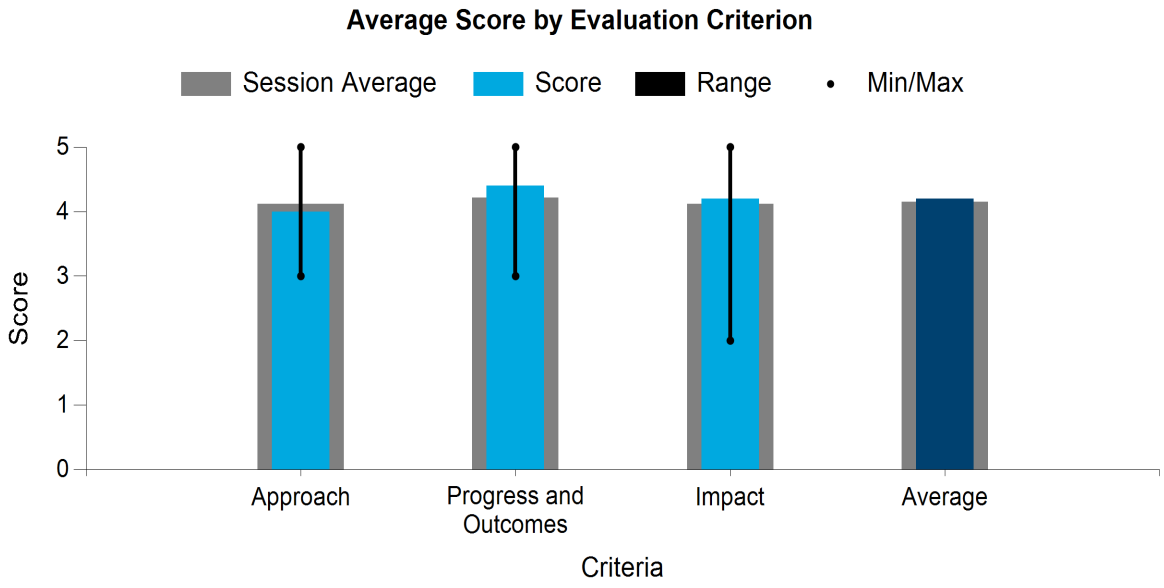
### PROJECT DESCRIPTION

| | |
|---|---|
| WBS: | 2.2.3.701 |
| Presenter(s): | Xiaowen Chen |
| Project Start Date: | 10/01/2021 |
| Planned Project End Date: | 12/31/2025 |
| Total Funding: | $3,500,000 |

Early second-generation cellulosic ethanol plants have faced challenges with low fermentability of the sugar hydrolysates produced by dilute acid pretreatment. To address this issue, NREL has developed a scalable atmospheric pressure and low-severity biomass deconstruction and fractionation process called DMR. This process demonstrates superior performance in converting corn stover to high-concentration, low-toxicity sugars at high yields; however, the recovery of NaOH is a complex and expensive process that requires a significant initial capital investment.

To further reduce the minimum sugar selling price (MSSP) of the DMR process, the goal of this project is to develop a CRF-DMR pretreatment technology. This technology should produce highly fermentable cellulosic sugars at an MSSP of $0.20/lb. Additionally, the project aims to enhance the deacetylation step by using chemicals that can also function as fertilizers. This approach can further lower the overall DMR sugar cost by avoiding the chemical recovery step.

The project is a collaboration between industry and academic partners, with the objective of de-risking the downstream sugar conversion process by increasing its availability and performance.



## COMMENTS

- The project is focused on developing the DMR process for biomass pretreatment. A number of different pretreatment processes have been developed, all with different strengths and weaknesses. DMR is a promising one, assuming that NaOH can be recovered. It also nicely complements the work being funded by other programs within DOE focused on other approaches. Even though there has been significant prior work on developing the DMR process, this is a new project, so there are few results. That said, the project is exciting and appears to have a solid technical and management plan.

- This project is one of the jewels of the BETO program. Whether or not this technology becomes viable, it is very promising and worthy of investigation and investment. Deconstruction is still the major bottleneck, particularly at the pretreatment stage. DMR is a unique and promising technology, so it should be pursued.

- The technology approach involves considerable environmental and sustainability benefits over the predominant technologies in use.

- The presentation of this project was very clear and deliberate in explaining the context for and potential impact of the successful execution of this research. The project explicitly addresses circularity in the utilization of lignocellulosic feedstocks: It includes the proposal that byproducts of the CRF-DMR process may go back into the soil for fertilization and carbon sequestration benefits. The TEA and LCA may not adequately capture the potential opportunities and risks of this circular element of the technology. This fertilizer opportunity is an interesting proposed co-benefit of the CMF-DMR process. If successful, this use could increase carbon sequestration and reduce the GHG footprint of the CMF-DMR process; however, significant research would be required to ensure that this would not have unintended consequences, including potential introduction of toxins into soils. Few details on this research were provided because it is largely in the domain of USDA collaborators on the project. It is encouraging to see a potential interface between BETO- and USDA-funded research.

- The overarching goal of this project is to develop a cost-effective method with low GHG emissions to produce sugars and lignin from corn stover and other biomass feedstocks by improving the DMR process. This project has strong potential to add value across the BETO portfolio and support the programmatic goals of producing SAF, improving the economics of biofuels/biochemicals, and reducing GHG emissions associated with manufacturing these end products.

- From a project management perspective, a project strength is its leveraging of knowledge gained from scaling up dilute acid pretreatment processes, including challenges with continuous solids handling, and previous work developing on a DMR process. Insights from each of these areas are being used to guide the work plan, which is expected to contribute significantly to reducing both R&D and scale-up risks.

- The team has proposed a simple but innovative solution that involves a two-stage deacetylation process using ammonia and potassium-based salts during pretreatment, which eliminates the need for energy-intensive NaOH recycling. Although the project is still in its early stages, previous work on a two-stage deacetylation process using sodium carbonate supports the technical feasibility of the overall process. Using sodium carbonate in the first stage, the project has demonstrated over 90% glucose yields and achieved a modeled $1/GGE reduction in minimum fuel selling price (MFSP), as well as a modeled 23% reduction in GHG emissions. Further, the resulting sugars were of suitable quality for use in microbial fermentations. If successful, the proposed ammonia-/K-salt-based approach may further decrease the MSP by 25% and GHG emissions by a modeled 48%. Therefore, the economic and environmental advantages of the technology could be substantial and warrant investigation.

- A critical assumption in this project is that the waste stream comprising N/P/K will be an effective fertilizer. The LCA and TEA presented are hinged on this assumption. Toward that end, the reviewer suggests that the level of effort placed on fertilizer replacement testing be substantially increased—for example, through industry collaborations and additional academic partnerships. If the results are positive, it would significantly de-risk this aspect of the project and increase its impact; however, if the results are negative, addressing this challenge during the project lifetime would be critical.
- Efforts to support DEI were not presented, and attention should be paid to ensure that the project incorporates one or more mechanisms to support this program objective.
- A process yields comparison between DMR and CRF-DMR is needed. The PI interchanges process yields and conversion yields, and it is confusing.
- A TEA comparison between DMR and CRF-DMR side by side would be very helpful.
- No risks are outlined for the new CRF-DMR process.
- It is challenging to review the CRF-DMR process when, as of today, everything is just TEA based. Were assumptions used to generate the TEA? If so, on what basis were these assumptions selected in the absence of lab data?
- The black liquor has toxic components. The PI should look at the black liquor's toxicity from the CRF-DMR process before assuming it will be good to be added to the soil as is without testing for potential issues.
- At what scale has CRF-DMR been tested as of today? No data as of today. Everything is just TEA based. DMR is the process that has been mostly tested as of now.
- The project is just starting the initial verification, and thus there is no real project progress to evaluate; however, the presenter did an excellent job explaining the project background and the approaches. The disadvantages of dilute acid pretreatment and the problem with NaOH treatment are clearly explained. The success of the previous project with two-stage DMR is also presented. The use of ammonia and alkali salts for DMR and the use of the residues for fertilizer is well justified. Although the waste stream containing lignin and nutrition elements from the DMR treatment differs from the lignin-based fertilizers synthesized in previous studies, the overall concept is novel, and the approach is logical. The project management and the industry engagement are excellent. Collaborating with academia, national labs, and industries to evaluate the fermentability of DMR hydrolysate for multiple products and fertilizer applications is also commendable. The project risks and mitigations are not discussed in the presentation; thus, additional information will be helpful. The biggest concern is about directly applying the waste liquor in soils. Any foreseen challenges and mitigation strategies could be elaborated. A DEI plan should also be described.

## PI RESPONSE TO REVIEWER COMMENTS

- We greatly appreciate and welcome the feedback from the reviewers regarding our CRF-DMR project. It has been noted that the project has the potential to contribute value to the BETO portfolio and support programmatic goals related to SAF, improved biofuel/biochemical economics, and reduced GHG emissions associated with end product manufacturing. Incorporating the suggestions and concerns of the reviewers is a priority for us as we move forward with the project. We recognize that the toxins produced during pretreatment and the direct application of waste liquor in soil could be a major concern. To address this issue, we plan to conduct a comprehensive compositional analysis of the liquor, including both organic and inorganic components, using inductively coupled plasma spectroscopy, X-ray fluorescence, gas chromatography–mass spectrometry, and other relevant methods. Further, we intend to

collaborate closely with USDA, Washington State University, and the University of Connecticut to assess the impact of potential toxins on soils. Additionally, we will increase our efforts to test the replacement of fertilizer, as suggested by one of the reviewers. One reviewer has pointed out that there needs to be more elaboration on the risk and mitigation strategies of the project. One of the major risks associated with the project is the possibility of not achieving equivalent high sugar yields as the two-stage DMR process that uses $Na_2CO_3$ and NaOH. This could result in high MSSP and GHG emissions from the produced sugar and derived fuel/chemical products. To mitigate this issue, we propose using $SO_2$ or oxygen to oxidize the lignin after the ammonia and potassium hydroxide pretreatment to solubilize more lignin and increase sugar yields. Another risk is that toxins produced during pretreatment may reduce the value of waste liquor for fertilizer replacement. As mentioned, our mitigation strategy is to conduct a comprehensive compositional analysis of the liquor and collaborate closely with relevant experts to assess its impact on soils. Despite the promising preliminary results from the TEA and LCA, it is important to note that the project is still in the verification stage. This is because the TEA and LCA were based on several assumptions, particularly regarding the sugar yield obtained through the use of NaOH and $Na_2CO_3$. As the project progresses, we plan to update the TEA and LCA results accordingly.

# SPERLU SELECTIVE PROCESS FOR EFFICIENT REMOVAL OF LIGNIN AND UPGRADING

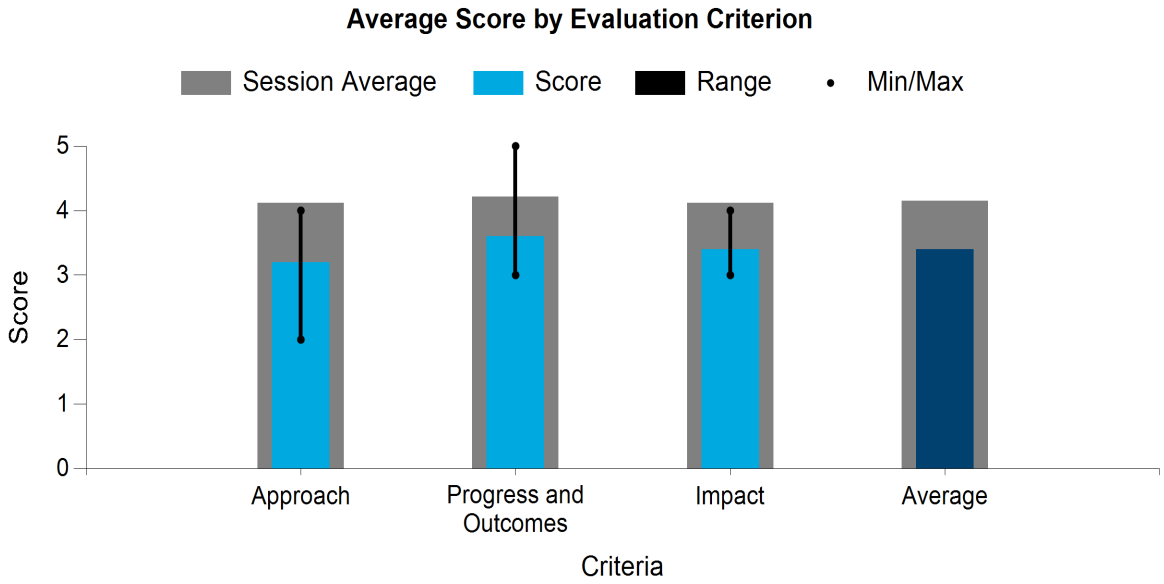
## Spero Renewables, LLC

### PROJECT DESCRIPTION

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|---|---|
| WBS: | 2.3.1.212 |
| Presenter(s): | Ian Klein |
| Project Start Date: | 10/01/2018 |
| Planned Project End Date: | 03/31/2023 |
| Total Funding: | $2,077,875 |

To meet the growing demand for bio-based chemicals and improve the profitability and efficiency of the emerging biorefinery industry, lignin is an abundant and attractive feedstock. Spero Renewables, LLC has developed a proprietary and patent-pending technology, the Selective Process for Efficient Removal of Lignin and Upgrading (SPERLU). The technology is a catalytic deconstruction and upgrading of lignin in lignocellulosic biomass or commercially available technical lignin to produce biophenol products. The SPERLU process has been demonstrated and vetted on a lab scale to convert commercially available technical lignin to biophenols in yields of 60%–90% (based on lignin). The biophenol products can be separated and further upgraded through catalytic or biological means.

Spero performed a detailed kinetic study of the SPERLU process and used the resulting kinetic data to design and construct a mini-pilot-scale reactor for the routine production of hundreds of grams of biophenol products. Spero's lignin-based biophenols have been demonstrated for use as a drop-in replacement for bisphenol A (BPA) in the synthesis of thermoset polymers. Spero's lignin-based thermoset polymers exhibit excellent mechanical properties and can be used in many applications. Additionally, a collaboration with NREL was used to evaluate the feasibility of upgrading SPERLU products through biological means into valuable chemicals. A comprehensive TEA and LCA have been completed and support the case for commercialization of the SPERLU technology. Through this project, Spero has significantly de-risked the SPERLU technology and is actively planning larger-scale pilot projects.



## COMMENTS

- The goal of this project is to convert lignin into biophenols as potential replacements for BPA using a catalytic approach. The work looks interesting, but the project is difficult to evaluate due to the lack of technical details. The team appears to have achieved its milestones, but, again, progress is difficult to evaluate. In addition, it was unclear what assumptions went into the TEA/LCA. One suggestion would be to have the TEA at NREL provide this analysis because it would give an unbiased view of whether the SPERLU process will ever be economically viable. Another suggestion would be to have a closed session where the reviewers, having signed nondisclosure agreements, could better evaluate the technology and progress.

- Management seems to have worked, as adequate progress was made to achieve project goals on time; however, limited information was shared. Risk management steps were stated (monthly project meetings and TEA of process changes), but individual risks identified before experimentation began were not identified. Once experiments began, challenges were identified as they arose, and dealt with. In contrast to other presented projects, this project did not present diversity, equity, inclusion, and accessibility (DEIA) goals or activities. Limited information is available by which to understand the technical aspects of the SPERLU process. Evaluation would benefit from greater elaboration of risks and mitigation strategies.

- The pivot from biomass feedstocks to technical lignin shows dexterity in project management as well as the benefits of an initial project strategy that considered and evaluated multiple feedstock types. Few details are provided. It would be helpful to better understand the difference in financial, logistical, and other costs associated with sourcing technical lignin versus woody biomass.

- The PI asserts that the project has met its goals on time with the end date of the project. As such, appropriate progress has been made. The project met the goal of producing >200 g of biophenols/day as of February 2023.

- The approach seems expensive relative to the potential impact. It would be valuable to better understand existing and anticipated future demand for BPA, be it from petroleum or renewable feedstocks. The SPERLU process is proprietary and patent pending. Should this project succeed, to what extent would this technology be broadly available for use, and to what extent would it result in sustainability gains in the production of petrochemical replacement products?

- There are gaps in the information presented regarding the purity of the monomer and dimer biophenols resulting from the SPERLU process. High monomer purity is crucial for generating polymers of commercial quality. It is expected that some byproduct formation may occur during processing, which would need to be addressed during monomer purification.

- The data presented on the lignin-based polymer specs do not clearly indicate if these polymers are true drop-in replacements for epoxidized bisphenol A (DGEBA). While parameters such as glass transition temperature and storage modulus are important, they alone are insufficient to claim that these polymers meet downstream user requirements. Therefore, the project could benefit from additional collaborations with industry and national labs to explore different applications of the output polymers in order to better understand their potential.

- The calculated net present value of 12% for the plant sizes required indicates a significant degree of scale-up and commercialization risk. If assumptions are overly aggressive or process performance diminishes with increasing scale, the overall economics of the project may be jeopardized. For example, the relatively long process times (e.g., 6 hours reported with technical lignin) expose the project to increased risk if this metric fails to scale.

- Overall, the impact of the technology on BETO's overall portfolio and long-term goals may be diminished due to the technical difficulties encountered with using lignin from poplar and other biomass feedstocks. Although these challenges may be addressed to some extent through increased catalyst loading or solvent volume, the process economics may diminish while the environmental impact increases. Further research and collaborations may be necessary to overcome these challenges and fully realize the potential impact of the technology.
- By using technical lignin data, show that the lignin/solvent ratio is reduced to 5:1. That is achieved without a solvent recycling loop. One recommendation would be to investigate the effect of solvent recycling on further reducing the ratio to an acceptable number that is economically viable. Another recommendation would be to investigate if the recycled solvent could potentially be toxic to the microorganism.
- The global market size of BPA is over $20 billion and will continue to grow; thus, a low-cost, bio-based replacement of bisphenol for thermoset production will provide a sufficiently large market for lignin valorization. In this project, biophenols are obtained from lignin using a catalytic, solvent-based process. The bio-based epoxide developed in this project showed comparable Tg and storage modulus, proving their applicability. The TEA and LCA show positive net present value and GHG reduction. The project management and industry engagement are adequate. From the project title, it looks like the team originally targeted CRF lignin removal from biomass; however, it seems like the team's focus is shifted to technical lignin because they find that using whole biomass requires a significantly higher amount of solvent than lignin, hurting TEA. It was reported that technical lignin could yield much more biophenols than whole biomass and directly produce dimeric phenols. Surprisingly, nearly 80% of biophenols were produced from technical lignin. Because the presentation did not disclose their process, it is difficult to evaluate the scientific merit of the project. The biophenol purity and solvent requirement are identified as the risks. It would be helpful if the team commented on the purity requirement in polymer synthesis and the challenges in obtaining consistent polymer properties. Based on the gas chromatogram given in the presentation, considerable byproducts are presented. High-temperature vacuum distillation is mentioned as a purification method. It will be helpful if the team comments on its effectiveness. Lignin oils are thermally unstable, and constituent compounds have high boiling points. The purpose of Task 2 (biotransformation) is unclear. What are the input and output of this task? How is this task related to other tasks?

# RECYCLABLE THERMOSET POLYMERS FROM LIGNIN-DERIVED PHENOLS

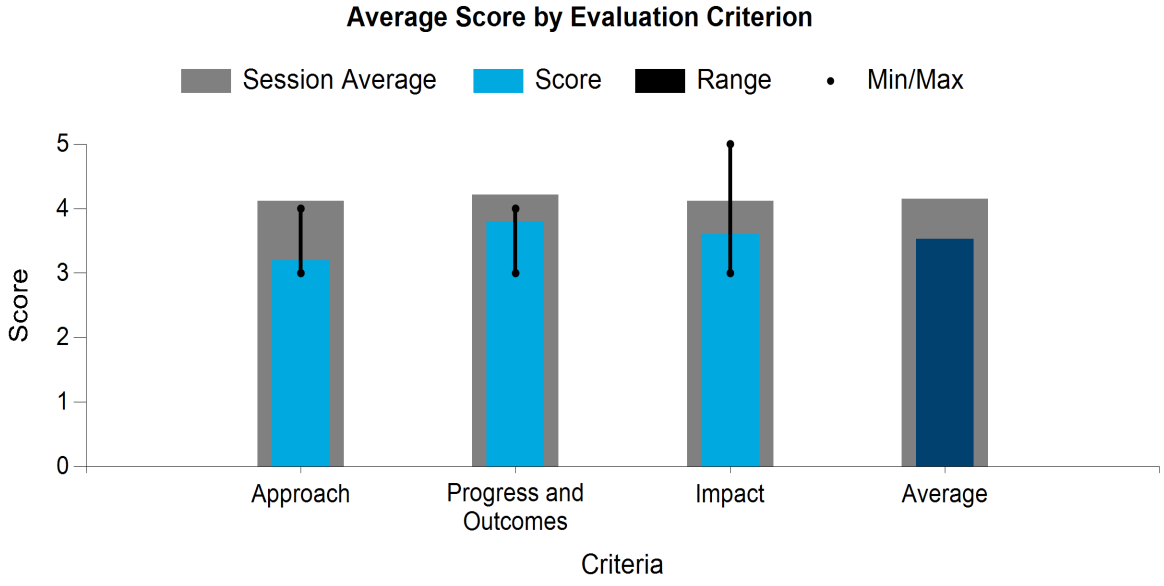
## Spero Renewables, LLC

### PROJECT DESCRIPTION

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|---|---|
| WBS: | 2.3.1.416 |
| Presenter(s): | Ian Klein |
| Project Start Date: | 10/01/2019 |
| Planned Project End Date: | 07/31/2024 |
| Total Funding: | $2,500,000 |

Recent innovations at Spero Renewables have resulted in a proprietary technology for producing decomposable and recyclable thermoset polymers. Thermosets are a class of polymers that are irreversibly cured from soft solids or liquid prepolymer, with the aid of heat or other action of energy. Because of the permanent cross-links, thermosets generally possess outstanding mechanical properties, chemical and thermal resistance, and excellent insulation. Thermosets are now commonly used as the plastic matrix in performance composites, also known as carbon-fiber-reinforced composites. Despite the outstanding material properties of carbon-fiber-reinforced composites, the non-decomposable and nonrecyclable nature of thermosets has limited the widespread application of thermoset composites. It is estimated that hundreds of millions of dollars are lost each year from the landfilling of thermoset composite wastes.

Spero Renewables' recyclable thermosets incorporate bio-based feedstocks derived from lignin, which can be incorporated at more than 50% of the mass of the polymer. Through this project, the technology is being refined to produce thermosets that can be decomposed under mild conditions yet possess comparable thermomechanical properties to conventional BPA-based thermosets. Spero's thermosets can be chemically recycled, with the thermomechanical properties of recycled thermosets comparable to virgin thermosets. Through collaboration with Argonne National Laboratory, a comprehensive LCA of the synthesis and recycling of Spero's thermosets and carbon-fiber-reinforced composites is underway. TEA is being used to validate the commercial potential of Spero's technology. At the conclusion of this project, Spero expects to have sufficient data to enter large-scale pilot production ahead of commercialization.



## COMMENTS

- The goal of this project is to produce carbon fiber composites using lignin-derived phenolics. The key strength of this approach is that it replaces BPA and yields a recyclable material that can be depolymerized. Like many industrial projects, this one was difficult to evaluate due to the lack of technical details. While impressive results are shown, it is unclear what the benchmarks are. This project was also materials focused with not much discussion of lignin.
- Overall, this appears to be an interesting project, but the feasibility and impact are difficult to evaluate due to the lack of technical details.
- Limited information is available by which to understand the technical aspects of the project. The evaluation would benefit from greater elaboration of processes, risks and mitigation strategies, LCA, TEA, and results.
- Progress seems to be promising; Spero seems to be hitting its targets in terms of material qualities and recyclability.
- In principle, the research is interesting. It would be valuable to have recyclable thermoset composites; however, there are major unaddressed questions regarding whether and how, if technically possible, the recycling of these thermoset polymers would actually take place. Enabling the recycling of small consumer goods is identified as a more near-term goal, and the recycling of larger industrial composites would be expected to be a longer-term goal due to the safety testing that would be required.
- The project is still in its early stages, making it challenging to predict the commercial impact of the product resins and technology as well as their final alignment with BETO's programmatic goals; however, there are promising indications that the product resins possess properties that could be attractive across a wide range of commercial applications, including carbon fiber composites. The project has successfully developed resins with excellent recyclability, minimal change in storage modulus, "welding" or adhering capability, and high fatigue resistance compared to reference BPA. These favorable properties make the resins a promising candidate for various industrial uses, as demonstrated by the project's successful engagement with potential commercialization partners.
- The technical approach of using a chemically recyclable resin based on reversible bonds is novel and attractive, but the level of detail provided is insufficient to appropriately assess the feasibility and approach. For example, more information about the catalyst used is needed to provide substantive input on the technical risks and the overall feasibility of the approach. Additional details would enhance the understanding and evaluation of the project's overarching technical strategy.
- The TEA indicates that the process may be profitable even at production volumes as low as 1,000 metric tons per year, which is typically considered as demo scale for most plants. This suggests that the project's technology has the potential to be economically viable and commercially scalable, further enhancing its potential impact.
- There is a need for more comprehensive material properties analysis to further validate the potential for commercial adoption of the polymers. Currently, only a limited set of parameters, such as glass transition temperature and storage modulus, have been reported. To gain wider acceptance in the industry, it will be crucial to generate more data on various material and mechanical properties as well as to evaluate the compatibility of the developed materials with existing BPA processing equipment.
- Overall, the project seems to be on track with projected milestones. Satisfactory results have been achieved with tasks 4 and 6.

- A recommendation would be to test how many cycles can be achieved without losing efficiency. Data only show up to two generations.
- Although thermoset polymers with reversible bonds are reported by others, making them using lignin is attractive. Recyclable thermosets will potentially reduce the disposal of waste plastics; thus, the impact of this project is multifold. The project management and approach are appropriate. The project targets recyclable carbon fiber composites, which is highly commendable. Because carbon fiber has high production costs and its recycling has been ineffective, being able to recycle both carbon fibers and polymer resin is highly attractive. The bio-based thermoset developed in this project showed high material performance and good recyclability. The results of the solvent-dissolution recycling, the welding-repaired resin, the separation of intact carbon fibers, and the fatigue tests of the recycled composites all suggest the excellent properties of the products. The TEA looks promising too. The team is proactively marketing its polymer by working with international companies.
- The potential risks and mitigations are not mentioned in the project. Due to the limited information given in the presentation, it is difficult to fully evaluate the potential risks of this project. While it mentions that the product contains more than 50% lignin, polymer synthesis is not disclosed; thus, the potential risks in polymer synthesis are unknown. The recycling solvent is also not disclosed; thus, its relevance to LCA and potential environmental pollution cannot be determined. Polymer recycling has been demonstrated using virgin resin; however, the project should evaluate how environmental degradation and impurities in post-application resins affect the recycling chemistry.

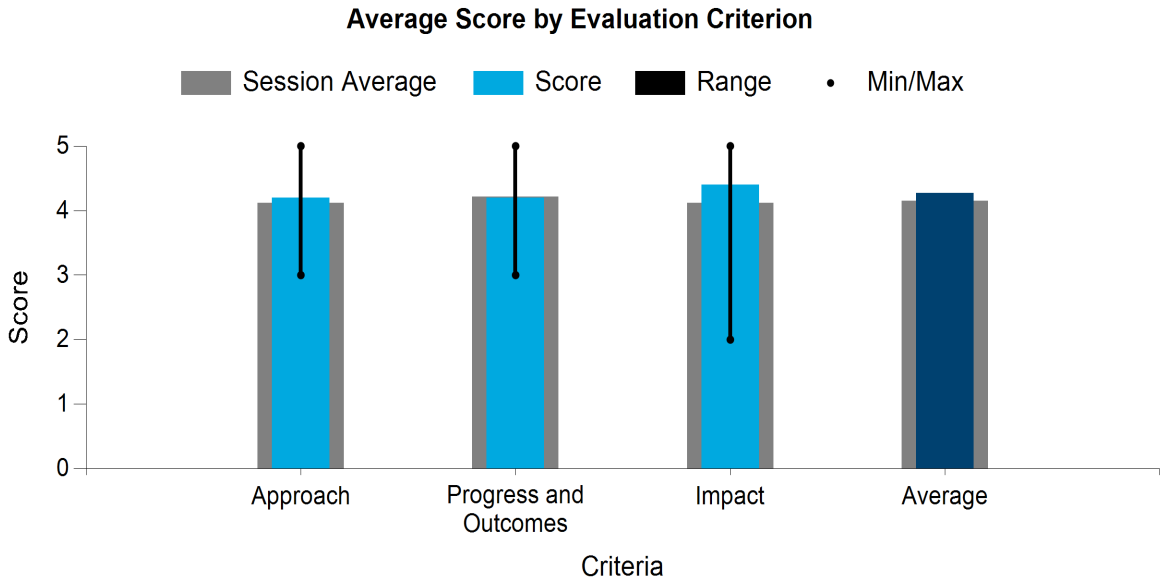
# BIOLOGICAL LIGNIN VALORIZATION

## National Renewable Energy Laboratory

### PROJECT DESCRIPTION

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|---|---|
| WBS: | 2.3.2.100 |
| Presenter(s): | Davinia Salvachua; Gregg Beckham; Laura Hollingsworth; Megan Krysiak |
| Project Start Date: | 10/01/2020 |
| Planned Project End Date: | 09/30/2023 |
| Total Funding: | $2,100,000 |

The Biological Lignin Valorization project develops microbial strains and associated bioprocesses to convert lignin-derived aromatic compounds into value-added bioproducts. Our main objective in the project is to achieve industrially relevant bioproduction metrics that can directly contribute to the economic viability and improved sustainability of the integrated lignocellulosic biorefinery in collaboration with complementary BETO-funded lignin valorization projects. Specifically, the project works closely with the BETO-funded Lignin Utilization project, which provides bioavailable aromatic compounds from chemo-catalytic lignin depolymerization. We use the robust soil bacterium *Pseudomonas putida* as our primary microbial host for the conversion of lignin-derived compounds to bioproducts. To date, we have focused on atom-efficient bioproducts that can be used as either direct replacement chemicals or performance-advantaged bioproducts, including cis,cis-muconic acid, beta-ketoadipic acid, and 2-pyrone-4,6-dicarboxylic acid. From model aromatic substrates, we have achieved titers of each of these compounds approaching 40 g/L and productivity values ranging from 0.5 to more than 1 g/L/h, all at 90% molar yield or higher. From real lignin streams, we have thus far achieved 24 g/L, 0.66 g/L/h and theoretical yield of beta-ketoadipic acid. A major pursuit now is to reach industrially relevant performance metrics on an expanded slate of lignin-derived streams.




### COMMENTS

- The goal of this project is to develop strains of *P. putida* to convert lignin monomers into value-added chemicals such as adipic and muconic acid. The technical work is outstanding and provides a unique route for lignin valorization. The team are world leaders in the area. The basic strategy is to exploit *P. putida*'s ability to metabolize aromatic compounds by engineering strains unable to completely oxidize

the substrates so that valuable intermediates are produced instead. This requires the addition of a second carbon source to provide the energy for lignin monomer bioconversion.

- The team is making good progress, and the project is well managed. The only criticism is that the details of the TEA were vague—it was unclear what the MSP was actually measuring.
- This project is one of a suite of lignin projects supported by BETO and led by NREL. This portfolio of projects fits together well in direct support of BETO's goals for lignin valorization. This project approaches lignin valorization via biology, developing strains and bioprocesses to produce single coproducts from lignin. Management and technical approaches are reasonable. The team has identified risks and appropriate mitigation strategies.
- These may have been identified in the project management strategy, but the presentation would have benefited from greater elaboration of risks and mitigation strategies, particularly around potential toxicity and other variables affecting strain performance.
- The integration of this project with other ongoing lignin projects at NREL is a strength, as are collaborations with industry partners.
- The project aims to develop *P. putida* strains and bioreactor processes to valorize diverse monomeric compounds derived from varied lignin deconstruction methods, including alkaline, lignin-first, and hydrodeoxygenation (HDO). This approach leverages the host microbe's native aromatic compound import, catabolism, and tolerance, lightening the biological engineering load and allowing the team to focus on strain and bioreactor process optimization. Toward that end, the team has already made good progress toward increasing acetate, aromatic byproduct, and salt tolerance.
- In addition to valorizing lignin, the project's impact stems from the development of a manufacturing technology that is environmentally advantaged and cost-competitive with nonrenewable adipic acid. As described, the conversion of lignin to muconate or beta-ketoadipate has the potential to deliver substantial decreases in GHG emissions (GGE) compared to petroleum-derived adipic acid (i.e., 40%–50% reduction, dependent on lignin conversion to adipic acid). Likewise, the modeled future production cost for lignin-derived beta-ketoadipate is on par with petro-based adipic acid and, in an integrated biorefinery context, can contribute substantially to lower fuel selling prices (i.e., MFSP reduction greater than $2/GGE).
- The performance metrics presented (titer, rate, and yield) are important indicators of process efficiency, and achieving high conversion yields and volumetric productivities are oft-cited challenges across most conversion technologies utilizing microbes. While achieving a 40-g/L titer is an important technical goal (FY 2023 goal), this metric alone provides limited insight into the actual commercial feasibility of the technology.
- Of note, the strain may be catabolizing a large fraction of glucose to $CO_2$ to generate adenosine triphosphate (ATP) to cope with substrate and/or production inhibition. At low pH—i.e., at or below the organic acid's (or acids') pKa—organic acid diffusion into the cell may necessitate ATP for re-export. Because the rate of diffusion will increase with concentration, a titer ceiling may be encountered. Data presented on the effect of glucose-to-aromatic compound ratio indicate this could be occurring.
- Last, the project could take additional, incremental steps to support programmatic DEI goals.
- Conversion yields (lignin to bioavailable monomers) seem to be solely driven by the monomers' bioavailability. It would be beneficial to have a sense of what the overall process yields are as well as the process viability.

- The heterogeneity of lignin and lignin-derived products is the most challenging problem with lignin valorization. In this context, the chemical deconstruction of lignin followed by biological funneling to single compounds is scientifically meritorious and highly attractive. In this project, the team works with other BETO-relevant projects to obtain bioavailable lignin compounds for bioprocessing. The risks are identified, and mitigation strategies are reasonable. The results are impressive—the team reports nearly 100% conversion based on model monomers and corn-stover-derived bioavailable monomers. Bioavailable monomer mixtures extracted from poplar are also converted in high selectivity. Acetate was also demonstrated as a carbon source to reduce or eliminate glucose costs. The industry collaboration is strong. Working with industry for strains, bioprocess development, upstream separations, and industrial lignin streams will facilitate a commercially viable technology. DEI focused on creating a healthy and safe research environment. Overall, the project team did a great job and is on track to success. The presented results are based on model compounds and high-purity extracted bioavailable monomers. Considering lignin-derived aromatics can be strong imitators, it would be helpful if the team could comment on the purity requirement in extracting bioavailable monomers and the toxicity of the non-bioavailable aromatics if they co-present.

## PI RESPONSE TO REVIEWER COMMENTS

- We thank the review team for the positive feedback! For MFSP (slides 2, 7, and 12), this is the economic contribution of a lignin-derived product to a fuel molecule produced from the carbohydrate fraction of biomass in dollars per GGE. This metric therefore measures the extent to which a lignin-derived product contributes positively to biorefinery economics; thus, the MFSP results presented (slide 7) indicate that the production of β-ketoadipic acid from lignin can offset the selling price of carbohydrate-derived fuels by $2/GGE, increasing biorefining economics. Regarding risks and mitigation strategies for "potential toxicity and other variables affecting strain performance," we agree that this is a risk (slide 5), and thus are actively identifying and overcoming toxicity and titer challenges (slide 16). To this end, we have used adaptive laboratory evolution, systems biology tools, and genome-wide screening tools (e.g., randomly barcoded transposon insertion sequencing [RB-TnSeq]) to identify nonintuitive targets for strain tolerance improvements, and we have adopted fed-batch bioreactor cultivations as a primary means to ameliorate substrate toxicity. A related reviewer's comment also asked about the toxicity of non-bioavailable aromatic compounds. This is an excellent point, and we very briefly showed this on slide 11, for example, where acetovanillone accumulates in a bioreactor cultivation. Wherein our strain does not have catabolic capabilities, including in this case, we are actively engineering the catabolic pathways, which will both reduce substrate toxicity and increase overall carbon conversion efficiency. For the glucose consumption and ATP generation, we agree that glucose (or another supplementary carbon and energy source) will generate energy for non-growth-associated maintenance (e.g., coping with toxic compounds). To quantify this, we are actively engaged in fluxomics experiments with β-ketoadipate and muconate production strains that are measuring ATP/adenosine diphosphate (ADP) ratios, $CO_2$ generation, import/export rates, and carbon flux from glucose across the bioreactor cultivation phases; however, we note that the glucose loading is quite low, at an 8:1 aromatic compound:glucose molar ratio, and we achieve equivalent strain performance to a 2:1 molar ratio. We have also quantified the economic impacts of glucose feeding on the overall economics, as shown on slide 7. We agree with the comment that "the project could take additional, incremental steps to support programmatic DEI goals" and plan on formally incorporating DEI milestones in the proposal for future project work; however, we have many DEI activities that were not mentioned on slide 5, including mentoring interns for workforce development, allocating funds for early-career staff to present at conferences (promotes networking and scientific outreach), and following recruiting DEI best practices. In terms of the overall process yields, this is driven by both deconstruction and bioconversion yields. As we have achieved strains with 100 mol % biological conversion of aromatic monomers to product (slides 8–13), the reviewer is correct that overall process yields are now principally driven by the generation of bioavailable monomers from lignin. Monomer yields vary by the substrate and deconstruction approach

and are shown on slides 4 and 10–14. The Lignin Utilization project and other projects in the BETO-funded lignin portfolio are working toward higher yields from several lignin streams. Excitingly, we are now able to obtain approximately 50%–80% yield of bioavailable monomers from lignin streams, the extent of which depends on the upstream catalytic process, and we are engineering and adapting strains for 100 mol % conversion of these monomer mixtures to this end now (slides 13–14).

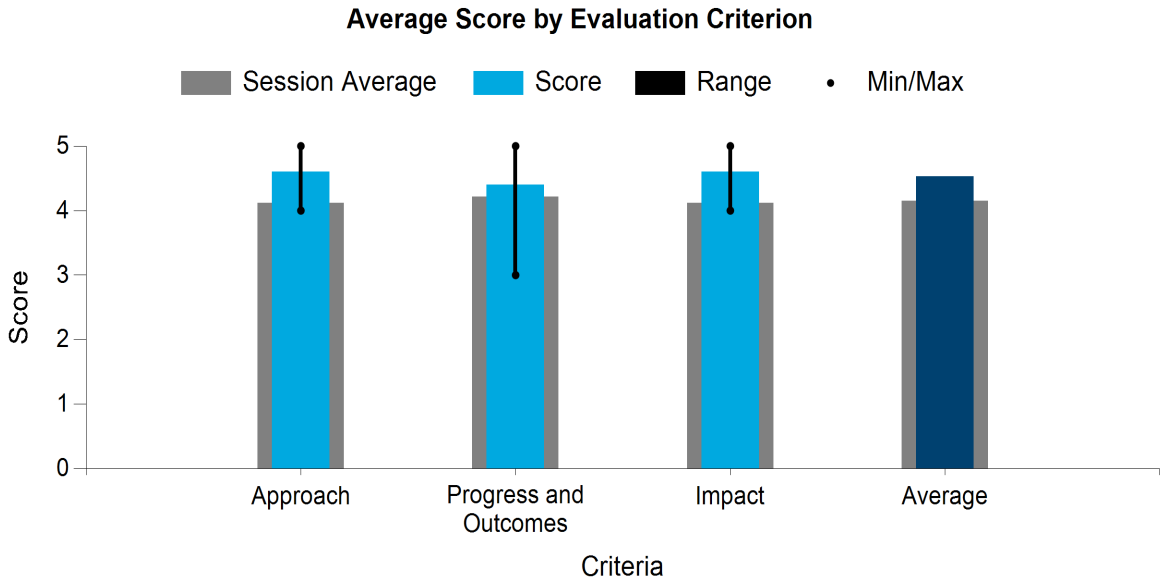
# BIOLOGICAL UPGRADING OF SUGARS

## National Renewable Energy Laboratory

### PROJECT DESCRIPTION

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|---|---|
| WBS: | 2.3.2.105 |
| Presenter(s): | Jeffrey Linger; Laura Hollingsworth; Megan Krysiak |
| Project Start Date: | 10/01/2020 |
| Planned Project End Date: | 09/30/2023 |
| Total Funding: | $2,850,000 |

The Biological Upgrading of Sugars project aims to produce SAF from lignocellulosic feedstocks through the anaerobic conversion of sugars into butyric acid, which can be further upgraded into various fuels and chemicals. In this presentation, we showcase a fully integrated bench-scale process that utilizes *Clostridium tyrobutyricum* as the biocatalyst for the production of bio-butyric acid. We discuss the genetic improvements made to the biocatalyst that have led to process enhancements as well as the design and initial operation of a pilot-scale reactor capable of 100-L fermentations coupled with *in situ* product recovery, which enables the production of 5–10 kgs of bio-butyric acid and other carboxylic acids per run. Additionally, we share our recent progress in commercializing this technology through collaborations with external partners. Our integrated approach to R&D has significantly advanced the technology of carboxylic acid production and has brought us closer to achieving our goal of cost-effective SAF production.




### COMMENTS

- The goal of this project is to provide a pilot-plant process for producing butyric acid from lignocellulosic hydrolysate using *in situ* product recovery. A key strength of this project is a focus on downstream separation, which is a major cost in biofuel production. In this regard, a pilot plant could provide a nice platform for de-risking this keystone project for the BETO portfolio.

- Overall, this program appears to be well managed with a solid risk management plan in place. The team is also making good progress, both on strain development (e.g., co-sugar utilization) and developing the pilot-scale process. It would have helped to provide more detail on the solvent selection and separation

train, but progress looks to be good. That said, the team is able to produce purified butyric acid while running the fermenter above the pKa, which is an impressive accomplishment. Another key strength is multiple partnerships with companies, which demonstrates the impact of the project. The next key step will be to integrate the different unit operations and demonstrate sustained production. No major weaknesses were noted.

- This project supports BETO's goals for SAF production.
- The project management is strong; a large team is in place to lead the complementary components of the project. Risks and mitigation strategies are clearly identified. The project benefits from multiple collaborations with BETO-funded consortia and commercial companies. The program considers DEI goals.
- In terms of the technical approach, it is interesting to see the use of three approaches to strain development (genetic engineering, mutagenic selection, and adaptive laboratory evolution) in parallel, each with different preliminary successes. The PI indicated that future areas of opportunity could include greater emphasis on new genetic tools to emphasize new and more complex products.
- The project is demonstrating adequate progress toward goals.
- The project has demonstrated significant advancements in TRL through a combination of *C. tyrobutyricum* strain engineering (i.e., addressing challenges in xylose utilization and addressing potential intracellular pH challenges via arginine decarboxylase expression) and process development (i.e., hybrid extraction distillation). The net economic impact of the estimated production costs for the hybrid process at scale is substantially below that for competitive bio-based butyric acid (i.e., approximately $1/kg compared to $1.80/kg).
- The project has demonstrated the potential to deliver low-cost, bio-based butyric acid (and derived compounds) commercially, as evidenced by collaborations with industry partners working toward SAF, chemicals, and derivates beneficial for human health and wellness.
- Of note, there is some degree of risk that the production of butyrate at extracellular pH values at or below the pKa will lead to technical challenges, limiting the overall fermentation performance. At low pH, extracellular butyric acid (protonated form) may diffuse into the cell, upon which butyrate is formed (due to the neutral cytosolic pH). This phenomenon may lead to high levels of ATP being expended, resulting in a titer ceiling that makes achieving yield and productivity targets challenging.
- Looking forward, the project may benefit from a shift in the level of effort from strain engineering and fermentation process development toward integrated process development, including *in situ* product removal. A number of downstream processing challenges may be encountered as the process shifts from batch toward continuous that can only be accurately identified through long process runs.
- With the project ending in 6 months, what is the project's current TRL? Solvent toxicity is an issue for both processes (liquid liquid extraction and fermentation). What steps are taken to address that issue? Will that be part of the units of operations integration scheduled for the next 6 months?
- This project includes strain development, fermentation with online product separation, and pilot process development, all in a single project. If successful, low-cost butyrate produced using C5/C6 sugar mixtures demonstrated in a fully integrated pilot system will significantly impact biofuels and biochemical production. The project approach for identifying key differences between carbon resources and pHs in carbon metabolism and engineering stains to co-utilize mixed sugars is logical. The progress in developing the integrated system is excellent. The team designed and constructed a fully integrated

pilot-scale system to combine fermentation and online product separation/recovery. Testing DMR hydrolysate feedstock in the pilot system allows the team to identify potential scaling problems. The team's efforts for industry engagement and commercialization are excellent. Overall, the project is on track. Although the scientific approach is justified, the progress of strain development is somehow unclear. It will be helpful if the team can comment on the performance of the current strain (e.g., titer, production rate). TEA estimates an MSP of $0.99/kg compared to the current $1.8/kg. What titer and production rates were used in this TEA?

## PI RESPONSE TO REVIEWER COMMENTS

- "It would have helped to provide more detail on the solvent selection and separation train, but progress looks to be good." When we pivoted from an NaOH back extraction to the hybrid extraction and distillation mode, we changed solvents from 20% trioctylphosphine oxide with 40% 2-undecanone and 40% mineral oil to 70% Cyanex 923 (a commercially available phosphine oxide extractant) in mineral oil. The TEA presented was based on using Cyanex; however, the toxicity of this is higher than desired, so we are currently, as our highest priority, screening other blends of extractants, seeking a balance of high extraction efficiency with reduced cytotoxicity. "That said, the team is able to produce purified butyric acid while running the fermenter above the pKa, which is an impressive accomplishment" and "Of note, there is some degree of risk that the production of butyrate at extracellular pH values at or below the pKa will lead to technical challenges, limiting the overall fermentation performance. At low pH, extracellular butyric acid (protonated form) may diffuse into the cell, upon which butyrate is formed (due to the neutral cytosolic pH). This phenomenon may lead to high levels of ATP being expended, resulting in a titer ceiling that makes achieving yield and productivity targets challenging." Thank you for the thoughtful commentary on this topic. It has certainly been the key challenge of this project to optimize pH and parameters that are influenced by pH. In particular, we are looking for pH conditions enabling sufficient growth and productivity but low enough to enable sufficient extraction. As pH increases, which helps *C. tyrobutyricum*, extraction is reduced, which, of course, means additional butyric acid in the reactor, which then adds to the toxicity. Given that we are operating close to the pKa, small reductions in pH lead to large swings in the ability of the acid to be extracted. It is a fine balance, but something we view as key to improved overall process performance. "It is interesting to see the use of three approaches to strain development (genetic engineering, mutagenic selection, and adaptive laboratory evolution) in parallel, each with different preliminary successes." We believe in taking an "all-of-the-above" approach, using both classical methodologies and more modern synthetic-biology-based approaches. There are positives and negatives to both, and we have seen positive shifts from both approaches. "Looking forward, the project may benefit from a shift in the level of effort from strain engineering and fermentation process development toward integrated process development, including *in situ* product removal" and "Solvent toxicity is an issue for both processes (liquid liquid extraction and fermentation). What steps are taken to address that issue? Will that be part of the units of operations integration scheduled for the next 6 months?" We agree with the reviewer completely and have essentially stopped all strain development work for the remainder of the fiscal year so that we can focus efforts on system optimization, modification, and safety enhancements. Regarding future solvent selection, we are in the midst of an experimental campaign to test some new organic blends. Additionally, we have some potential system modifications we are experimenting with that would help alleviate some of the trouble of balancing *C. tyrobutyricum*'s physiological challenges with process efficiencies. "With the project ending in 6 months, what is the project's current TRL?" We would estimate that we are in the TRL 6–7 range based on the following descriptions. TRL 6: System/process model or prototype demonstration in a relevant environment. Beta prototype (system): Prototyping implementations are partially integrated with existing systems. Engineering feasibility fully demonstrated in actual or high-fidelity system applications in an environment relevant to the end user. TRL 7: System/process prototype demonstration in an operational environment. Integrated pilot (system): System prototyping demonstration in operational environment. System is at or near full scale

(pilot or engineering scale) of the operational system, with most functions available for demonstration and test. The system, component, or process is integrated with collateral and ancillary systems in a near-production-quality prototype. “Although the scientific approach is justified, the progress of strain development is somehow unclear. It will be helpful if the team can comment on the performance of the current strain (e.g., titer, production rate). TEA estimates the MSP of $0.99/kg compared to the current $1.8/kg. What titer and production rates were used in this TEA?” We apologize for the lack of clarity on this front, but it is a bit of a convoluted discussion because of the numerous process configurations we are using. With the bench-scale pertractive setup (where we get the highest overall process metrics), the experimental throughput is slow and personnel heavy. Accordingly, we do the vast majority of our fermentations in batch or fed-batch mode (without pertraction) to assess strain performance, which leads to reduced titers and yields. We are preparing now to evaluate our top strains in the bench-scale pertractive setup and will then be able to accurately describe the overall improvement. For a detailed discussion on the TEA and productivity metrics, please see https://doi.org/10.1016/j.xcrp.2021.100587.

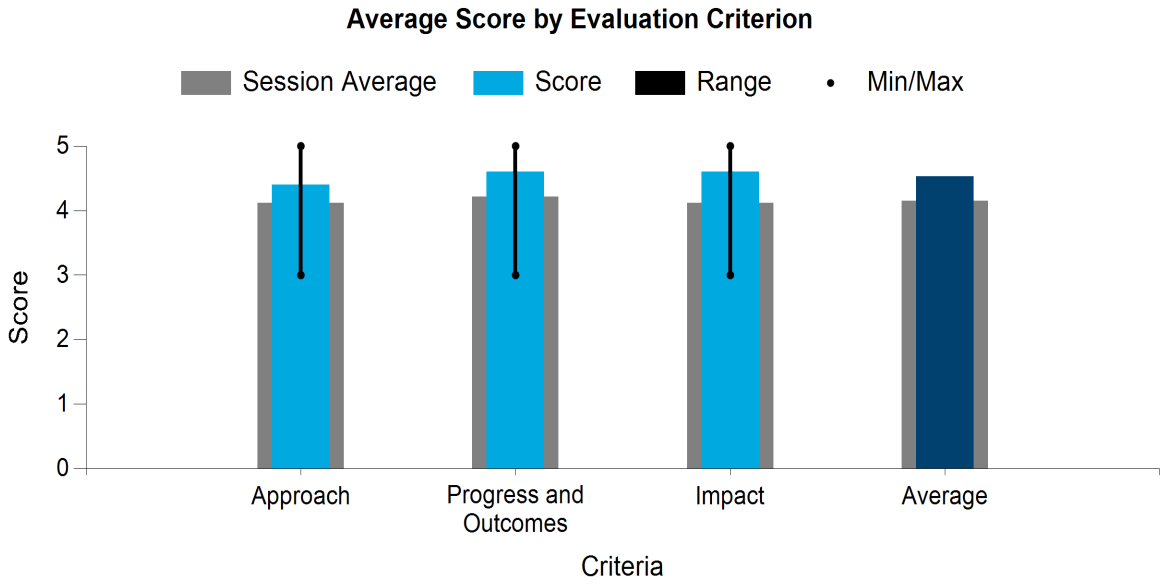
# LIGNIN UTILIZATION

## National Renewable Energy Laboratory

### PROJECT DESCRIPTION

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|---|---|
| WBS: | 2.3.4.100 |
| Presenter(s): | Gregg Beckham; Laura Hollingsworth; Megan Krysiak |
| Project Start Date: | 10/01/2022 |
| Planned Project End Date: | 09/30/2025 |
| Total Funding: | $1,875,000 |

Lignin depolymerization to aromatic monomers is a primary route for myriad lignin valorization strategies. To date, there are many strategies able to cleave aryl–ether linkages in lignin, but the lignin polymer, in both its native and processed forms, contains a substantial fraction of refractory carbon–carbon linkages between aromatic units, which typically limits aromatic monomer yields to about 30–40 wt % or lower, depending on the feedstock. To that end, the Lignin Utilization project addresses the critical challenge of lignin depolymerization catalysis with emphasis on C–C bond cleavage. Being able to achieve cost-effective C–C bond catalysis in lignin depolymerization would enable a substantial increase in the accessible aromatic monomer yields from lignin. Among the catalysis strategies that have been investigated in the project, we have made substantial progress in the use of autoxidation catalysis, inspired by the industrial conversion of p-xylene to terephthalic acid, for C–C bond cleavage in lignin. Using multiple substrates, we have demonstrated that autoxidation catalysis can produce mixtures of bioavailable aromatic monomers for conversion to exemplary bioproducts, such as cis,cis-muconic acid, in collaboration with the Biological Lignin Valorization project. Prior to FY 2023, the Lignin Utilization project also included lignin analytical chemistry method development, lignin analytics for BETO-funded projects, and model compound syntheses, which will also be presented.




### COMMENTS

- The goal of this project is to depolymerize lignin with a focus on cracking C–C bonds. The team is making good progress even though the milestones are aggressive. The project is well managed with a clear plan for dealing with risk. A key strength is the development of methods for lignin analytics, which is shared with multiple projects. The technical work is outstanding, in particular the demonstration of C–

C bond cleavage. Progress is outstanding and may lead to a high-yield process for generating monomers. The work also nicely complements a number of existing projects within the BETO portfolio. Overall, the lignin work is the "jewel" within the BETO portfolio.

- This is a strong project that addresses an important problem. Basic feasibility has been established using multiple approaches. The next step is to develop an economically viable process.
- This project is one of a suite of lignin projects supported by BETO and led by NREL. This portfolio of projects fits together well in direct support of BETO's goals for lignin valorization. The primary aim of this project is to develop catalytic processes for generating aromatic monomers from lignin. The project has identified C–C bonds as an underutilized target for catalytic deconstruction. There are challenges to overcome, but should project goals and scale-up be achieved, the impact of this would be substantial.
- The technical and management approaches are reasonable. The project is making good progress toward its goals. Collaborations with other NREL-led lignin research, as well as industry partnerships, strengthen the research.
- Project impacts are enhanced by efforts to make the analytical tools and protocols it develops publicly available online. It will be interesting to see to what extent these online resources are accessed. Such data may provide insights into whether (1) the posted materials are in demand by other researchers (and by which researchers) and (2) an outreach plan may be necessary to increase awareness that the tools and protocols are available for use.
- The project aims to address a critical technical need in the pursuit of BETO's goal of valorizing lignin by focusing on C–C bond cleavage in lignin, overcoming the approximately 30%–40% yield limitations faced by current C–O bond cleavage technologies. An innovative approach, autooxidation, is used to generate water-soluble, bioavailable aromatics that can be microbially catabolized. Because the technology should be feedstock agnostic and the output monomers are readily catabolized microbially, this project provides a versatile method to connect upstream lignin to diverse downstream bioconversion technologies (e.g., Biological Lignin Valorization projects).
- Regarding progress to date, in collaboration with the Process Modeling and Simulation project, the team developed analytical methods to track lignin-derived compounds throughout the process. The team also demonstrated proof-of-concept autoxidation of C–C-linked acetylated oligomer, methylated oligomers, and hydrodeoxygenated oligomers. This work sets the stage for introduction of flow-chemistry-based approaches to improve reaction performance.
- The project aims to address the technical problems related to phenol inhibition through three different methods: methylation, acetylation, and HDO. All three routes appear to be viable from a downstream biological perspective, with acetylated products being likely catabolic enzyme substrates; however, concerns were raised about the LCA challenges associated with HDO, and it was intimated that methylation may pose technical challenges. Looking forward, the project would be well served by a rapid determination of how HDO or methylation compete with acetylation from TEA and LCA perspectives. If these approaches are not viable, R&D efficiency cannot solely be increased (i.e., the sole focus is on acetylation); downstream bioconversion projects will have greater clarity on what their input substrates will look like.
- As presented, work to foster DEI is minimal, and additional steps could be taken to support program DEI goals.

- The autooxidation catalytic work performed in collaboration with University of Wisconsin, Madison seems to still be at the proof-of-concept stage, and it is not clear if it has the potential to be economically viable at prepilot or pilot scales. Are plans being made to eventually scale this process up?

- This project is one of the BETO-relevant projects for lignin valorization and complements other projects. The project goals address important aspects of analytical science and lignin valorization. The lack of analytical capability for identifying lignin compounds has been the bottleneck in the field. Gas chromatography/mass spectrometry and nuclear magnetic resonance are insufficient for complex lignin chemistry. Although high-resolution mass spectrometry could provide accurate masses of oligomeric compounds, it is difficult to determine molecular structures. In this context, the energy-resolved mass spectrometry method, Lignin Wrangler library (in collaboration with the computational project), and open-source protocol developed in this project enable the rapid and accurate identification of lignin compounds, broadly benefiting the community. The difficulty of producing chemicals from lignin in high selectivity is addressed by converting lignin into bioavailable monomers and then biologically funneling down to single molecules. Catalytic autooxidation and masking phenolic OH were highly effective in depolymerizing lignin into aromatic acids. The project concept is novel. The risks and mitigation strategies are adequate, and the project management is excellent. The collaborations with multiple parties for TEA/LCA, product separations, product upgrading, and flow-through reaction are commendable. DEIA activities are appropriate. Overall, the project made excellent progress.

## PI RESPONSE TO REVIEWER COMMENTS

- We thank the reviewers for the positive feedback on the Lignin Utilization project! We are extremely excited about the C–C bond cleavage efforts and analytics method development efforts in this project. The point about monitoring the views and citations of the posted protocols is excellent. We posted these protocols immediately before the FY 2023 BETO Project Peer Review, and we will monitor these closely going forward to understand if the community is using them. We will also include these posted protocols in external presentations as a resource for the community as well. One reviewer raised the TEA- and LCA-oriented question regarding the choices between acetylation, methylation, and HDO, which we fully agree with as the major question to answer in the C–C bond cleavage chemistry. To that end, we are now actively doing what the reviewer suggested. Regarding the scale-up of autoxidation, we note that the same catalyst system and solvent are used in the Amoco process to make terephthalic acid from p-xylene at 80 million metric tonnes/year, and thus there is considerable precedent for this chemistry at the industrial scale. We intend to conduct TEA and LCA on the autoxidation process in parallel with the experimental transition to flow-based reactor systems. For the comment on future DEI work, we agree, and are working on a larger DEI plan between the NREL lignin projects to make a larger impact.

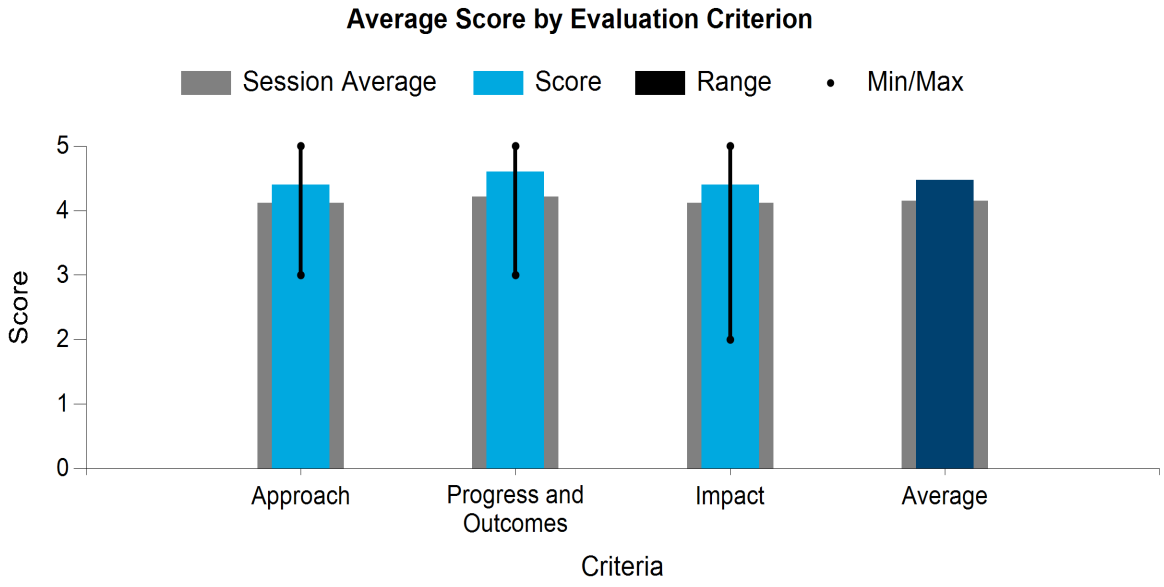
# LIGNIN CONVERSION TO SUSTAINABLE AVIATION FUEL BLENDSTOCKS

## National Renewable Energy Laboratory

### PROJECT DESCRIPTION

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| WBS: | 2.3.4.104 |
| Presenter(s): | Gregg Beckham; Laura Hollingsworth; Megan Krysiak |
| Project Start Date: | 10/01/2022 |
| Planned Project End Date: | 09/30/2025 |
| Total Funding: | $3,600,000 |

The Lignin Conversion to Sustainable Aviation Fuel Blendstocks project focuses on the conversion of lignin-rich streams to deoxygenated aromatic and cycloalkane blendstocks in the jet fuel range. This work is done in close collaboration with the BETO-funded Lignin-First Biorefinery Development project and industrial scale-up partners, and the work is closely guided by analysis to develop cost-effective and sustainable routes to produce lignin-based SAF blendstocks. To date, we have demonstrated the continuous catalytic conversion of a lignin oil from poplar to deoxygenated aromatic products at approximately 85% C-mol yield. This HDO process uses a stable, earth-abundant catalyst and requires no solvent. We have also established a baseline process model and associated TEA and LCA that together demonstrate the potential to achieve cost parity with fossil carbon-based jet fuel at approximately 70% reduction in GHG emissions. Current work is focused on expanding the slate of feedstocks for HDO to include lignin oils from softwoods, agricultural residues, and grasses, as well as from hydrolysis lignin substrates from biochemical conversion and pulp-and-paper processes. We are also undertaking catalyst development efforts to tune the reaction selectivity from aromatic compounds to cycloalkanes. Last, we are investigating reaction engineering strategies to slurry solids for HDO reactions.




### COMMENTS

- The goal of this project is to convert lignin monomers into aromatic and cyclohexane molecules for use in aviation fuel. The key challenge is achieving this goal is to reduce the oxygen content with these molecules. The proposed solution is HDO. The team is making excellent progress, and the technical

component is outstanding. The project appears to be well managed with a strong risk management program and is making good progress toward achieving their milestones. A key strength is that TEA and LCA are central to the project goals. The potential impact is huge because it provides a potential route for lignin valorization by targeting the important problem of developing SAF.

- Overall, this is an exciting project and one of the highlights of the BETO portfolio.
- This project is one of a suite of lignin projects supported by BETO and led by NREL. This portfolio of projects fits together well in direct support of BETO's goals for lignin valorization and the SAF Grand Challenge. This project uses substrates from the lignin-first biorefinery project, among other sources, to develop HDO processes to produce performance-advantaged and SAF-relevant products efficiently, economically, and sustainably.
- The approach is well considered. The numerous collaborators involved in the work allow the project to cover a lot of ground in addressing the project's core goals. As with other BETO-funded projects, DEI goals could be strengthened if additional support and guidance were provided.
- Affirming the relevance and potential impact of this work, the project demonstrates multiple collaborations with industry and academic laboratories. The project has achieved early goals and set ambitious future targets. Improving the practicality of the HDO process will require overcoming a number of challenges. The team has identified risks and appropriate mitigation strategies.
- The project has a well-defined management plan that is guided by TEA and LCA. In addition, the combination of product testing through ASTM blending analysis, industry collaborations, and engagement with stakeholders ensures that the project outcomes are relevant and advance the SOA, aligning with BETO's goal of producing SAF.
- From a technical perspective, the decision to focus on developing a benchtop-scale continuous system, rather than a batch reactor, is a strength because it allows for a thorough understanding of how key process performance parameters, such as catalyst total turnover and reactor kinetics, impact the technical feasibility and commercial viability of the technology; however, it is notable that there is no explicit target for reaction kinetics, and it is recommended this be addressed alongside the 500 hours of cumulative time-on-stream target.
- While the project is relatively new, significant progress has been made in completing foundational experiments that set the stage for continuous processing. The data presented in the review are promising, with high C-mol yields (e.g., 86% of theoretical) and low residual oxygen achieved in a two-pass HDO process at the benchtop scale using different feedstocks. Moreover, the output HDO oils show potential as a performance-enhancing component of SAF blends, with approximately two-thirds of the output HDO oil from poplar falling within the jet fuel range and exhibiting superior performance parameters compared to traditional fuels. The LCA results also demonstrate impressive reductions (54%–81%) in GHG emissions associated with the production of RCF HDO oil compared to petroleum-derived Jet A fuel. While the TEA results indicate reasonable MSSPs, further improvement may be targeted in future years to make the sugars cost-competitive with those derived from cane and corn. In sum, if successful, this project has the potential to significantly contribute toward achieving BETO's long-term goals of producing SAF.
- The project is taking steps to support DEI programmatic goals through internships with minority students.
- The retention times seem to be very long. One recommendation would be to find ways to shorten the retention times and subsequently the cost associated with the process as it currently stands.

- The process seems to be expensive as of today. One recommendation would be to put action items in place to find ways to significantly reduce the process cost to meet BETO's metrics and cost targets.

- The project aims to convert lignin to SAF via catalytic HDO. Lignin HDO is an extensively explored research area; however, upgrading RCF oil makes this process an interesting and promising pathway. The team identifies flow reactor setup, catalyst development, and feedstock dependency as the major risks and barriers. The project approaches are very thoughtful and strongly focus on scaling up. Industry engagement is strong. Engaging companies for lignin substrates, reactor scale-up, and end product development will facilitate technology transfer when it is ready for commercialization. Other than SAF, using HDO oil as bio-based lubricants and working fluids is mentioned, which can diversify the product applications. Although the project started recently, the team has already achieved impressive HDO conversion and a high percentage of SAF-range molecules. TEA and LCA support the feasibility of the technology. Overall, the project approach is logical, and the management plan is excellent. DEI activities, such as recruiting interns and holding DEI training workshops, are adequate. It would be helpful if the dependency of HDO conversion and catalyst deactivation on lignin feedstock is elaborated, especially when technical lignins with high molecular weights and impurities are used.

## PI RESPONSE TO REVIEWER COMMENTS

- We thank the reviewers for their positive feedback on the Lignin Conversion to Sustainable Aviation Fuel Blendstocks project. We appreciate the comment on reaction kinetics, and we are pursuing this with real RCF oils as substrates by tracking the reactivity of monomers that can be readily tracked. Regarding the comment about retention times and cost, we showed the estimated economics of the HDO process on residual lignin streams in the presentation at the current residence times, and we note that the estimated economics are quite attractive already. We will continue to use TEA and LCA to evaluate the most impactful process variables, including residence time, to ensure that our focus is maintained on the most important process parameters for beneficial economics, reduced energy use, and reduced GHG emissions. For the comment about using technical lignins, we fully agree that this will be an interesting area to pursue, and we are doing so. In the short time that the project has been running, we have been able to fully conduct HDO on poplar and pine RCF oil, and technical and biorefinery residual lignins are slated to be studied in the future after we are able to reach the scaled-up Tier Beta level for these initial, high-priority feedstocks. For the comment referring to our DEI goals, we would like to elaborate that more work is going on at all institutions than could be highlighted on a slide. All project institutions have the support of institutional DEI plans for hiring, equipment purchases, and workshops. We also focus on the next generation of scientists by allocating funds for early-career researchers to travel and present at conferences as well as funding for research technicians to attend trainings to continue to build their skill sets.

# FERMENTATIVE PRODUCTION OF TULIPALIN A: A NEXT-GENERATION, SUSTAINABLE MONOMER THAT DRASTICALLY IMPROVES THE PERFORMANCE OF PMMA

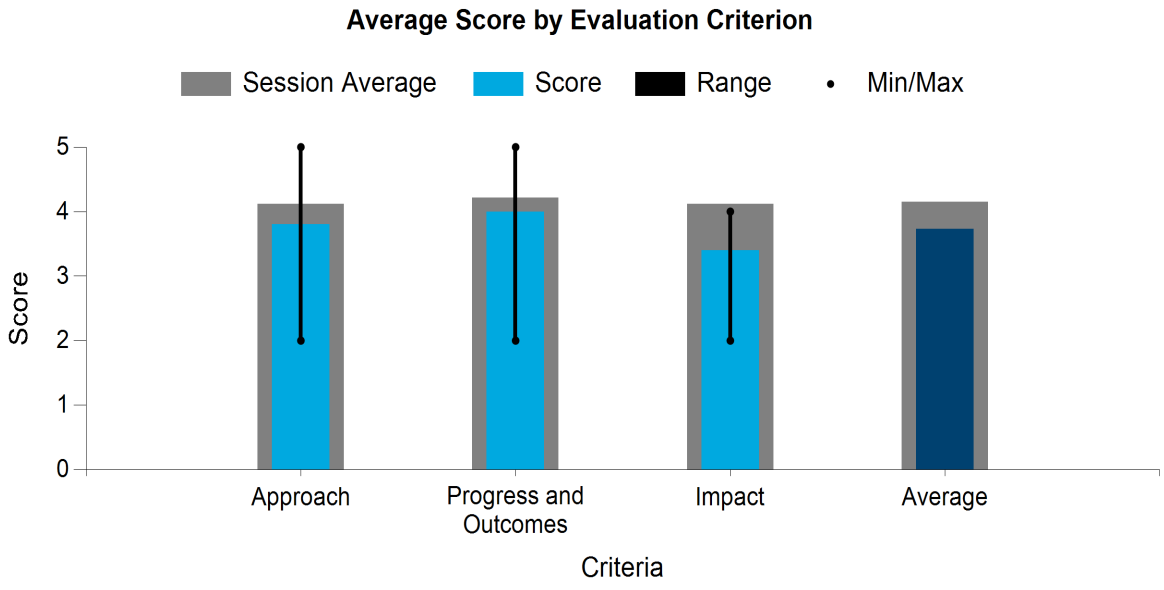
## Arzeda Corp.

### PROJECT DESCRIPTION

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|---|---|
| WBS: | 2.3.4.208 |
| Presenter(s): | Alex Zanghellini; Aaron Korkegian |
| Project Start Date: | 10/01/2018 |
| Planned Project End Date: | 12/31/2022 |
| Total Funding: | $3,704,351 |

Methyl methacrylate (MMA) is a large-volume petrochemical monomer with a $6 billion/year global market. Its homopolymer, polymethyl methacrylic acid (pMMA), is a transparent plastic with applications in surface coatings, automotive and aerospace casts/sheets, and optical devices. Tulipalin A (i.e., alpha-methylene butyrolactone [MBL]) is a sustainable monomer that as a homopolymer or copolymer with MMA yields materials with similar properties as pMMA but significantly higher Tg (105°C for pMMA, 195°C for poly[alpha-methylene butyrolactone] [pMBL]) and improved scratch and mar resistance, weatherability, and birefringence in optical applications. MBL occurs naturally in small amounts in tulips, the metabolic pathway is only partially known, and the molecule is not produced by any microorganism. Chemical routes are too expensive to reach target prices, so today there are no scalable, cost-effective production routes.

Using proprietary computational pathway and enzyme design techniques, Arzeda has developed an MBL catalytic route. The implementation and development into a fermentation host has produced titers of 5 g/L. Bioprocess development has further demonstrated that the process is amenable to use with sustainable lignocellulosic material. Arzeda has developed a downstream process for the extraction and purification of MBL monomer from fermentation broth at high purity and yield and has demonstrated the ability to polymerize monomer into the clear plastic pMBL. Desirable thermal, mechanical, and optical properties that rival those of its competitor pMMA have been confirmed in collaboration with Pacific Northwest National Laboratory.




## COMMENTS

- The goal of this project is to produce tulipalin A, a potential replacement for methyl methacrylate. Details were vague, so it was difficult to evaluate progress; however, the underlying science/technology is impressive and represents the SOA with regard to enzyme and pathway design. While the team is on track, the current results do not suggest that this work will yield an economic process. They are currently making 150 mg/L of tulipalin A, which is at least two orders of magnitude less than required for a commodity chemical. There are not many examples where such increases are possible.

- The reviewer also questions the overall logic of this project. Most successful biological products work with a native producer that for some reason likes to produce high amounts of a fermentation product. It is questionable whether you can start with product and then work backward. Yes, you will be able to establish proof of principle, but generating a strain that will produce a commodity at scale requires high productivities and titers and is an extremely difficult task. There are hundreds of examples of such failures in the literature.

- In summary, this is an interesting project utilizing cutting-edge science that is unlikely to yield a viable product. The key strength is the use of computational enzyme engineering. The key weakness is the lack of TEA or any justification for why tulipalin A can be produced biologically at costs necessary for a commodity chemical replacement.

- The project aims to develop a microbial strain that can produce, via fermentation of lignocellulosic material, a performance-advantaged bioproduct that is otherwise produced in nature via an unknown metabolic pathway. The project is interesting in that the market price for tulipalin A is high, and chemical routes are not cost-effective. Should the team succeed in refining its fermentative production, the product would be valuable. The approaches utilized in this project could expand opportunities to design pathways for other new products.

- The approach of combining computer modeling with wet lab experimentation to develop the catalytic route, strain, and processing approaches is sound.

- The project has achieved some project goals: It has succeeded in producing the target product in small quantities and high quality. The project will need to increase the titer for practical applications. Many technical and management details were unavailable due to the proprietary nature of the research.
- The project aims to develop a biological route to tulipalin A, a monomer used for the production of pMBL as a potential substitute for pMMA. The project has made significant progress to date, achieving key technical milestones such as pathway design and screening achieving, high-throughput optimization, and meeting initial process performance targets with over 99.9% purity monomer precursor at titers of 5 g/L. That said, a more thorough discussion on yield and productivity is warranted because these are typically the primary cost drivers during fermentative small-molecule production.
- The project structure is well developed, leveraging the biodesign capabilities of Arzeda along with the expertise of the national lab (Pacific Northwest National Laboratory) in monomer and polymer characterization. The project's staged management plan, involving design, high-throughput testing, and integrated process development, has contributed to its success by strategically stage-gating resource-intensive aspects such as fermentation process development after achieving key pathway performance targets. The parallelization of certain aspects of the project, such as downstream process development using mock mixtures, has also been pursued to increase overall project efficiency.
- The absence of specific technical details, such as graph axes and the naming of metabolic intermediates or fermentation products, makes it challenging to fully assess the feasibility of future development plans, particularly in achieving commercial yields, titers, and productivities. There may be flux imbalances between different parts of the pathway, and the nature of the remaining technical challenges may be fundamentally different from those addressed in the project to date.
- The potential impact of the project is high because pMBL is a sustainable polymer with potential performance advantages over pMMA, such as increased solvent resistance, high glass transition temperature, and high elastic modulus. The project has also made progress toward industry partnerships and manufacturing agreements, as demonstrated through sampling agreements and a manufacturing letter of intent, which may decrease commercialization risk. Additional integration of downstream partners from industry or national labs could benefit the project because there may be areas where pMBL is advantaged compared to pMMA (such as solvent tolerance) but also areas where it may be deficient (such as elongation at break). While a snapshot of aspirational business development efforts was provided, it is still unclear to what extent pMBL is a true substitute for pMMA in the identified application spaces.
- The project's approach has some potential to provide a more sustainable pathway to producing acrylate.
- Risks/challenges are listed in the presentation, but no mitigation strategies are outlined. How will the challenges outlined be addressed? It would have been helpful to propose potential mitigation to the challenges outlined.
- There is no TEA to support the economic claims. Can a TEA be provided to assess the economic impact, if any?
- No DEI plan was outlined in the presentation. How and where does DEI fit in this project?
- Some collaboration with contract manufacturing partners and potential partners at various scales are mentioned, and 2 kg of tulipalin was shipped to 15 companies. What is the current TRL? Is the project still at such a small scale since 2018?

- What is the intermediate metabolite? It seems to be more concentrated than the desired target product. Future work should look at ways to produce more of the target products and less of the intermediate. Maybe focus on another pathway that produces less intermediate metabolite and more of the product? What is the benefit of focusing on the current pathway if it is not quite satisfactory?
- The project presents a structure with no timeline. It is difficult to assess progress with no timeline.
- It is challenging to accurately review the data presented when the axes in the graphs have no units and are not labeled, the culture times are not disclosed, and the metrics are not clear. What is the productivity? What is the process yield? How much does the natural enzyme produce versus the designed enzyme?
- What is the commercial-scale viability for this project?
- The project goal is to deliver a commercially viable synthesis of bio-based pMBL. pMBL can be a performance-advanced polymer counterpart of MMA; however, tulipalin is difficult to obtain in sufficient quality at low costs. Thus, biosynthesizing tulipalin from sugars for cost-competitive pMBL makes this project attractive. The risks are thoroughly discussed. The project approach is adequate, and the progress is on track. The proof-of-concept enzyme is developed, and pMBL is synthesized to characterize its polymer properties. The resulting MBL and pMBL show excellent properties; MBL at 99% purity was obtained, and pMBL had impressive thermal, mechanical, optical, and solvent resistance properties, better than that of pMMA and the literature-reported pMBL. MBL was also demonstrated using deacetylated and disc-refined hydrolysate, which can greatly improve its commercial potential. The team is actively developing commercial partnerships for product manufacturing and market development. Overall, the project approaches and progress are appropriate. Although the product titer is still much lower than the commercially viable titer level (5 g/L compared to 20 g/L), the results are encouraging. It would be helpful if TEA is given to discuss the cost of pMBL compared to pMMA.

## PI RESPONSE TO REVIEWER COMMENTS

- The vagueness in describing the specific pathway and strain utilized was done intentionally to protect confidentiality, and we understand and sympathize with how that can make a thorough review difficult. Details of the pathway were shared with and reviewed by the DOE technical team privately. As a point of clarification, our current achieved experimental titers are at 5 grams of tulipalin A per liter of broth and not 150 mg per liter. This is still short of our estimated 20 g/L needed for initial engagement; however, we are off by four times and not orders of magnitude. I would agree that the goal of creating a novel strain to produce a product that is not produced biologically in nature by any known microorganism is indeed challenging; however, having reached 5-g/L titers while still operating from plasmid without significant optimization of expression, strain, or fermentation, or encountering any issues with toxicity from production in the current host, I believe there is reason to be optimistic that there is a path forward to continue to improve titers. Granted, this is a significantly more difficult problem than optimizing the production of a native pathway in a given host. We appreciate and agree with the concept that the approaches described in our presentation are generalizable to any biologically produced performance-advantaged product, and we are always looking for new potential applications of our platform. We agree that titers will need to be further increased by at least fourfold for any practical commercial engagement and tenfold to further open up the market opportunities to a broader range of applications. Lab-scale research is still required before it would make sense to attempt any scaling for manufacture, although TEA should be taken into account. We agree with the reviewer that measurements of yield and a clear TEA of market opportunities are needed metrics for evaluating the likelihood of success or identifying key risks to be addressed. We are tracking these; however, for the purpose of confidentiality, we were not able to share them in a public setting. We recognize that this answer is not particularly satisfying. Similarly, we understand that not disclosing specifics with regard to our pathway, strain, or production

rates makes suggestions and constructive criticisms at times difficult. For each slide presented, we considered what we felt we could share at the time given the public nature of the presentation. We agree that there is risk in pathway engineering for losing yield due to issues of relative flux or the production of off-target products, and as a means of mitigating that risk, we are measuring yield and carbon utilization to monitor for off-pathway products. Although we were not able to share the specifics of those results for confidentiality, we have not yet encountered an insurmountable issue to date, and we have shared that information privately with the DOE technical team. We recognize that additional research in the application space is required before it is clear if applications of tulipalin are commercially viable, and we are in negotiations with several potential application business partners to supply them with sufficient bio-acrylate products for them to conduct their testing for evaluation once fermentation titers and purified yields are sufficient to supply. Potential project risks were identified and used as a means of crafting the project plan, which was reviewed and accepted by the DOE technical team. As part of the de-risking effort, a TEA was produced and shared with the DOE technical team; however, for purposes of confidentiality, we did not wish to disclose the findings in a public setting. The project TRL is still in a research phase to prove feasibility, which will require sufficient (kilogram) quantities of bio-produced MBL to be supplied to potential business partners. We are currently engaged with partners for the applications testing required before they would commit to purchasing at a certain volume and price point. Once a commercial agreement is reached, the project will move to a TRL for reaching the price point required to sell with adequate margin for that application space. The intermediate metabolite is a natural product generated by an engineered strain and is the starting point for the engineered tulipalin pathway. The identity of the intermediate metabolite is confidential and was thus not shared publicly but was shared and vetted with the DOE technical team. This reviewer astutely points out that the intermediate is at higher concentration in the production broth than the target. This is an indicator that the pathway producing intermediate is faster than the pathway producing product. Further refining the full pathway will be the primary focus for improving production by increasing flux through the pathway leading to the target from intermediate to increase target titers while decreasing intermediate titers. We believe that the current engineered pathway to tulipalin is satisfactory, as demonstrated *in vitro*; however, the enzyme production and/or activities need refinement to improve flux through to the final target over the intermediate. We understand the difficulty in critically evaluating the project progress and timelines when information such as productivity and yield are not disclosed in the presentation due to confidentiality. We have presented those metrics to the DOE technical team, and they are part of our validation review process. Currently, the pathway flux is pulled by the designed last-step enzyme from the two initial-step natural enzymes. The natural enzymes are not annotated as having this specific substrate to product conversion; however, we demonstrated *in vitro* that they were capable of conducting the reaction to completion at viable rates, though the production/rate of the enzyme in strain likely needs further refinement. Commercial scales depend on application, and we gave examples of low-, mid-, and high-volume potential applications for the product that would require a range of production scales and production yields/rates. Initial commercial engagement could be done in a low-volume application where tulipalin is used as a copolymer or is used for a high-value application such as photoresist polymers for chip manufacturing. Further refinement of yields, rates, and processing could reach additional application spaces, requiring higher production volumes and lower costs. As the reviewer highlights, there is a gap between the current production and the production required for commercial engagement. Therefore, the current focus of the project is to further improve production titers/rates/yield to achieve the amounts needed for initial commercial engagement. That number was determined through the production of a TEA for some of the lower-volume, higher-value applications identified for which we are in discussions with business partners. A TEA was shared with the DOE technical team as part of the validation but was not shared during the public presentation for reasons of confidentiality.

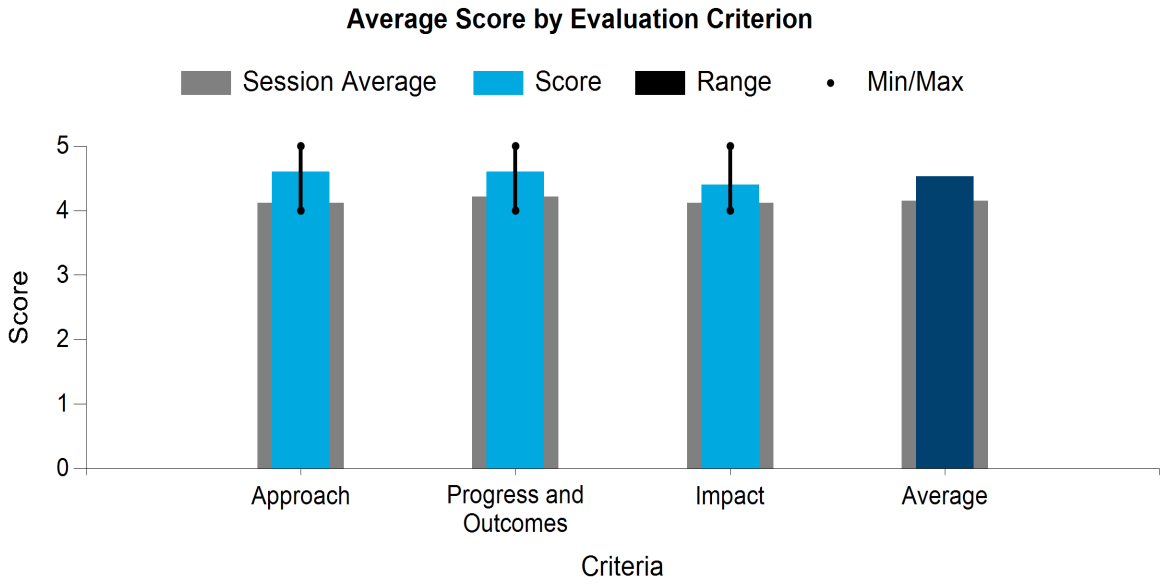
# BENCH-SCALE RESEARCH AND DEVELOPMENT

## National Renewable Energy Laboratory

### PROJECT DESCRIPTION

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| WBS: | 2.4.1.100 |
| Presenter(s): | Laura Hollingsworth; Megan Krysiak; Nancy Dowe |
| Project Start Date: | 10/01/2015 |
| Planned Project End Date: | 09/30/2023 |
| Total Funding: | $2,250,000 |

Bench-Scale Research and Development develops and optimizes fermentation processes to produce bio-based fuels and chemicals for commercial scale-up. The project uses fermentation science to achieve high titers and rates and develops online control strategies for better fermentation operations. For this review, we continued developing a commercial-ready 2,3-BDO fermentation from biomass sugars using NREL's proprietary *Zymomonas mobilis* microorganism. 2,3-BDO is a versatile, low-carbon chemical that can be catalytically upgraded to a variety of hydrocarbon fuels and chemicals. The project had three goals: Evaluate the technical feasibility of using whole-slurry pretreated corn stover, optimize a liquor-based fed-batch fermentation for high titer, and develop strategies to enable scale-up. After evaluating different iterations of a whole-slurry fermentation, a go/no-go decision was made to pivot to liquor only with new TEA performance targets, the main one being a 140-g/L titer. We successfully met this goal, producing 170 g/L of 2,3-BDO at 1-g/L-h productivity and 86% process yield. We developed a near-infrared spectroscopy method for rapid analysis, which was used to maximize 2,3-BDO production. We are preparing to scale by mapping the oxygen transfer coefficient (kLa) and oxygen transfer rate in larger vessels to match our optimized bench system. Our end-of-project goal is to meet the design target 2,3-BDO titer (140–150 g/L) from corn stover liquor at 1,000 L to demonstrate scalability.




### COMMENTS

- The goal of this project is to produce 2,3-BDO from hydrolyzed, DMR-pretreated corn stover. 2,3-BDO is a potential platform molecule. The only weakness is that 2,3-BDO is a fairly easy target in the field of metabolic engineering: it has been produced at high titer in a number of microorganisms. That said, it is a valuable target and one with potential for commercialization, whether in *Zymomonas* or any number of

other microorganisms. Perhaps it would help for the team to benchmark their work against other approaches in the literature.

- With regard to this specific project, it appears to be well managed. The team is making good progress reaching various metrics. The development of the near-infrared probe is a key advance, and it will greatly help with process optimization and scale-up. The latter is also a key strength of the project, and it is good to see that the team is considering these challenges in their plans.
- Overall, this has been a productive though not especially exciting project. The team has exceeded their milestones and demonstrated that a process based on *Zymomonas* can be used to produce 2,3-BDO at high rates and titers. It is unclear what fermentation parameters are needed for an economically viable process.
- This project focuses on scale-up and optimizing fermentation to produce 2,3-BDO from corn stover in support of BETO's SAF goals. The potential for impact is high.
- The project approach is sound, with risks and mitigation strategies appropriately identified. The team reached a go/no-go decision on starting material and pivoted to focus on liquor fermentation only rather than continue to pursue high-solids hydrolyzed DMR and non-hydrolyzed DMR corn stover.
- The team exhibited creativity when addressing challenges related to monitoring reactor conditions in fermentation scale-up. A handheld, near-infrared spectrometer allows for the rapid analysis of ongoing fermentation. They report successes in using this device. Questions remain regarding how reliable the data from such a device will be should scale-up continue.
- The progress and outcomes are positive. The project has achieved remarkable improvements in titer, exceeding project goals.
- Overall, the team has demonstrated significant advancements in the fermentative production of 2,3-BDO using engineering *Z. mobilis* strains with both purified glucose and DMR-derived substrates, achieving best-in-class product titers and yields. The project has also exhibited, or plans to pursue, collaborative projects with other NREL groups (e.g., separations and upgrading consortiums) to develop integrated processes.
- The management approach (e.g., risk register) appears to have done an effective job guiding the R&D objectives. For example, the use of DMR streams comprising solids presented challenges with materials handling, water content, or enzyme loading requirements that made them unfeasible. By focusing on concentrated DMR liquors, the team was able to circumvent these issues and achieve both high product titers and yields.
- The project has the potential to help facilitate the development of 2,3-BDO as a novel bio-based chemical intermediate, as demonstrated by industry partnerships with BioBrincipia (process scaling) and partners working to convert 2,3-BDO to finished chemicals and products. This could be particularly impactful given bio-based 2,3-BDO's negative carbon intensity value.
- The reviewer encourages the project to embrace fermentation goals other than titer, particularly because 2,3-BDO is nontoxic and exhibits little to no end product inhibition. Particularly as the team now explores process intensification, it may be more appropriate to include a combination of yield and productivity goals.

- The project has made significant progress since the last Project Peer Review and has maintained the process efficiency on pure sugars and cellulosic hydrolysates while increasing 2,3-BDO titer. A 170-g/L titer on pure sugar is impressive, and the PI foresees a similar performance with cellulosic sugars.
- We look forward to reading what the focus on de-risking the fermentation technology for scaling will achieve.
- This is an important project in the BETO portfolio for developing commercially relevant technology and enabling low-cost SAF. Choosing 2,3-BDO as the product is well justified (not too toxic to industrial enzymes, not too many byproducts). A multidisciplinary team consisting of chemical/mechanical engineers, microbiologists, and analytical chemists work together, and their roles are reflected in the project approaches and tasks. The project management and industry engagement are adequate. The challenges in scaling up are logically presented, and various mitigation strategies are developed. The team's approaches to overcoming low sugar concentration and insufficient titer are commendable. 2,3-BDO titer increased to 170 g/L using glucose, showing their effective approach. The online analytical capability was developed to aid process control and optimization. The oxygen transfer rate/kLa maps have been developed for reactor scales from 500 mL up to 1,000 L, suggesting the near-infrared analysis can be extended in a commercial-scale process. Overall, the project approach is excellent, and the project is on track. It will be helpful if the team comments on how the 2,3-BDO productions were affected by the different reactor scales they tested. This information is important in developing reliable TEA to guide commercialization.

## PI RESPONSE TO REVIEWER COMMENTS

- We thank the reviewers for their positive comments and appreciate their acknowledgement of the significant advances the project has made in developing a commercially viable fermentation process to produce 2,3-BDO from lignocellulosic sugars for SAF. For future work, we agree with the reviewers to include other fermentation metrics beyond titer, like yield and productivity, as we work with our industry partner to scale the process and develop a relevant techno-economic model. The near-infrared rapid analysis will continue to play a role in scaling, and we are producing data on 2,3-BDO production in different reactors to validate the oxygen transfer rate/kLa mapping work for scale-up.

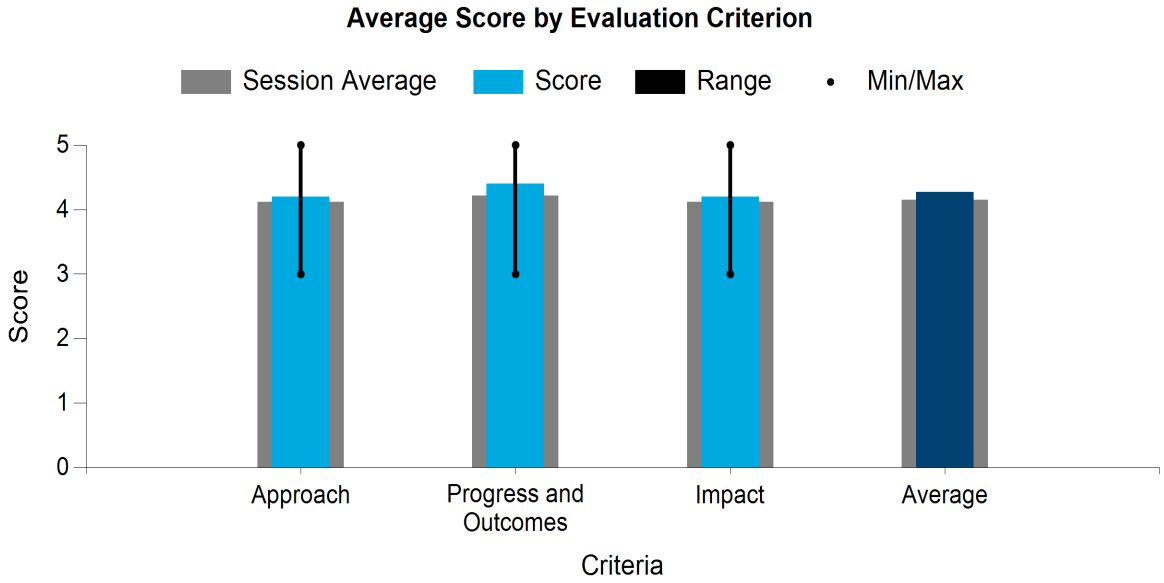
# CONTINUOUS ENZYMATIC HYDROLYSIS DEVELOPMENT

## National Renewable Energy Laboratory

### PROJECT DESCRIPTION

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|---|---|
| WBS: | 2.4.1.101 |
| Presenter(s): | Mike Himmel |
| Project Start Date: | 10/01/2017 |
| Planned Project End Date: | 09/30/2023 |
| Total Funding: | $1,650,000 |

The Continuous Enzymatic Hydrolysis Development project aims to reduce the cost and commercialization risks of second-generation biorefinery sugar/lignin/ethanol production through the development of a readily deployable CEH process. Recent changes in the technical landscape of the commercial enzymatic hydrolysis of second-generation pretreated biomass dictate that the existing hybrid SSF approach be reconsidered. Most importantly, the current practice of "finishing hydrolysis" in SSF must be abandoned. We have recently demonstrated at the bench scale that CEH permits optimal saccharification performance with NREL's DMR solids, because unlike simultaneous saccharification and fermentation with yeast or *Zymomonas*, the pH, temperature, oxygen tension, lytic polysaccharide monooxegenase mediator concentration, and removal of end product inhibitors can be optimized precisely. In scale-up, the goal is to use existing commercial cross-flow ceramic membrane filtration external loops coupled to enzymatic hydrolysis reactors. Pretreated biomass solids and enzymes are retained for reaction while solubilized product sugars are removed *in situ*, with high extents of conversion and longer enzyme lifetimes achieved through a series of reactor-membrane unit stages. The project is focused on advancing CEH as a transformational, process-intensified, lower-cost method for producing soluble clarified biomass sugars and insoluble lignin-rich streams. In summary, the CEH project's primary objective is to reduce the cost of enzymatic hydrolysis by optimizing operating conditions acellularly. Our provisional goals are 10% lower in Year 1 and 20% lower in Year 3 (end of project). This project was reviewed in FY 2020. It is now in its third year of a new 3-year plan spanning FY 2021–FY 2023.




### COMMENTS

- This project addresses a key challenge in biochemical conversion by reducing the cost of sugars obtained from biomass through the development of a continuous/intensified process. The development of the pilot

plant connecting multiple unit operations is a strength of the project, as are detailed costs calculated. The team has made excellent progress, as evidenced with a cost reduction of 25% through the combined effect of higher yields and lower enzyme loadings. While the science is not especially exciting or cutting edge, the project addresses an important problem that could have wide impact. In these regards, it is an important problem that addresses a central problem in biofuel production: enzymatic hydrolysis.

- Overall, the project has been successful. Ideally, further support could be provided so that long-term runs could be performed to establish both the stability and viability of the pilot plant.
- The project supports BETO's goals with respect to cellulosic sugars.
- The project exercised due diligence in reviewing and refining the project approaches and goals with the onboarding of a new PI. The project demonstrates interest and engagement of industry partners. The project exemplifies creativity and consideration of DEI opportunities with its emphasis on supporting women- and minority-owned industry partners and vendors. As DEI goals become more common among BETO-funded projects, this may serve as an example among many possibilities of how to further DEI goals.
- The project review shows careful consideration of risks and successes in overcoming some and beginning to overcome others.
- The successful development of CEH at larger scales has the potential for significant impact.
- The project aims to develop a CEH process to address issues associated with batch enzyme hydrolysis (i.e., rate limitations and product inhibition of enzyme activity). The goal of the project is to improve the economics of an integrated process from DMR feedstocks through finished end products, including traditional biofuels/chemicals and SAF, by reducing enzymatic hydrolysis operating and capital expenses through process intensification. Overall, the project has the potential to significantly contribute to addressing the challenges associated with enzymatic hydrolysis and promoting sustainable processes for biofuel production from DMR slurries in the future.
- The team has made significant progress in implementing the CEH process. Utilizing a benchtop-scale unit, they have achieved a 24% reduction in MSP associated with CEH, primarily through reduced hydrolysis and conditioning costs, as well as cellulase enzyme cost. The team has also shown that even enzymes evolved for reduced product inhibition still benefit from diafiltration, suggesting that kinetic phenomena and increased time to failure of the enzyme may be contributing factors.
- The project's achievements include a near-theoretical yield of glucose and close to 90% xylose yield with low enzyme loading in a mock or semicontinuous process. Here, the collaboration with Novozymes for enzyme production should also be highlighted as a positive technical approach indicator as the limitation in xylose production is being addressed through enzyme formulation adjustments. At the pilot scale, the assembly of a skid comprising a high-capacity tangential flow filtration membrane with a 1,000-L saccharification tank is a significant milestone because it allows for processing the slurry with high rheological value, addressing the challenges associated with DMR slurries at a smaller scale.
- Regarding DEI, it would be nice to see a stronger commitment (i.e., more definitive language around these goals), but it is noted the project is taking positive steps toward supporting this objective.
- During enzymatic hydrolysis, enzymes tend to bind to the solids, and some go with the liquid. The PI mentioned that most of the enzymes stay with solids in the CEH process. Is that percentage known? Is enzyme recycling being considered? If not, it would be something to consider given the enzyme dosage used in the process.

- The project results demonstrate several advantages of CEH over batch enzymatic hydrolysis. The TEA shows that reduced enzyme costs and increased sugar conversion are the main contributors to the reduced MSSP. Increasing glucan yield by 20% using a lower enzyme loading is impressive. There was a recent project leadership change. The new management plan is in place, and it seems to be reasonable. The modified risks and mitigation strategies are adequate. Several approaches are employed to improve the technology transition in the commercial process, such as using commercial enzymes and a commercial-scale membrane for CEH demonstration, identifying new stakeholders for generating reliable TEA and performing the preliminary TEA. It seems like solid mass loading and the rheology of the slurry are the problem. Because it was mentioned that dilute acid pulp could achieve a higher solid loading than DMR pulp due to the lower viscosity, it will be interesting to know how this affects their TEAs.

## PI RESPONSE TO REVIEWER COMMENTS

- We appreciate your support. I do consider the diafiltration-assisted enzymatic hydrolysis of pretreated biomass to be the next level of process evolution; however, as I mentioned, fully relieving even the best commercial enzyme formulation of end product inhibition will deliver the best economics for this second-generation sugar production unit operation. And the macro/microporous ceramic membranes available today make this possible. We are planning, in the next 3-year funding cycle, to demonstrate the reliability and feasibility of the long-term CEH operation at a large scale.

- Thank you for your support.

- Very thorough and accurate summary, thank you. We are indeed working on bringing more focus on DEI to the project. The only student working on the project is a woman, and we have made a commitment to employ only woman-owned companies, where possible, to outside services when needed for reactor repair/modification. Going beyond this planning, I do see opportunities to reach out to students with underrepresented backgrounds for rotating assignments working with the CEH system, especially those from the NREL Student Training in Applied Research (STAR) program.

- This is a very interesting topic and one that is not entirely known at the molecular scale. It is true that the most active individual enzymes are thought to remain bound to the substrate until hydrolysis is complete; however, the “duty cycle” of cellulases is to function processively on a strand of cellodextrin until further processing is not possible, and then release from the surface. It is assumed that each enzyme then quickly reacquires another binding site and continues on, releasing more cellobiose and glucose. Once the enzyme has been sufficiently “damaged,” it can no longer bind to cellulose and is considered inactive. So, this is really an effect defined by the time period of observation. In the process-level timescale, active enzymes remain associated with biomass and inactive enmesh do not. This also means that attempts to recycle cellulases by classical means fail. Of course, one must be certain that enzymes are not lost through membrane pores that are too large or to membrane surfaces that bind proteins (such as PVDF polyvinylidene difluoride membranes).

- Yes, this is true, but overall the DMR pretreatment is much superior to the dilute acid pretreatment. Specially, DMR results in a much more hydrolysable substrate. When acid hydrolysis is used, there is always some replating of lignin onto the cellulose microfibrils. Even with the best process control, this is unavoidable. When alkali pretreatments are used, lignin remains fully soluble and is more completely removed through a series of washing steps. Put another way, 0.1 M NaOH is essentially a true theta solvent for lignin. The other concern for acid pretreatment is the degradation observed for lignin at pH 1.5 and 160°C compared to DMR. Finally, acid pretreatments often result in the generation of fermentation inhibitors from reversion and other reactions. So, we took on the challenge of adapting ultrafiltration with small-scale but commercially relevant systems to handle the rheology exhibited by DMR solids. We also note that when these ultrafiltration systems are scaled to the true commercial scale,

the larger piping, pumps, membranes casings, etc., will reduce most of these concerns. This is the dilemma of doing bench-scale or small pilot-scale work with slurries—some of the flowability issues will disappear upon final scale-up.

# SUGAR IS THE NEW CRUDE

## AVAPCO LLC

### PROJECT DESCRIPTION

| | |
|---|---|
| WBS: | 2.4.3.201 |
| Presenter(s): | Kim Nelson |
| Project Start Date: | 10/01/2021 |
| Planned Project End Date: | 05/31/2025 |
| Total Funding: | $3,500,000 |

The Sugar is the New Crude project will demonstrate the production of lignocellulosic sugars with three levels of purity from three low-cost feedstocks—construction waste, energy cane, and forest residues—using AVAPCO's patented AVAP biorefinery process. These feedstocks have been identified by DOE, USDA, and the bioeconomy industry as offering cost-advantaged production of biofuels and bioproducts, as well as additional advantages, including:

- Reduction of escalating, costly forest fires (forest residues)
- Landfill $CO_2$ emissions (construction waste)
- Exceptional carbon sequestration (high-productivity energy cane).

With the growing commercial interest in sustainable aviation and marine fuels, a wide variety of low-cost biomass feedstocks will be required to satisfy extremely large aviation fuel markets.

AVAP's downstream partners—BASF, Arbiom, and Corbion—will evaluate the quality and convertibility of the sugars to a proprietary biochemical, a second-generation feed protein, and lactic acid to gain a diverse understanding of the quality of the substrates and market potential. In addition, an established alcohol-to-jet technology partner will evaluate the technical specifications of cellulosic ethanol produced by AVAPCO from the various AVAP sugars to confirm if the ethanol from each source meets their quality requirements for conversion to SAF. Students and professors from Clark Atlanta University—a historically Black University—will perform data science analysis of project data.

Goals of the project include:

- Reducing the specific manufacturing cost of AVAP sugars to <$0.20/lb through utilization of three low-cost feedstocks and process intensification
- Confirming the TEA and LCA benefits of low-cost feedstocks and AVAP process intensification for production of high-conversion second-generation sugars
- Incorporating underserved communities on the project team and STEM mentorship.

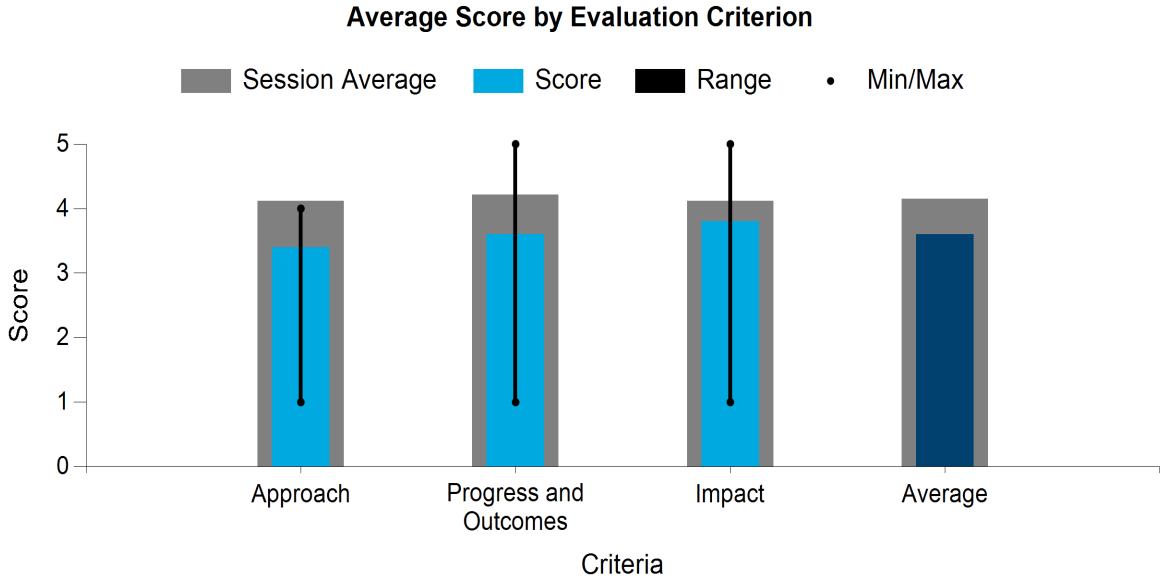
The project passed the initial verification gate in December 2022. The baseline LCA considering hardwood chips gives an 82% reduction in GHG emissions (g $CO_2$eq/kg sugar) for AVAP sugars over corn dextrose sugars. The baseline MSSP estimate of AVAP sugars falls below $0.20/lb for all feedstocks to be evaluated in this project. Process intensification is anticipated to further decrease the prices by about $0.02/lb.

Potential challenges that the project may encounter include:

- Inflation-related price escalation of equipment
- Extended equipment delivery time due to COVID-related backups.

By proving the technical and economic viability of low-cost residual feedstocks, this project will help AVAPCO reach its goal of securing 3% of the world's anticipated SAF demand by 2050, which would require approximately the same daily consumption of biomass as currently utilized by the U.S. pulp and paper industry.

The low-cost residual feedstocks and AVAP process intensification could reduce the MSSP of alcohol-to-jet SAF by 30% compared to current estimates using woody biomass.




## COMMENTS

- This project mostly seems to be about the verification and benchmarking of the AVAPCO process. They are already up and running. They will then validate their process for the production of different grades of sugars. The project also includes detailed TEA and LCA for their process. It is not clear why the milestones are necessary because they can already produce the target sugars.

- Overall, this is a solid project that supports an existing industrial process and will provide detailed data regarding the AVAPCO process. This will enable their pretreatment process to be compared with other processes. This is a strength of this industrial project, which lacks technical details like the others. Looking forward, these sorts of validation projects are the ideal ones for BETO to support. In particular, provide the support so that companies can demonstrate their existing technology rather than develop new technologies.

- AVAPCO has long produced cellulosic sugars using its proprietary biorefinery process. This project aims to refine this process for low-cost and waste feedstocks: energy cane, construction waste, and forest residues. This objective aligns with BETO's sustainability goals. The project advances DEI goals through AVAPCO's long-running collaborative relationship with an MSI, which is analyzing project data.

- The presentation was light on technical details, and as such it is challenging to evaluate technical concerns. The project is demonstrating on-time progress toward its goals.

- Should this research succeed, questions remain regarding scale-up. For example, AVAPCO has identified a local supplier of aggregated and sorted construction waste with sufficient material to supply their process. To what extent are similar supplies available nationally, both currently and if demand grows?
- To date, this project has demonstrated an ability to produce high-quality sugars with excellent process metrics (e.g., 87% sugar yields); now, the project aims to build on the SOA through process intensification (chip pre-steaming and post-fractionation separation) and demonstration of the technology with low-cost, abundant biomass feedstocks.
- There is no mention of process yields in the presentation, just conversion yields. Because the process is feedstock agnostic, what are the overall process yields for each feedstock to be tested? What were the TEA model predictions, and do they match the results at lab scale?
- TEA/LCA and DEI are carried out, but experimental work has yet to be started because the project is working on equipment acquisition and installation. The company has a successful history of producing cellulosic sugars from various biomass based on the AVAP process. Third parties previously demonstrated the utilization of sugars for chemicals. The risks are identified. The project company partners with several feedstock suppliers and chemical companies for sugar utilization, which is commendable. The project goals are excellent: developing a commercially relevant process using non-ideal, low-cost feedstock and developing TEA to include the interplay among the feedstock inhibitor profile, sugar purification costs, and sugar performance in its conversion. Including construction and demolition wastes in their feedstock lists will expand the impact of the project from biomass conversion to waste recycling. AVAP sugars are compared with corn sugars to show a significant GHG reduction. How do AVAP sugars compare to other cellulosic sugar processes? In the TEA, construction and demolition feedstock had the lowest MSSP due to the lowest feedstock price from using the waste material. It would be helpful if the team could comment on what levels of preprocessing and decontamination are required for this waste to become an acceptable feedstock in this project. Was the cost for preprocessing construction and demolition waste considered in the TEA?

# BIOCHEMICAL PROCESS MODELING AND SIMULATION

## National Renewable Energy Laboratory

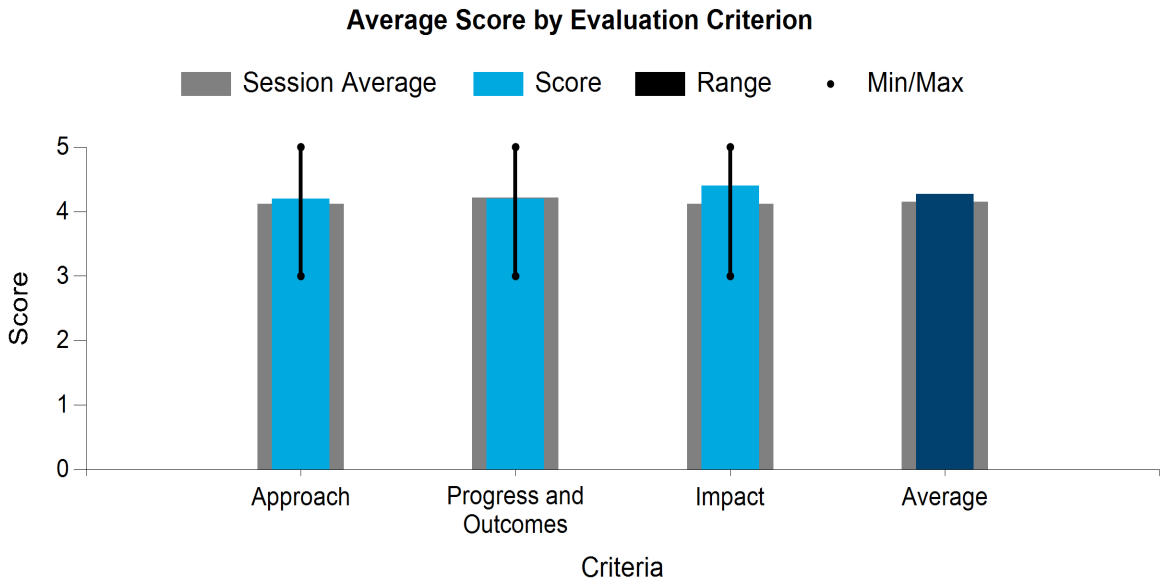
### PROJECT DESCRIPTION

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|---|---|
| WBS: | 2.5.1.100 |
| Presenter(s): | Yannick Bomble |
| Project Start Date: | 10/01/2021 |
| Planned Project End Date: | 09/30/2024 |
| Total Funding: | $3,000,000 |

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

We work closely with experimental projects to identify problems and iterate with experiments to find and refine solutions. By working with experimentalists, we decide on problems that can be solved with simulations that could otherwise not be solved or would take too long with experiments alone to reach BETO's targets. Over the years, we have produced solutions that have resulted in determining the most likely fatty acid derivative for passive transport out of bacteria that upgrades biomass, and we have also designed enzyme mutations for enhanced lignin upgrading. Metabolic models have been developed to tune the activity of 2,3-BDO production for the 2022 target. A computational method to deliver understanding of how complex omics data can be interpreted in the metabolic pathways of organisms is used in the Agile BioFoundry. We have found methods to overcome specific barriers and continue to develop those methods. Our reactor studies have guided the design of both the microbes and reactors for aerobic and micro-aerobic production at all scales and have been instrumental in improving the accuracy of TEA models. This project is essential in the process of selecting the final processes for 2040 SAF production targets.

More specifically, we have recently (1) predicted the strength of the basic structural interactions in commodity plastics to provide guidance for plastic upcycling strategies; (2) developed computational tools to improve the characterization of lignin-derived compounds; (3) developed new methodologies to enable machine learning-based directed evolution for protein engineering; (4) developed machine learning methods to predict protein promiscuity and mutations to further improve microbial and enzymatic-driven processes and demonstrated the utility of machine learning approaches to engineering proteins from sparse experimental datasets; (5) developed new methods to enable high-fidelity simulation of aerobic fermentation at the industrial scale and resolve the mismatch of timescales through subcycling/operator splitting; and (7) identified the difficulty in preventing local high-oxygen conditions in industrial bubble columns, which leads to less desirable acetoin production, suggesting future research directions in alternative reactor configurations (e.g., loop reactors, shallow-channel reactors).




## COMMENTS

- This project provides computational tools to support projects within the BETO/NREL portfolio. While computer modeling still has not achieved equivalent success in biotechnology as it has in other technical fields, it is a potential game changer in that it can dramatically accelerate progress. BETO, in particular, can take a leading role in developing accurate computer-aided design tools for biofuel/chemical production. Another key strength of the program is the development of tools from the molecular to macro scale.

- The key outcome of the program is the development of the LigninWrangler tool. This will greatly aid lignin analytics. The other is the use of computational fluid dynamics (CFD) for assessing the performance of different fermenter configurations. The use of timescale coupling is a key outcome because it addresses some of the challenges of CFD simulations over longer timescales. While the work on machine learning and pathway modeling is useful, it is less impressive than the other two initiatives compared to what has been done in the past.

- This project is extremely useful. Its main impact is to provide foundational tools for bioprocess development. While modeling still has a far way to go in biotechnology, it can potentially be a game changer like in other fields. One suggestion is to increase support for CFD modeling because this area is generally underfunded in the United States compared to molecular and pathway modeling. Bioprocess scale-up is still one of the major barriers to commercialization, and this is where CFD can have a huge impact. Another suggestion would be to develop a plan for the long-term support of these computational tools, either by making a source or at least having a plan for eventual loss of support.

- The project provides a valuable service in developing modeling and simulation approaches to challenges in biochemical processes. The project has developed a strategic approach to providing modeling and simulations that will be of most value to the community they serve, within the constraints of available financial, human, and capital resources.

- The project has made remarkable progress with limited funding and significant staff turnover. The project managers have identified staff turnover as a significant risk and ongoing concern. Increasing emphasis on recruiting graduate students is a reasonable near-term mitigation strategy. Ultimately, the

project will need to consider how to recruit and retain more senior staff to maintain continuity of operations and provide strategic direction going forward.

- The project's impacts are clear. High among these impacts is the project's development of LigninWrangler, a publicly available computational tool that has seen substantial uptake by the community. It would be useful to see a strategy for developing and making publicly available additional tools and knowledge. Providing opportunities for public feedback or consultations on what tools are most needed can assist in shaping future directions.
- This project has the potential to significantly impact biofuel/bioproduct R&D across BETO's project portfolio. When successfully employed, modeling- and simulation-based guidance can increase R&D efficiency by shortening timelines and reducing overall project cost. While some examples of direct impact were provided, more detailed information would be beneficial to assess the full extent of the project's effectiveness in this regard. An increase in interproject engagement would help magnify the impact of the tools developed.
- One weakness of the project's approach is that its focus is quite diffuse, with work spread across four different research areas. This may be exacerbated by challenges in attracting and retaining top talent in this competitive field. To address this, it may be appropriate to prioritize a smaller number of research areas that address unmet needs in industry or academic research. For example, LigninWrangler appears to be a successful example where a unique tool was developed that addresses a challenging technical problem in the area of biomass deconstruction and lignin valorization.
- A project strength is that its models, tools, and libraries have been made publicly available, providing a way for this work to support the advancement of biofuel and bioproduct research outside of just the national labs. This open approach to sharing resources may promote future collaborations and further increase overall project impact.
- Regarding DEI, the team has made some steps to increase minority student engagement by working with the Graduate Education for Minority Students (GEM) program.
- The project was able to successfully develop tools that facilitate machine learning techniques to guide enzyme engineering with model-based data that have been reproduced at the small scale to validate the model predictions.
- The project has also been able to predict reactor models for 2,3-BDO fermentation yields at the small scale that translate in experimental data. One recommendation would be to improve the model predictions to match the fermentation yields at the large scale.
- The team's approach to working with BETO overall to determine where modeling is applicable and using multiscale approaches to assist and provide guidance for the BETO-relevant processes is excellent. The benefits and impact of this project are clear. Using modeling to overcome experimentally inaccessible space and to guide the system designs improves scientific understanding and can significantly reduce the cycle of technology development. The team supports projects with a wide bandwidth, from bio-based SAF and plastic degradation to cell-free synthesis. The project management is adequate, and the outcomes are commendable. For example, LigninWrangler, a publicly available computational tool developed by the team in collaboration with another BETO project, resolved the long-standing challenge of identifying lignin compounds using a computational and experimental combined approach. Overall, the project made great progress.

## PI RESPONSE TO REVIEWER COMMENTS

- We would first like to thank the reviewers for their effort to carefully evaluate this project. Their feedback will help us redirect some of our efforts and consider details we had overlooked. We agree that we always need to remind other projects of the value of modeling and what improvements were or could be obtained from modeling to improve processes. CFD is indeed an area of our project that we think has a lot of promise, notably to help with reactor design for atypical production routes, including complex fermentations and cell-free biocatalysis. We do have budget constraints in terms of our total funding, but we do intend to increase the share of CFD in the next few years. We realize that it can really help evaluate scale-up potential for a variety of processes. We do aim to focus on research projects where modeling could have the most impact, and to do so we are in constant discussion with experimental project leads to demonstrate that modeling can help improve their bottom line. We have tried to refocus over the years and have made progress doing so by wrapping up several ongoing projects. We believe that in 1–2 years we will be able to be more focused than we are now and therefore quicker to deliver on the most important problems. Additionally, regarding the development of publicly available tools and getting public feedback, we try to focus on the tools for which there is a clear need and lack of development; however, we must also be aware of the fact that we have limited funds to achieve our goals and help with near-term experimental projects. Nevertheless, we will make an effort to reach out to the community and see if we have the bandwidth to help. Finally, we are indeed trying to develop more robust approaches to predict the fermentation yields at a large scale using a combination of metabolic modeling and CFD. This approach shows promise when used to predict 2,3-BDO yields, so we are hopeful that we can further improve and apply it to other processes and products.

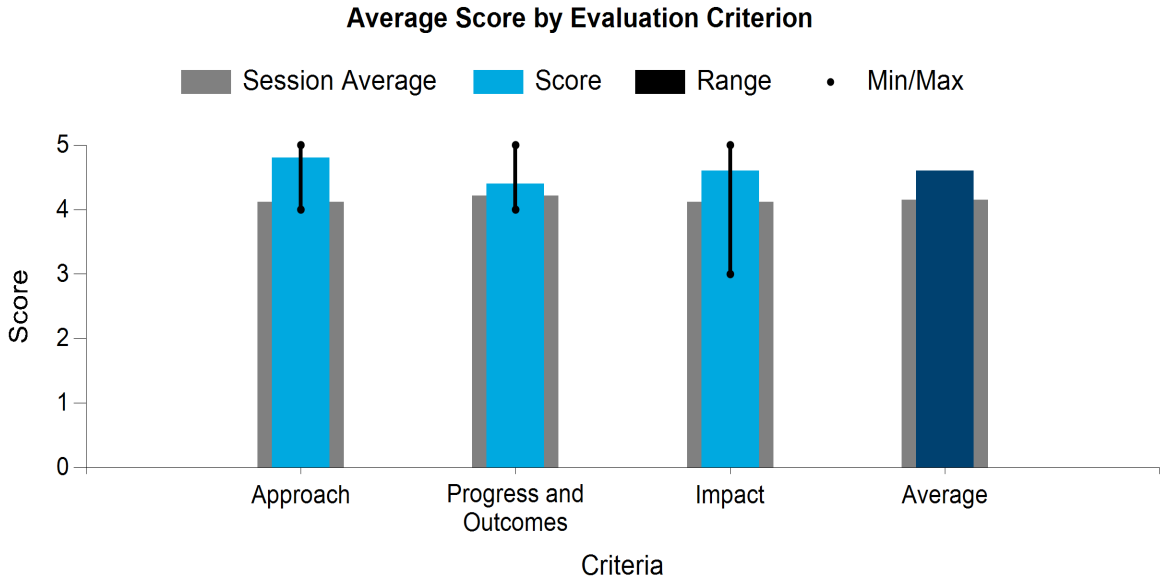
# ANALYTICAL DEVELOPMENT AND SUPPORT

## National Renewable Energy Laboratory

### PROJECT DESCRIPTION

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| WBS: | 2.5.1.101 |
| Presenter(s): | Justin Sluiter |
| Project Start Date: | 10/01/2021 |
| Planned Project End Date: | 09/30/2024 |
| Total Funding: | $2,025,000 |

The objective of the Analytical Development and Support project is to produce and maintain the critical analytical methods and tools that enable the evaluation of emerging biofuels R&D within BETO and within the broader biofuels community. Our project is divided into two parts: The development task develops novel analytical techniques and improves existing methods, and the support task maintains existing analytical capabilities at NREL and provides outreach to the wider community. The project is world-recognized for our laboratory analytical procedures, which provide detailed procedures for the compositional analysis of biomass and have been adopted as the de facto standards within the biofuels community, largely due to the transparency of the methods and the high reputation of NREL's research. Our dialogue with stakeholders allows us to provide robust, precise, accurate, and publicly available analytical procedures for better valuation of scientific tools. Our focus is on developing characterization methods to support the conversion of cost-advantaged feedstocks to a variety of fuels including SAF. This project also has four ongoing industry-led agreements for method development that will have a direct impact on biofuel production.




### COMMENTS

- The goal of this project is to provide analytical support for 20 different projects within the BETO/NREL portfolio. In addition, the program develops new methods and protocols that benefit the field at large. A key strength is that the team provides detailed merits demonstrating their progress and impact within the field. Another key strength is that the team is working with a number of companies, demonstrating their impact to the field. A final strength of note is the project management and resource allocation: The team has a clear vision on the type of problem that they wish to develop and their unique skill set. Overall, this is an impressive, productive, and well-run project.

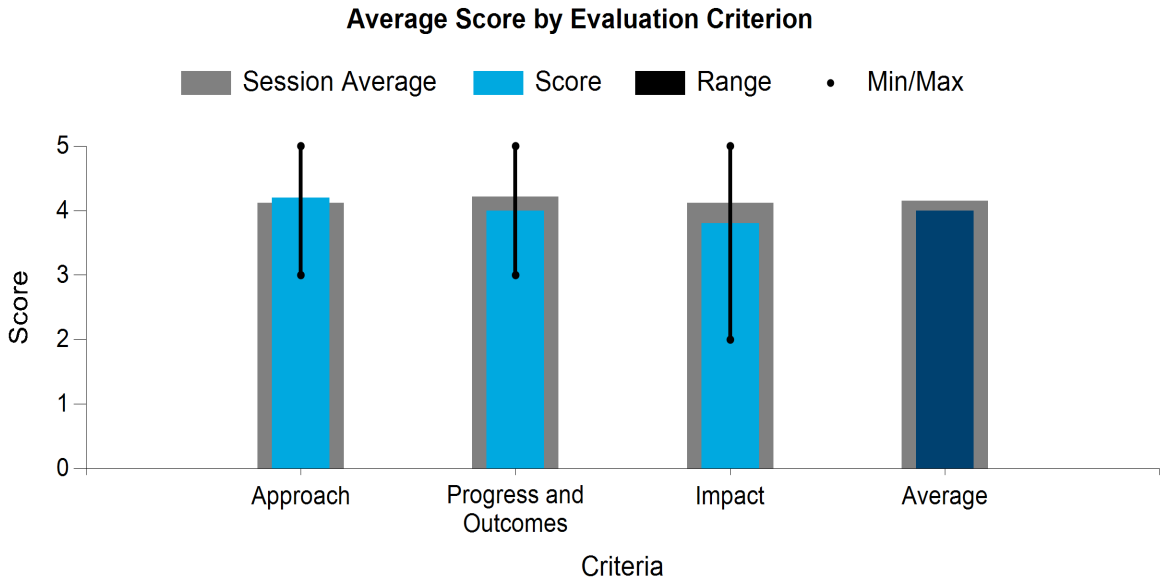
- This is a very important project that supports multiple BETO projects. In addition, this project also develops standards for the broader community and has an impressive working relationship with industry and other federal agencies.
- The project serves a valuable crosscutting purpose, providing support to existing projects, improvements of existing analytical techniques, and the development of new analytical techniques. The project approach maximizes impact for its small team and limited budget. Most collaborative work has been with NREL. The project approach is sound and forward-looking. For example, the group's forecasting anticipates growth in interest in using municipal solid waste as a feedstock, and it has applied appropriate emphasis to prepare for this. A small team implements the project, and as such, staff turnover is a risk. Cross-training staff and developing partnerships are appropriate mitigation strategies. The project has successfully developed partnerships with industry and other groups to maximize impact. Successful industry collaborations demonstrate the value of the project's activities.
- The project's DEI strategy leverages its long-standing relationship with an MSI, and it works to deepen the opportunities that those ties provide.
- The project has a demonstrated track record of delivering novel analytical methods in support of new technologies, both in industry and at NREL, and it serves as a central and shared analytical, quality assurance/quality control, and instrumentation resource to BETO projects. This approach adds value to the BETO (and specifically NREL) portfolio of projects by providing centralized quality and control, coordinating samples between projects, maintaining instrumentation, and training/supporting staff using the equipment.
- The management approach is well thought out and developed, ensuring that the methods being developed are (1) not duplicative with ongoing method development work within BETO or industry and (2) broadly applicable (through work with ASTM) and can be practiced outside the national labs.
- Regarding the progress made, the team has demonstrated excellent progress toward developing a suite of analytical methods enabling good (90%) mass closure on a set of municipal solid waste substreams (e.g., papers and dairy products) and has developed a set of actionable steps to help increase mass balance closure in areas where it is currently lacking. More broadly, the team has demonstrated success supporting both BETO's internal goals (e.g., SAF) and addressing challenges in industry (e.g., cellulose assay for Gen 1.5 ethanol).
- Of note, the collaborations and partnerships are heavily skewed toward NREL projects, which is likely due to project collocation (i.e., it is easy to transfer samples between labs). Project impact could be increased if the team's unique capabilities were being leveraged by projects at other national labs; this could be realized, for example, by continued development of unique and/or specialized analytical methods unavailable at other sites.
- Regarding DEI, the project could elaborate further on how it is engaging Metro State University (a Hispanic-serving institution) and the potential impact of this effort.
- This three-person team has achieved satisfactory milestones and developed new methods that are useful and industry relevant.
- Focusing on wastes streams, this funding cycle is very useful and is helping the current efforts aimed at valorizing those streams.
- Analytical capability and data accuracy are essential parts of research. In this context, this is an important project for the BETO portfolio. The number and diversity of the projects this project supports

are impressive. Another important contribution of this project is developing standard analytical methods and making them available to the public at no cost. The team's approach to creating procedure FAQs and training videos is also an excellent way to help the industry and academia adopt the procedures. The project impact is visible; the laboratory analytical procedures are broadly downloaded internationally. The project also develops analytical capability for low-cost, complex feedstocks (e.g., municipal solid wastes, organic wastes). Such work aligns well with BETO's goal for low-cost SAF and supports increasing efforts in waste utilization. The project also helps industries solve analytical challenges, which is commendable. Overall, this is a high-demand project, and the team did an excellent job. The project approach, risk identification, and mitigation plans are appropriate. The DEI activities are adequate.

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

## Northwestern University

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| WBS: | 2.5.3.206 |
| Presenter(s): | Shivani Garg |
| Project Start Date: | 10/01/2019 |
| Planned Project End Date: | 03/31/2023 |
| Total Funding: | $2,000,000 |




## COMMENTS

- The goal of this project is to produce a range of promising chemicals such as hexanol from syngas by reversing beta-oxidation in *Clostridia*. A key component of the project is the use of cell-free systems for pathway optimization, which is a promising application of this technology. The team appears to be making good progress, though few technical details were provided, making it hard to determine whether this work will result in a viable process. The LCA results are also questionable: How can hydrocarbon-based process require more water than a biological one? This makes no sense, and the team was unable to answer this question.

- Overall, the main impact of this project is to demonstrate the utility of cell-free systems for pathway optimization. Whether its resulting designs yield an economic process is unknown. To answer this question, more details would be required about titers, yields, etc.

- The project aims to develop *Clostridia* to ferment syngas into a variety of advanced bioproducts at the pilot scale. This goal supports sustainability goals. It combines *in vitro* (cell-free) and *in vivo* approaches to assist in pathway design, validation, and production. A project strength is the collaboration among university and industry researchers to advance interdisciplinary objectives.

- The project is continuing to pursue the final milestone under a no-cost extension, after achieving all other project milestones on time.
- To date, the project has efficiently and effectively demonstrated the team's ability to translate an R&D objective (i.e., rapid pathway prototyping for new product biosynthesis) from design through near-pilot-scale application with a potential near-term commercialization with an industry partner. This is likely a testament to the high-quality management of the project and the interdisciplinary team (including expertise in techno-economics). While the team has not yet achieved their end-of-project goal (due August 2023), this appears to be more of a logistical challenge and not due to a technical hurdle.
- The work has the potential to be highly impactful in at least two areas. First, the *in vitro* pathway prototyping approach has the potential to serve as a general model for metabolic pathway optimization, an impact facilitated by the recent publication. Second, the engineered strains provide a route to access a relatively broad suite of small-molecule products from an abundant, inexpensive feedstock.
- As the team aims to wrap up their research, it may be helpful to disseminate more information on where there's room for additional optimization in this pathway prototyping approach. For example, it appears there were some issues translating enzymes from *E. coli* (where they may express well) to *Clostridium* (where they may not), or where specific enzymes may function only in aerobic contexts with glucose.
- Even though the TEA seems not to have been a requirement, it would have been very beneficial to have a sense of the project's economics and have some TEA output to assess if the project meets BETO's cost targets. How economically viable is this process?
- One highlight of this project is producing multiple platform molecules from syngas based on a cell-free system approach. Because syngas is less dependent on feedstock type, developing diverse advanced biochemicals from syngas has a clear advantage compared to other approaches. Although biomass gasification-derived syngas is mentioned in this project, this technology can be extended to syngas based on waste resources (municipal solid waste, waste plastics). The project is scientifically meritorious. By combining the reversal b-oxidation and cell-free system, versatile molecules can be available at high production rates. These molecules could be used as drop-in fuels, fuel additives, and chemical building blocks. The project is currently under a no-cost extension. The progress toward the new project's ending date is excellent. It is impressive that the team far exceeded the milestone targets. The project demonstrated 10 biochemicals but narrowed the products down to butanol and hexanol for pilot-scale development. LCA of the two molecules is completed, and the assessment of infrastructure and biomass supply chains is also performed. Overall, enabling versatile platform biochemicals and the ability to utilize diverse, low-cost feedstock make this project highly attractive. The presenter mentioned that syngas composition is currently standard $CO_2$, CO, and $H_2$ but may vary in commercialization depending on conditions. Discussing the potential risks associated with syngas conditions would be helpful. The project is ready for pilot-scale production. Thus, a TEA to evaluate commercial viability will be helpful.

## PI RESPONSE TO REVIEWER COMMENTS

- "Subject to disclaimers on the associated presentation." Response: We appreciate the reviewer's comments and questions. Regarding water consumption, an important aspect of LanzaTech's process is water recycling to reduce freshwater use. Further, because LanzaTech's process is decoupled from photosynthesis, the input water requirements are very low. Moreover, the use of water in petroleum extraction is included in the hydrocarbon-sourced chemicals. The LCA system boundary is well-to-chemical. The project has indeed leveraged cell-free systems for pathway validation and optimization; however, in this instance, a whole-cell biocatalyst is advantaged when gases are used as feedstock. Considering the stage of the project, we are not reporting titers and yields because we have yet to begin pilot-scale fermentations. We thank the reviewer for highlighting that all the milestones on the project

(except for the final piloting milestone) have been achieved on time. At this time, it remains LanzaTech's intent to assess the performance of our top strain at the pilot scale. Pilot-scale performance is a more accurate representation of strain performance at the commercial scale (versus continuous stirred-tank reactors). We want to thank the reviewer for the encouraging and positive feedback. The reviewer brought up a very valuable point, and the teams are working on further strengthening the correlation between cell-free data and *in vivo* data. This will further help us to predict future experimental designs. Looking ahead, there is an opportunity to develop anaerobic cell-free systems. The advantages and disadvantages of the current cell-free system are captured in previously disseminated manuscripts (e.g., https://doi.org/10.1038/s41589-020-0559-0). LanzaTech appreciates the reviewer's comment. Even though TEA is not a project deliverable, TEA was used to help define the project's performance targets. Further, LanzaTech uses TEA to quantify the impact of performance metrics such as selectivity and productivity on the cost of production. TEA is a tool and one of many inputs into specific business case development scenarios, which ultimately will help define the economic viability of a commercial project. This effort will be carried out downstream of the DOE project. We thank the reviewer for pointing out the scientific merit of the project and for acknowledging the excellent progress that the project has made so far. One strength of LanzaTech's bioprocess is the robustness of the biocatalyst to variable gas flows, gas component ratios, and even potential contaminates. Additionally, LanzaTech has significant experience evaluating myriad gas sources and has developed intellectual property and know-how to address risks that may be attributed to the gas sources. We have operated our ethanol process with a wide range of syngas feedstocks, and we understand the impacts. Another example is the previous DOE-funded acetone project, DE-EE000756, that resulted in the scaling up of acetone production from syngas at the pilot scale. Regarding TEA, as mentioned in the response to a prior question, LanzaTech uses TEA to quantify the impact of performance metrics, such as selectivity and productivity, on the cost of production. TEA is a tool and one of many inputs into specific business case development scenarios, which ultimately will help define the economic viability of a commercial project. This effort will be carried out downstream of the DOE project.

# CELL-FREE AND IMMOBILIZATION TECHNOLOGIES TO PRODUCE SUSTAINABLE AVIATION FUELS AND OTHER BIOPRODUCTS

## National Renewable Energy Laboratory

### PROJECT DESCRIPTION

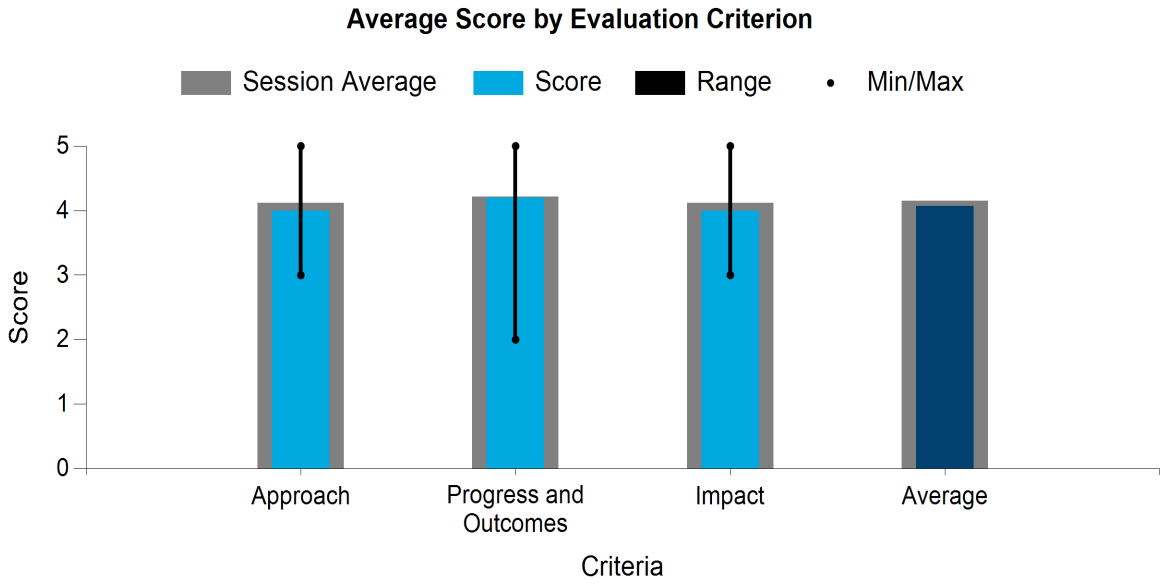
| | |
|---|---|
| WBS: | 2.5.4.101 |
| Presenter(s): | Yannick Bomble |
| Project Start Date: | 10/01/2021 |
| Planned Project End Date: | 09/30/2024 |
| Total Funding: | $3,685,000 |

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

More specifically, we are developing new technologies and routes that could be used to produce high-value biochemicals such as 2,3-BDO and terpenes (among many others) from biomass-derived C5/C6 sugars or lignin but also from waste byproducts such as glycerol. This project will lead to significant innovation and also new concepts and the rational design of pathways and enzymes. Within this project, we will develop new metabolic enzyme cascades that will represent natural or artificial combinations of enzymes to produce the desired biochemicals from a variety of feedstocks. We are also developing basic design principles for constructing synthetic metabolons, using fusion proteins and synthetic protein scaffolds, to promote substrate channeling and stability while conserving peak activity. Additionally, our efforts include a TEA of cell-free approaches to provide the sensitivities of the process to enzyme loading, activity, pH, reactor volumes, and cofactor recycling rates. Finally, we are focusing on further increasing the stability, operating lifetime, and efficiency of the pathway enzymes by immobilization on support surfaces. We are also focusing on immobilizing pathway enzymes or combinations of enzymes on several different conducting polymers and evaluating the effect on stability and operating lifetime. As more combinations become available, we will conduct a more systematic study of the means of immobilizing these enzymes. This preliminary work will enable the in-depth study of cofactor recycling at these interfaces using mediators for electron transfer. Together, these approaches will enable process intensification, continuous operation, lower capital and separations costs, and end product flexibility, and thus they have the potential to significantly contribute to BETO's goals of cost-competitive biofuels and bioproducts.

To date, we have (1) demonstrated the conversion of pyruvate to 2,3-BDO (four enzymes with cofactor recycling; no additional cofactors needed) at >100 g/L (>3 g/L/h) using our enzyme tethering approach; (2) generated mutants of a key redox enzyme with >180-fold improvement in nicotinamide adenine dinucleotide (NADH) utilization over wild type to >85% of NADPH utilization; (3) produced mevalonate at >10 g/L from glucose, limonene at >10 g/L from mevalonate, and >5 g/L from glucose with crude enzyme preparations and complete cofactor recycling; (4) successfully generated fully active cross-linked aggregates of NOX, a

cofactor-regenerating water-forming oxidase that is key to our process with increased stability; and (5) engineered and identified enzymes able to use the biomimetic cofactor NMN and synthesized new synthetic cofactors.




## COMMENTS

- The goal of this project is to produce terpenes using a cell-free system. In addition, 2,3-BDO is being pursued, presumably as a proof of principle because it can already be produced at high titers using microorganisms. With regard to terpenes, the team is making good progress with a strong risk mitigation plan. The work on immobilization and biomimetic cofactors is really nice and exciting science, though this is still a low-TRL project relative to others within the BETO portfolio.

- One question concerns the impact of cell-free technologies. There is no question that these technologies can be useful for pathway optimization. The question is whether cell-free systems can be used to produce chemicals economically due to the cost of enzymes (the reviewer is skeptical). In the reviewer's mind, this is a key question. Rather than trying to produce complex chemicals, why not first see whether you can produce a redox-balanced chemical like ethanol or lactic acid from glucose at some intermediate scale (e.g., >100 L)? Such a project would be useful for demonstrating the potential of cell-free systems and would directly address these recurrent questions. Otherwise, this project is a bit of an outlier in the BETO portfolio and seems more appropriate for a different program more focused on basic science/technology. Despite these criticisms, the team is making good progress and developing some promising approaches. They are accomplishing what they proposed to do.

- This project aims to develop new science and technologies to produce fuels and chemicals in cell-free systems. This goal supports BETO's goals for SAF. This research has high potential for impact, but it must overcome many challenges to achieve that potential. The team has developed a reasonable approach and engaged in active management of the research to maintain focus on the most promising opportunities. Collaborations with other BETO-funded projects, academic researchers, and industry contacts strengthen the approach.

- Cell-free systems promise advantages over traditional biological conversion, including the potential for improved predictability and yield and elimination of toxicity concerns; however, cost and scale-up are

ongoing concerns at this early stage in the development of the technology and into the future. This project acknowledges those concerns and uses TEA to guide the research. Details of the TEA were not presented.

- The strategy of focusing on terpenes as products ripe for production in a cell-free system, due to their toxicity, reasonably explores a need unmet by traditional biological conversion.
- The project provides an innovative technical approach, cell-free biosynthesis, to address issues with traditional, microbial production systems (e.g., limitations on productivity and titer). In addition to enabling the production of molecules that may be too toxic for living cells (e.g., many short-chain alcohols), the technology has the potential to achieve substantially higher productivities than typically witnessed in microbial fermentations. Thus, long-term, cell-free technologies may help not only expand the range of products accessible from lignin and sugars but also decrease production costs and simplify process scale-up.
- The team has demonstrated that they are actively managing the project to ensure that the research directives are advancing the technology (i.e., increasing TRL level) and/or addressing major cost centers associated with cell-free systems (e.g., cofactor and enzyme cost). As an example, management discontinued work on cofactor recycling via electrochemistry approaches and increased partnerships with industry experts to help address specific areas of risk.
- Substantial progress has been made to date, including directed evolution of a NAD-utilizing GapN (achieving 80% of activity compared to the wild-type enzyme with NADPH) and the production of terpene pathway intermediates (mevalonate) and products (limonene) at >90% yields. The planned work to produce synthetic mimetics of NAD(P)H has the potential to address one of the most significant disadvantages to current cell-free reaction schemes; if successful, this alone would be broadly useful given the ubiquity of NAD(P)H-dependent redox reactions in microbial metabolism.
- The project would benefit from a rigorous assessment of enzyme performance parameters, namely, the total turnover number and the specific rate to determine what, if any, additional work needs to be performed to engineer (or select for) more stable variants.
- The project has outlined specific steps to be taken to support programmatic DEI objectives, mainly along recruiting students and staff from underrepresented communities.
- The proposed project could help reduce costs, but the entire enzyme pathway seems to be more intensive for capital expenses. An apples-to-apples TEA comparison of both processes (conventional and cell-free and immobilization technologies) would be helpful to assess the process viability.
- How is the product separated at the end? What does the downstream process look like?
- One recommendation would be to test more cellulosic hydrolysates to test the process robustness. Will sugars/hydrolysate cleanup be necessary or not?
- This project aims to demonstrate the viability of cell-free-based approaches by producing terpenes as SAF and diesel intermediates or as direct replacements. One novel aspect of this project is their research approach, which will greatly advance conventional cell-free technologies. The project approach has strong scientific merit. The project team carries out very thoughtful research, investigating nearly all aspects of the cell-free system—from pathway, enzyme engineering, cofactor engineering, immobilization, and cofactor recycling—to TEA-assisted technology development. The approaches for reducing cofactors and enzyme costs are excellent. The project has made excellent progress. A computational approach was adopted for cofactor synthesis, and several low-cost synthetic cofactors

were developed with high yields. The impact of the successful project is significant—it will not only enable SAF molecules but also greatly advance science for cell-free systems. The project also interacts with other BETO-relevant projects and collaborates with academia, national labs, and industry. Risks are identified, and multiple mitigation strategies are discussed. The DEI plan is adequate. Because no TEA was given, it is difficult to understand how far the technology is from being commercially viable.

## PI RESPONSE TO REVIEWER COMMENTS

- We thank the reviewers for all their efforts to carefully evaluate this project, its strengths, and its weaknesses. It is very clear that these reviewers carefully considered our project and took the time to provide valuable feedback. We also appreciate the fact that the reviewers pointed out the promise of cell-free biomanufacturing and the impact it could have on the bioeconomy. We do realize that there are still significant hurdles for the technology to be viable, and this is exactly what our project is trying to help overcome. The reviewers have raised several important and valid points that we would like to address. Regarding enzyme production and costs (and therefore if we could produce chemicals economically using cell-free systems), we are confident that scale-up production can be achieved using dedicated production strains. We have now demonstrated extracellular production of some of these enzymes at high titers without optimization in a complementary Technology Commercialization Fund project. Therefore, we are confident that experts in enzyme expression would be able to greatly improve these titers. Of course, some enzymes will need to be expressed intracellularly, but they would only require crude purifications such as heat precipitation when thermophilic enzymes are used. The contribution of enzyme cost to the overall process can also be mitigated by increasing their operating lifetime, which we are trying to achieve using a combination of enzyme prospecting, engineering, and immobilization. We already know that some enzymes remain active for days in solvents without immobilization, which bodes well for the prospects of enzymes operating for 30+ days once stabilization approaches are put in place to enable continuous operation. Regarding concerns about scale-up, we appreciate those concerns, and they are legitimate; however, we believe that this technology can scale with a few changes to the way the reactions are conducted—for example, our collaborators have now shown that high titers of terpenes can be achieved when scaling up to 10 L, which is, of course, still pre pilot but significant. Additionally, there are other processes in Japan and Scandinavia that have been shown to scale for the cell-free production of biodiesel (though these processes did not have the challenge of using cofactors). Regarding the benefit from a rigorous assessment of enzyme performance parameters, we agree with the reviewer, and it is something that we continuously do to pick the best biocatalysts for our applications. We may not go into as many details for all enzymes, but we do for the key and problematic enzyme targets. We realize that this work was not part of the presentation due to the limited amount of time allocated. Regarding the fact that the TEA model was not presented, we do realize that a full TEA would be useful, especially to assess whether a low-TRL technology would be viable. We have been developing a TEA model for terpene production, and even though this model is preliminary and does not include all the benefits that cell-free systems can offer, this model shows that if scientific and technological advances are made to lower the cost of cofactors and increase the operating lifetime (and production cost) of enzymes, then our technology would lower the MFSP for the production of terpenes compared to microbial counterparts. Additionally, we are also working on an LCA model that we believe will also show the benefit of cell-free systems over microbial systems for terpene production. Regarding post-processing and the utilization of hydrolysates, we are currently using an organic overlay for extraction. Of course, we know that this is difficult to realize at scale, and we are currently evaluating liquid-liquid extraction methods with the separation consortium, which are more industrially relevant. We believe that these approaches will be compatible with our processes and products. The reviewer is correct to point out that hydrolysates represent a challenge. In the previous iteration of this project, the use of hydrolysates led to a reduction in titers (for 2,3-BDO in this case). We will be testing hydrolysates for our current pathway in FY 2024, and we will try to identify the compounds that could be inhibitory.

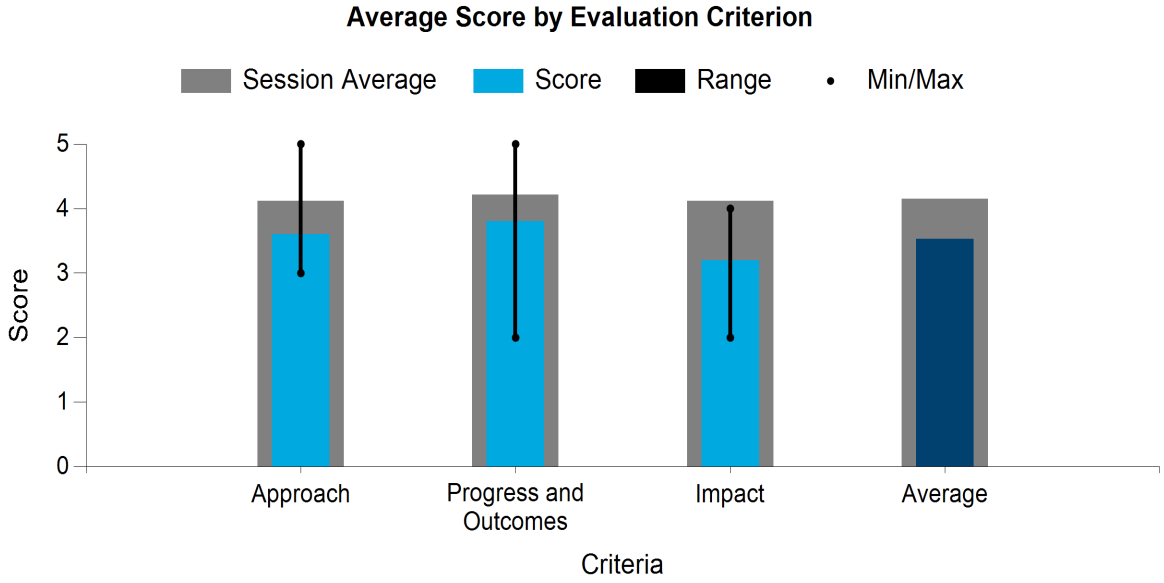
# TOWARDS ECONOMICAL CELL-FREE ISOBUTANOL PRODUCTION

## Invizyne Technologies Inc.

### PROJECT DESCRIPTION

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|---|---|
| WBS: | 2.5.6.203 |
| Presenter(s): | Paul Opgenorth |
| Project Start Date: | 10/01/2019 |
| Planned Project End Date: | 03/31/2023 |
| Total Funding: | $2,628,610 |

Cell-free enzymatic systems are an increasingly promising alternative to performing biochemical conversions in live cells. In this approach, metabolic pathways are reconstituted using purified enzyme activities (not necessarily pure enzymes) in bioreactors. This presentation will highlight two projects that advance the concept and TRL of cell-free systems. The first project is titled "Towards Economical Cell-Free Isobutanol Production." This project pushes the upper limit of productivity demonstrated in cell-free systems by utilizing sugars found in hydrolysates for conversion into isobutanol. The second project is titled "Cell-Free Biochemical Production of Terpenoid Chemical Astaxanthin Using Crude Cofactor Lysates." This project focuses on utilizing low-cost cofactor mixes from microbial waste steam lysates to produce long-chain terpenes.




### COMMENTS

- The goal of this project is to produce isobutanol and astaxanthin from sugar (both pure glucose and hydrolysate). With regard to isobutanol, the team has made excellent progress producing this chemical at high titers and yields, thus demonstrating the potential of cell-free systems. The choice of astaxanthin is puzzling. Why not first demonstrate that they can scale up the isobutanol process to 100 L before embarking on a new target? The major criticism of cell-free systems is that they will be too expensive at scale. Why not directly address this concern by developing a pilot-scale process? A number of promising results—namely, low enzyme loadings—suggests that this may be possible. Criticisms aside, this team is making excellent process. The reviewer also appreciates that unambiguous results were presented so that a technical evaluation is possible.

- With regard to the management of the project, the team is making excellent progress and meeting their milestones/objectives. The science and technology supporting this work are impressive. In these regards, the project is on track toward successful outcomes; however, the impact would be greatly enhanced if they focused more on the issue of cost because this is the big unknown with cell-free systems being used to produce commodity chemicals and fuels.
- Invizyne presented two cell-free projects in this session. The first project, nearing a close, aims to develop a new route to producing isobutanol. The second project, which began last year, aims to produce a long-chain terpene using microbial waste stream lysates. Cell-free technologies have high potential for impact, but they must overcome many challenges to achieve that potential.
- The presentation included few details on the project management approaches (an additional slide details a management overview for the first project) and certain technical details. The slides were difficult to follow (e.g., unclear sequencing of slides for Project 1 versus Project 2; charts are generally helpful, but font sizes are too small; many slides are too busy). The project strengths include an ongoing and long-term university collaboration. Progress is on track for all, and the team states that they have published in high-impact journals (citations?).
- This project provides an innovative approach to address the issues with more traditional, microbial production systems (e.g., limitations on productivity and titer). This is well demonstrated by progress made to date on isobutanol production, including yields (>95%), titers (>250 g/L), and productivities (4 g/L/h) from glucose that far exceed what microbial systems are capable of. The control systems used (i.e., NADH rheostat and ATP purge valve) provide a novel and broadly useful approach to addressing issues with maintaining sufficient ATP while ensuring the pathway is redox balanced.
- From the information presented, it was difficult to determine if the team has addressed the stated goal of de-risking cell-free, small-molecule production processes and economics. First, insufficient detail was provided in the presentation on cofactor optimization to address the challenges with cofactor cost. Second, there appears to be challenges producing sufficient enzyme to run reactions at greater than the 200-mL scale. The overall impact of the technology and project could be increased by addressing these two limitations.
- Last, the project may benefit from greater detail and/or sensitivity analyses on the various cost centers in the TEA to help better assess progress toward achieving higher TRLs and cost-competitive economics.
- No DEI plan was outlined in either project.
- Project 1: Project completed.
    - Experiments with pure glucose are performed at a 200-mL scale. It is not clear at what scale cellulosic hydrolysates are performed. Bigger or smaller scale?
    - What action items will be put in place to improve the titer with cellulosic sugars? Does the PI foresee challenges/risks associated with scaling this process to the bench scale and subsequently at process development unit and/or pilot scale?
- Project 2: Terpenes production.
    - It would be insightful to have the TEA for this project and assess how the project meets BETO's target metrics and cost.
    - This is a project for a cell-free system, and the target product is isobutanol. The project aims to reduce production costs by achieving high titer and productivity of isobutanol (in the first project)

and developing low-cost cofactors (in the Small Business Innovation Research project). The team demonstrated isobutanol production exceeding the target titer (60 g/L versus 40 g/L) using a C5 and C6 mixture. The scaled system is currently being tested. The team also demonstrated comparable isobutanol titers using lower-cost and standard higher-cost cofactors. ATP production using microbial waste stream is an interesting idea that can reduce the cofactor costs. The project included a product cost estimation to show the effect of cofactor costs on the product cost, which justifies the importance of developing low-cost cofactors. The project approach is adequate. The risks and mitigations are not specifically discussed, but I assume they are related to enzyme engineering and developing cheap cofactors. The end project goal is 500 g/L from glucose, while the current titer is 250 g/L. Because the project is ending, it would be helpful if additional information is provided to explain how the gap will be addressed.