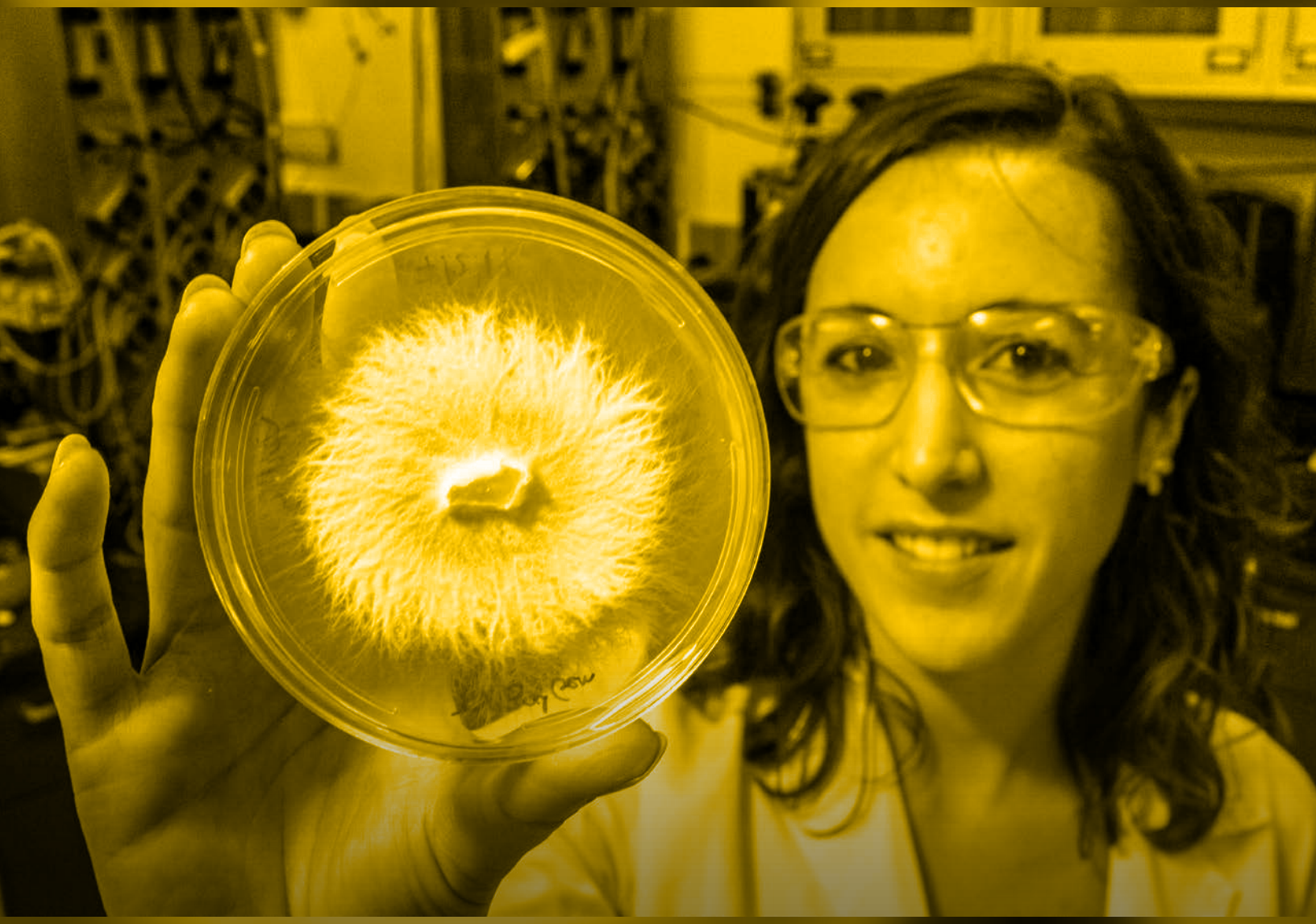


BIOCHEMICAL CONVERSION AND LIGNIN UTILIZATION

TECHNOLOGY AREA

CONTENTS

INTRODUCTION

Biochemical Conversion and Lignin Utilization is one of 12 technology areas that were reviewed during the 2021 Bioenergy Technologies Office (BETO) Project Peer Review, which took place virtually March 8–12, 15–16, and 22–26, 2021. A total of 26 presentations were reviewed in the Biochemical Conversion and Lignin Utilization session by five external experts from industry, academia, and other government agencies. For information about the structure, strategy, and implementation of the technology area and its relation to BETO's overall mission, please refer the corresponding program and technology area overview presentation slide decks, which can be accessed here: https://www.energy.gov/eere/bioenergy/2021-project-peer-review-biochemical-conversion-and-lignin-utilization.

This review addressed a total U.S. Department of Energy (DOE) investment value of approximately $54,311,484, which represents approximately 8% of the BETO portfolio reviewed during the 2021 Peer Review. During the Project Peer Review meeting, the presenter for each project was given 30 minutes to deliver a presentation and respond to questions from the Review Panel.

Projects were evaluated and scored for their project management, 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 Review, including scoring information for each project, comments from each reviewer, and the response provided by the project team.

BETO designated Ian Rowe as the Biochemical Conversion and Lignin Utilization Technology Area Review Lead, with contractor support from Chidiebere Agwu (BGS). In this capacity, Ian Rowe was responsible for all aspects of review planning and implementation.

BIOCHEMICAL CONVERSION AND LIGNIN UTILIZATION REVIEW PANEL

Name	Affiliation
Christopher Rao*	University of Illinois at Urbana-Champaign
Christopher Gerkin	ICM, Inc.
Charles Abbas	iBioCat, Inc.
Joseph Bozell	University of Tennessee, Knoxville
Daniel Noguera	University of Wisconsin–Madison

* Lead Reviewer

BIOCHEMICAL CONVERSION AND LIGNIN UTILIZATION REVIEW PANEL SUMMARY REPORT

Prepared by the Biochemical Conversion and Lignin Utilization Review Panel

INTRODUCTION

The BETO program in Biochemical Conversion and Lignin Utilization is focused on two primary aims: (1) developing new approaches to separate and valorize lignin and lignin-derived products with the goal of increasing their value when compared to current uses as fuel; and (2) developing biochemical conversion processes for transforming lignocellulosic biomass into different fuels and chemicals. The lignin valorization projects ranged from using different lignin fractions as a direct replacement for existing products to the conversion of lignin monomers into new and existing products using both chemical and biochemical approaches. The biochemical conversion projects include strain development and engineering, improved methods for biomass pretreatment, enzyme development for saccharification, fermentation process development and optimization, cell-free technologies, and the development of analytic and modeling tools for processes characterization and optimization.

The program incorporates both applied and fundamental objectives focused on answering the scientific and engineering questions necessary for reaching the overall goal of producing lignocellulosic biofuels at a cost of $2.50 per gallon gasoline equivalent (GGE) by 2030. This goal drives the research program, with each project within the portfolio displaying a clear understanding of the goal and directing their research—both fundamental and applied—towards achieving it. A significant strength of the BETO program is the handoff of these cost and operating goals to research teams within academia and industry. BETO, to their great credit, is supporting a number of parallel research approaches for meeting these goals, reflected in the 26 projects being evaluated as part of the peer review.

BETO's multifaceted approach to cracking these problems by supporting multiple approaches to lignin valorization and biomass conversion is commendable: catalytic, thermochemical, and biological transformations; whole-cell and cell-free fermentations; and batch and continuous processing, with outstanding science supporting the molecular-level understanding of these processes and technologies. The program also allows for both success and failure. This is consistent with the continued evolution of BETO from an industry-only focus to one that supports fundamental work and science as long as the case can be made for such work having an impact on the overall goals of the program. The close integration of science, economics, and application has become a hallmark of the program, able to provide significant advances in knowledge while still attracting commercial interest. As pointed out in the techno-economic presentation, BETO is not trying to put all of their eggs in one basket. The approach makes sense, and the management team should be congratulated for the directions they are taking in biomass conversion and lignin valorization.

While most projects are related to improving unit processes within specific technology paths, there is a solid mix of projects that are exploring new technologies. This is healthy since the currently envisioned critical path technology may not be the preferred one in the future. Specific examples of this are the "lignin first" concept, which would require rethinking current technologies for biomass pretreatment and deconstruction for the main purpose of improving how to make small chemicals from lignin, and cell-free technologies, which would bypass the challenges of using living organisms for biomass conversion.

Overall, the program is well managed with most projects meeting or exceeding their milestones, which all have clear relevance to the overall program goals. More importantly, BETO has course-corrected projects that got off to a slow start and incorporated feedback from previous reviews. This clearly indicates that BETO is taking an active role in managing their research portfolio.

STRATEGY IMPLEMENTATION AND PROGRESS

Techno-economic analysis (TEA) – The broad application of TEA is a key strength of the BETO portfolio, especially for the projects funded at the National Renewable Energy Laboratory (NREL), where it has been used to select different target products and processes. However, greater clarity around the methodology for TEA would be helpful. A number of the projects presented TEA results that discussed sales prices, current market values of products, or the minimum sales price needed to achieve or have a significant impact on the dollar-per-GGE target. What was not clear is what the *production* cost of the targeted materials would be. This would appear to be a critical component of the TEA methodology, but production cost estimates were only explicitly shown in the Texas A&M project. The market determines the sales cost, which varies depending on demand, but the technology determines the production cost, which may show much less flexibility. If a stakeholder is investigating potential new products or approaches, they would be most interested in how much it will cost them to make the new product in comparison to conventional approaches. The sales cost is of less interest.

In addition, there was a lack of information regarding mass balances. For example, only a small portion of the isolated lignin is being converted to a product, and a smaller fraction is captured in the final product. Do these lignin conversion efforts run into a problem of diminishing returns as more and more mass is lost in each step? Mass balances will provide not only a better understanding of the process, but also help understand the product and coproduct mix and identify plant discharges (air, water, solids for landfill). This type of information is consistently missing from the projects and can be provided in a simple Excel sheet or table.

If we use the model of distributed biorefineries as the baseline, how can multiple efforts and capital investment dispersed around the biomass-generating portions of the United States compete with a single plant near Houston grinding out huge amounts of material from a single location? Perhaps such assessments are buried within the TEA approach.

For some early-stage technologies, detailed TEA is unnecessary. Perhaps a hierarchical approach to process design and economics would be more useful, where gross calculations are performed for low-technology-readiness-level (TRL) projects and more detailed analyses for higher-TRL projects. Finally, greater transparency regarding the governing assumptions would be helpful.

Life cycle assessment (LCA) – The emphasis on using technical and economic targets is valuable to help compare projects and have them working towards a common goal. However, a perceived gap in the portfolio is the application of LCA as a guide for whether a given technology is sustainable and will have environmental benefits. Examples of this are included in the current portfolio (e.g., too much use of NaOH is not sustainable), but many other projects would benefit from LCA. In particular, the cell-free projects come to mind as projects for which LCA may be very useful to either promote the technology or curtail the extent at which it is evaluated as part of the BETO portfolio. Using LCA could avoid claims such as cell-free processes having no CO_2 emissions. This may be true for a specific pathway, but certainly not true when one considers the energy needed to make enzymes, cofactors, etc.

Cell-free technologies – BETO is funding three projects involving cell-free systems. These technologies are promising because they potentially avoid many of the complications associated with using living organisms. At the simplest level (as illustrated by the project led by Northwestern in collaboration with LanzaTech), these systems can be used to rapidly test different enzyme combinations for pathway optimization. The rationale and impact in this instance is clear: These systems accelerate the design process by minimizing the amount of tedious genetic engineering required. In addition, the Northwestern/LanzaTech team was able to demonstrate that results obtained with cell-free systems derived from *E. coli* lysates correlate well with whole-cell results obtained with *Clostridia*. In addition to providing an enabling technology for accelerating the design process, BETO also funded two projects using cell-free systems to directly produce 2,3-butanediol (BDO) (at NREL) and isobutanol (at Invizyne). Both teams have made remarkable progress and were able to obtain impressive results at the bench scale. The review team, however, was skeptical whether such processes could produce

their target products at competitive prices and at scale due to the associated cost of producing the requisite enzymes. To be clear, both teams were cognizant of these challenges. Lacking were any data to rationally frame this discussion. In other words, no one had a clear idea whether such systems would be economical or not. Going forward, if BETO plans to continue funding projects using cell-free systems as the production platform, they are advised to invest in projects focused on scaling up cell-free systems. The promise has been established, in no small part due to current investments from BETO in the promising technology. The next step should be to determine whether these systems can produce bulk chemicals and fuels economically at scale. In addition, identifying the right product targets, while challenging, will allow BETO to focus its resources on more near-term opportunities that can validate this approach and achieve early commercial successes.

Pretreatment – In contrast to the excellent variety of approaches being explored to reach the BETO GGE goal, one area where it appears that the choices have been limited is the front end of BETO's putative biorefinery. Corn stover has a long history at NREL, and has in more recent years been combined with deacetylation and mechanical refining (DMR) for initial pretreatment. Nonetheless, it would be helpful to know why other pretreatment processes do not seem to be in the mix or see very limited emphasis. It is not clear whether any commercial efforts outside of BETO use this technology for lignocellulosic conversion. What happens if this process is not what the general lignocellulosic biofuel industry ultimately chooses? It would be helpful to know of specific industrial interest in order to put this concern to rest.

Enabling technologies – BETO funds a number of project within NREL that provide supporting services. These services (analytic, TEA, modeling, scale-up) provide clear value by taking advantage of the unique expertise and capabilities within the national labs. They are also led by leaders in their respective fields and their work is recognized globally. The narrow focus of these projects is perhaps a missed opportunity because they could also provide equal value to the field in general and not just projects funded within NREL.

Chemicals to fuels – The review committee questioned the logic of converting cellulosic sugars into high-value chemicals (e.g. 2,3-BDO, butyric acid) and then downgrading them into fuels. Perhaps there is a reasonable and economically justified rationale, but it was difficult to see this from the presentations. The powerful TEA methodology in the program should be flexible enough to allow evaluation of multi-product scenarios, and to determine a profitable balance between fuels and chemical products from both carbohydrates and lignin.

Innovation – While many projects will advance knowledge, it is hard to gauge impact and outcomes without an active engagement by industry. Since many of these projects can be classified as a better fit by providing proof of concept and therefore have high inherent risk, they need to be evaluated based on their potential for disruption. By definition, a disruptive innovation is an innovation that creates a new market or can eventually disrupt an existing market, thereby displacing established market-leading firms, products, and alliances. Judging by the current 26 projects evaluated, some projects fit into one of these two categories. It has to be recognized that the path to commercialization goes beyond technological breakthroughs, as there are market, regulatory, and economic hurdles to overcome. While many projects identified companies or indicated interest by industry, there is little evidence that the industrial partner is actively engaged in these projects and are providing in-kind support or guidance to the projects. Projects that fund startup companies and businesses do not provide an indication of commercial plans and timeline. For this reason, it is hard to comment on commercial viability.

Fundamental versus applied science – From the plenary presentations, there is a clear philosophical change underway at the highest levels of BETO management. Specifically, DOE's focus on jobs, decarbonization, and climate change will increase the emphasis on applied science and demonstration projects. Historically, this type of change has been to the detriment of the fundamental science that BETO has worked so hard to incorporate. Statements during the plenaries such as "we have to get the scale-up going," "we will have to see the development of jobs," and "we have done enough fundamental and applied work to put us on the 2050 path," along with a rejuvenation of pre-pilot and pilot work, strongly suggest that fundamental work may be

reduced. Obviously, these high-level decisions are outside of BETO's control, but the program could strategize about how to maintain a science-driven effort while addressing potential new directives for deployment.

Moreover, it is clear that bioenergy is a unique field of study. By working in bioenergy, one is necessarily linked to the eventual development and deployment of technology for industry. But having industry development as a goal does not mean that the need for fundamental science is reduced. The entire experience of BETO at the national labs with their greater inclusion of fundamental science over the last 15 years graphically illustrates what can be learned and implemented in a science-driven program. BETO is in a unique position to make this happen. The Basic Energy Sciences program will not fund such work because it is not fundamental enough. Industry will not fund it because it is too risky and they do not want to be the first to invest in high-risk efforts. BETO provides the bridge that others cannot or will not develop and allows study of new technology for others to consider and implement. There is nothing wrong with demonstration efforts, but the only way that one reaches that point is through a strong scientific foundation, both in fundamentals and applications.

Therefore, this should not become an either/or for fundamental research versus applied science and large-scale demonstrations. The most powerful combination of activities for BETO would be the retention of the strong scientific program that BETO has built while advocating for new projects to support what appears to be a strong administration interest in large-scale demonstration.

Conclusion – BETO's program in Biochemical Conversion and Lignin Utilization has developed a strong portfolio with compelling scientific and engineering goals. The overall portfolio provides a good balance of fundamental and applied projects along with investments in a number of high-risk, high-reward projects. The targets based on techno-economic analysis, scaling up, and achieving future goals for the minimum selling price of transportation fuels is unique and fills an important niche in de-risking potential technologies for lignocellulosic biorefining. The portfolio is also well managed, with most projects meeting or exceeding their target milestones. Lastly, the program should strongly consider embracing LCA as an important metric to evaluate the sustainability of the proposed technologies since cost is not the only variable that will play an important role in the future of the lignocellulosic biorefining industry.

RECOMMENDATIONS

Recommendation 1: There is a perceived gap in the portfolio in the application of LCA as a guide for whether the technologies being explored are sustainable and will have environmental benefits. The panel recommends an increased emphasis on using LCA to inform direction.

Recommendation 2: There is a lack of clarity around the way in which prices, costs, and mass balances are communicated in BETO's economic analyses. There is also an opportunity for TEA capabilities in the portfolio to be used to assess product streams that go beyond simply generating fuels, and instead include other, more valuable products. The panel recommends greater clarity in TEA methodology and allowing flexibility in these analyses such that BETO can consider multi-product scenarios.

Recommendation 3: BETO has a long history of investigating DMR as the main method of biomass pretreatment for the front end of a biorefinery. However, the justification for the emphasis of this strategy at the exclusion of other pretreatment methods has not been clear. The panel recommends that BETO clarify the reasoning behind choosing DMR while also increasing the industrial involvement in the development of this pretreatment process.

Recommendation 4: BETO has built a portfolio that contains a significant amount of lower-TRL scientific research that is not being done by industry. The panel recommends that BETO develop a strategy to maintain a balance between more fundamental science work as it endeavors to increase technology deployment.

BIOCHEMICAL CONVERSION AND LIGNIN UTILIZATION PROGRAMMATIC RESPONSE

INTRODUCTION

The Conversion Program expresses its immense gratitude to the reviewers for their feedback, recommendations, and all the time and effort put forward in the 3-day virtual review process and the subsequent debrief sessions. The reviewers stated that the diverse investments made by BETO in Biochemical Conversion and Lignin Utilization research and development (R&D) are well structured and balanced between national labs, industry, and academia. The reviewers applauded the program's integration of science with economic analysis and commercial interest. The panel specifically identified the strong TEA capabilities and their broad application across the portfolio. However, they cautioned that at early stages, TEA can be an unnecessary activity that provides questionable information, and that transparency in such analyses is key.

The Review Panel specifically highlighted the willingness of the program to invest in fundamental technologies that have a longer time horizon. This was seen as a strength of the portfolio. Specifically, the reviewers pointed out the work in cell-free technologies for developing fuels and products as an example of early-stage technology that shows promise in no small part to BETO investment. Reviewers stated that these technologies were in need of exploration at larger scale to determine if they are industrially viable.

The panel pointed toward more strategic industrial involvement as an area that could help ensure that the innovative work occurring in the portfolio has an impact in the market. The commercial viability of many technologies go beyond just hitting certain cost targets, and digging into what makes a technology commercially viable in collaboration with stakeholders was seen as an area that needs improvement. Similarly, the panel suggested that analyses done within the program should allow for flexibility beyond just producing fuels from biomass and instead evaluate scenarios that explore converting lignin and sugars into chemicals and products.

Recommendation 1: The panel recommends an increased emphasis on using LCA to inform direction.

The program wholeheartedly agrees with this guidance from the panel. Most of the goals associated with BETO efforts are related to metrics like titer/rate/yield or more encompassing objectives like hitting a certain dollar-per-gallon target. BETO is actively exploring officewide targets that more accurately describe the sustainability benefits of BETO technologies, such as those related to reductions in the carbon intensity of fuels and products.

Recommendation 2: The panel recommends greater clarity in TEA methodology and allowing flexibility in these analyses such that BETO can consider multi-product scenarios.

The program concurs with the need for clarity in TEA methodology. The program endeavors to align TEA efforts across the portfolio such that assumptions are consistent and methodology is the same. The program acknowledges that clarity around selling price versus production cost is essential to understanding technology advances, and BETO commits to communicating this more accurately. Finally, the program strongly agrees with the reviewer recommendation to explore multi-product scenarios. BETO acknowledges that examining product choices other than fuels is essential to understanding the full potential of technologies advanced by BETO. The potential of products/coproducts to enable fuels is something that BETO is actively studying.

Recommendation 3: The panel recommends that BETO clarifies the reasoning behind choosing DMR while also increasing the industrial involvement in the development of pretreatment processes.

The program greatly appreciates the reviewers' feedback on biomass pretreatment and agree with their recommendation on industrial involvement. BETO considers that certain advantages of DMR, such as the quality of the resulting lignin and that it doesn't require high pressure, make it a method that avoids some barriers of other pretreatment strategies. Additionally, BETO agrees with the importance of industrial collaboration in pretreatment and is actively pursuing it. The recent Affordable, Clean Cellulosic Sugars for High-Yield Conversion topic area from the fiscal year (FY) 2021 Scale-up and Conversion funding opportunity announcement seeks applicants with technologies for producing cellulosic sugars and is open to a wide array of pretreatment strategies.

Recommendation 4: The panel recommends that BETO develops a strategy to maintain a balance between our more fundamental science work as it endeavors to increase technology deployment.

The program strongly agrees with and appreciates the reviewers' assessment of the importance of lower-TRL technologies. While the shift toward demonstration and deployment is a focus of BETO, the need for fundamental research on many early-stage technologies persists. BETO endeavors to balance the TRL of research needed to achieve its goals.

BIOCHEMICAL PLATFORM ANALYSIS

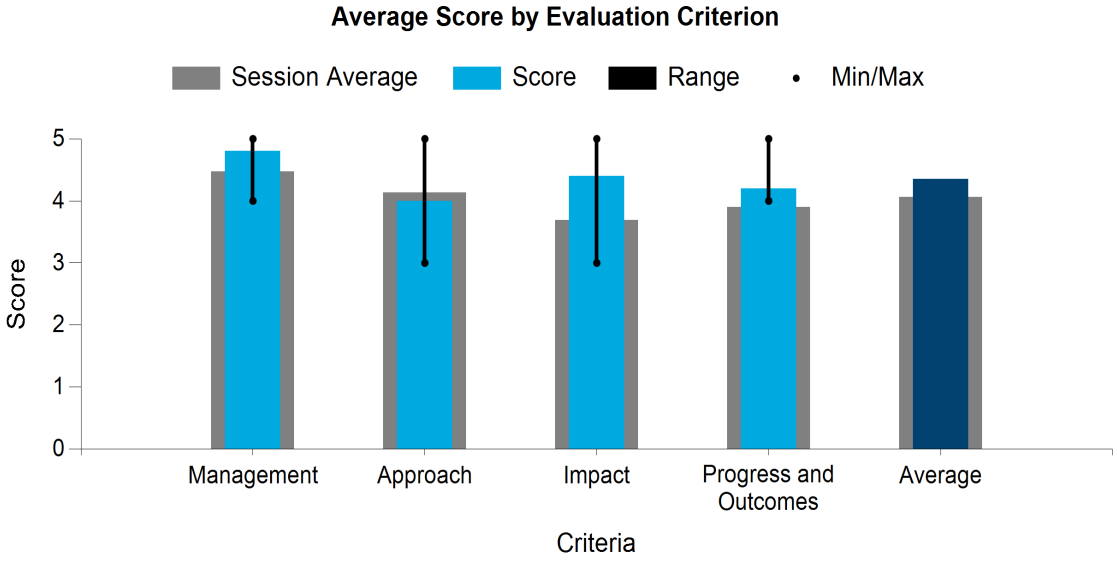
National Renewable Energy Laboratory

PROJECT DESCRIPTION

The objective of this project is to perform TEA to guide Biochemical Platform efforts, utilizing models for purposes of setting future R&D targets and tracking performance progress against those targets. Outcomes of our work are leveraged by BETO to guide program plans, as well as by other NREL/partner projects to quantify the impact of research on key technology barriers and to prioritize future efforts.

WBS:	2.1.0.100
Presenter(s):	Adam Bratis; Ryan Davis; Zia Abdullah; Courtney Payne; Jessica Krupa
Project Start Date:	10/01/2019
Planned Project End Date:	09/30/2022
Total DOE Funding:	$1,500,000

This project provides high impact and relevance by establishing “bottom-up” TEA models as a basis for understanding the technical feasibility to meet “top-down” BETO cost targets. By providing a framework to translate technical performance to cost reductions in a biorefinery, our TEA models may be leveraged to maximize the efficiency of research funding towards the most economically impactful priorities, ultimately in support of BETO’s 2030 fuel cost targets below $2.5/GGE. In order to mitigate a key risk/challenge to this project in overly constraining our analyses to a singular technology focus or TEA metric, our approach continuously reassesses opportunities for better optimization and alternative technology pathway options, while maintaining close interaction with other BETO analysis partners. We have made numerous recent accomplishments rooted around identifying and working with the researchers to solve key technical and TEA/LCA challenges, reflected through notable improvements in state-of-technology (SOT) updates over prior benchmarks.



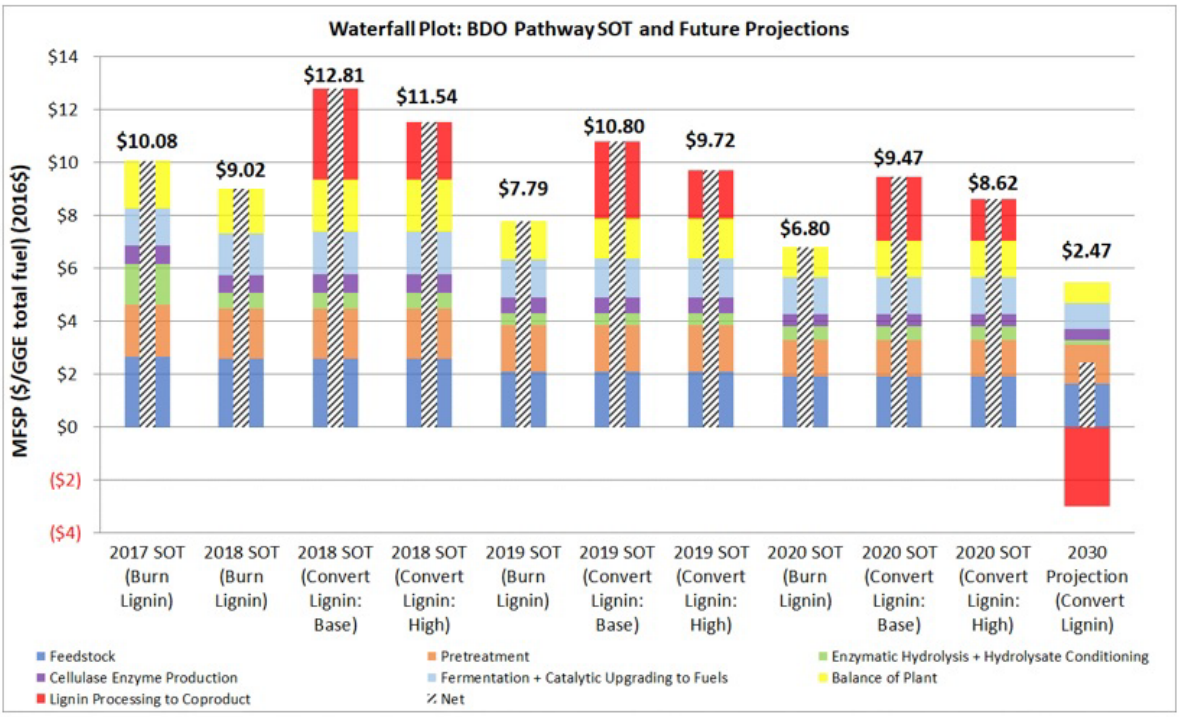


Photo courtesy of NREL

COMMENTS

- Good management team with relevant experience. Communication plan and collaboration in place. Risk mitigation strategy outlined. Approach is good in helping to address challenges to cost-effective biomass pretreatment. Biomass hydrolysis and fermentation production of 2,3-BDO and organic acid production used as models. Challenges are inherent in technologies that have not been demonstrated at pilot and commercial scale. There is a need to work with industry to address scale-up issues. Widespread impact to policy holders and technology enablers is anticipated from this work. Good analysis is provided on the modeled two cases that confirm that current approaches to lignin monetization are challenging. So far, the best-case economic scenarios support burning lignin as providing the best economics. The results also confirm that using NaOH is a costly approach with greenhouse gas (GHG) implications. The modified two-stage Na_2CO_3/NaOH is interesting but may not be viable. The new approach using methanol/H_2 using reductive catalytic fractionation (RCF) is a better option, as lignin is removed as an oil. Methanol is highly toxic to microbes, so would need to be stripped fully from solubilized fraction before fermentation. Are there better solvents to use (ethanol [EtOH], isopropyl alcohol)? These can be recovered and reused, and in case of EtOH is considerably less toxic to microbes. Overall, the team contributes well to the DOE program in highlighting costs and technology barriers. The downside is that the research work still needs to be done before good TEA/LCA models can be developed.

- Strengths: The Biochemical Analysis program provides a vital crosscutting service function by assessing economic progress and opportunities that roll up to achieve the higher BETO program directions. Since one of BETO's primary goals is to transfer the work to industrial stakeholders, a value proposition is necessary. The evaluation of each project provides that proposition and gives an entree for discussions with industry. The team notes that they try to keep all the eggs out of one basket, reflecting BETO's philosophy of setting a single high-level goal (the $/GGE price) and their willingness to study multiple, parallel routes to achieving that goal. The emphasis on better integration of LCA with TEA is an improvement over 2019 and shows good progress in program direction. The increased LCA emphasis is also in line with the administration's interests in decarbonization to reduce the effects of climate change.

Areas for improvement: The team relies primarily on Aspen Plus as a one-size-fits-all tool for TEA. This can take a long time to execute and is cumbersome for early-stage projects. Access to different levels of analysis would improve the program—for example, quick, back-of-the-envelope, best-case scenario evaluations that could be carried out in hours. The results generated by analysis need to be clearer and easier to compare to industrial standards. A primary issue is the lack of production costs for a fuel or chemical. Production costs (set by the technology) offer a much more useful means of comparing competing routes, and are easier to present to a potential stakeholder. Yet in this presentation and most others, various forms of sales costs (set by the market, such as minimum fuel selling price [MFSP] or the required sales price of adipic acid, sugars, BDO, etc.) are used for comparison. In some cases, sales costs for a single chemical product are applied to product mixtures (pyrolysis oils, Oak Ridge National Lab mixed alcohols), which will never generate the same sales cost as a pure material. Perhaps a production cost is buried somewhere in the analyses, but overall, the lack of a clear means of comparison can make the analyses appear muddled and less transparent. More information on the impact on $/GGE as a function of different balances between fuel and chemical production would be helpful. TEA should be flexible enough to move away from assuming that all carbohydrates will end up as fuel while lignin will supply chemical products to support the production of low-value hydrocarbons. The project would be strengthened if there were sensitivity analyses showing the effect on $/GGE if various levels of sugars were also sold as products (e.g., 10%, 25%, 50%), since the current fuel intermediates are marketplace products in their own right. The program would be further strengthened if the rationale behind the choice of chemical coproducts for inclusion in the evaluation was more clearly described (e.g., why adipic acid or BDO?).

- The goal of the project is to provide process modeling and techno-economic analysis for the BETO/NREL projects. Overall, this is an extremely valuable service and a key/unique strength of the BETO/NREL portfolio. These activities should continue because they can help direct the various technology-focused projects to more realistic processes and molecules. Another key strength is outreach and dissemination. The team has also published a number of high-impact reports and models on the economics of lignocellulosic-based fuels. These activities should continue, and ideally expand. In particular, this is where the team can have real impact beyond the activities within NREL. It will also address some concerns regarding the calculated numbers, which some reviewers thought were overly optimistic. It would also be great to see the team expand into more comprehensive LCA. Lastly, TEA can provide clear milestones for go/no-go decision points. To really highlight the impact of defining these benchmarks, it may help to provide explicit examples of processes that are not feasible given current technology or never will be. This is where the real impact of the project is: eliminating promising but infeasible projects.

- The project seems to be running quite well. The iterative approach TEA and optimization evaluations to drive R&D is a system with substantive merit. This systems bridge approach to guide technical targets is of value. Impact enhancement by generating tools, as opposed to reports, could perhaps be of consideration. There are many skillful and competent members within this consortium, and developing of a block model TEA tool for industrial applications, with discrete unit operation blocks tailored for a suite of processes, would be valuable and plausible. Acknowledging the challenges with TEA of novel technologies, which are not understood well at scale, well-understood feed streams such as excess wet mill, dry mill, cane sugar capacity could be modeled for near-term market impact. We can see that the lignin coproduct is a critical pinch point to the economics. Ideally, this could somehow be decoupled, as no viable pathways are currently operating to my knowledge. The technical and startup complexities with any of these proposed technologies are tremendous and are not transparent in the modeling effort. Content has to be limited due to the presentation window; however, more details related to the process would be helpful. In other words, was the TEA performed on the exact process that was deployed according to the process flow diagram? For instance, were commercial cellulases used in piloting while "on-site enzyme production" was modeled? Capital expenses (CapEx) reduction potential would also be insightful. The modeled processes shown generate revenue, and the returns on investment make sense on

paper, although $700,000,000 for a process producing 31 million GGE annually is a tough pill to swallow. Core technology changes might be necessary to decrease the impacts of economies of scale, allowing design of smaller systems. Industry will have a difficult time couching up that magnitude of capital. If possible, visibility of the "Credibility of Analysis" conclusions would be value added from a project review perspective. The nonlinear results for the 70/35 deacetylation catalyst loading slide, in my opinion, adds confidence to the data set, as a bad or off data point is believable. It is great to see that fuel targets were met in advance. Similarly, considering the new sugar catalyst pathway, it is great to see the higher GGE/ton yield due to higher carbon utilization. Good job team, thank you.

- This project provides the TEA of the different lignocellulosic biorefinery processes being studied. It effectively serves as a connection point for setting economic targets for other projects seeking to enhance the value of lignocellulosic biorefinery applications. The project management is well done and there is evidence of interaction and communication with personnel from other related projects. The impact of this team on the development of the cellulosic biorefinery concept is without question. The TEA reports produced often become benchmarks that are used by others to compare their alternative approaches. The project uses the production of 2,3-BDO and organic acids as baseline cases for comparisons of year-to-year progress against a 2030 benchmark of achieving an MFSP of $2.5 per gallon. These baseline analyses are effectively used to give targets to other projects such as those working on making coproducts from lignin, with adipic acid being the tested product. The analysis provides less clarity when attempting to display cases with coproducts, as assumptions are not easily identified. For instance, in order to achieve 2030 goals, there is a need to convert adipic acid production from adding cost to the overall cellulosic biorefining process to producing revenue. Productivity and percentage of lignin conversion are identified as the main drivers to creating a revenue-generating coproduct. Does this assume a fixed selling price for adipic acid? Is that selling price equal to the market price of adipic acid made from fossil fuels? It would be useful to have information of the production cost to compare to the market price more easily. Then, when evaluating the lignin-first process, the selling price of the lignin oil is a new target (in $/lb) instead of continuing using adipic acid as the target coproduct. The metric changes to the minimum sugar selling price (MSSP) (in $/lb) when evaluating NaOH replacement. Then, when evaluating sugar catalytic upgrading, the process diagram shows production of C14–C16 hydrocarbon fuels plus adipic acid. Is adipic acid production fixed to 2030 goals to make the estimates of MFSP for the hydrocarbons? On a more general note, reporting in this and other connected projects could be improved if the alternative metrics used in other projects are also emphasized in this project. For instance, if the MSSP is a more logical metric for some projects to use, the reporting could benefit from information about the 2030 goal for MSSP that this project uses.

PI RESPONSE TO REVIEWER COMMENTS

- We thank the reviewers for their insightful comments, and appreciate the recognition of the importance of this project in guiding NREL/BETO program directions. Regarding the comments around the TEA models solving for "production costs" versus "market values," we would like to provide clarification that the TEA work conducted here in fact does solve for "production costs" as driven by the technology, based on underlying technology performance, yields, and associated capital/operating costs. This can then be compared against market values; for example, MFSPs calculated from the TEA models can be compared to market values for fuels, which are currently set at a fixed $2.5/GGE basis per BETO guidance across all platforms. Any coproducts (such as adipic acid from lignin) are set at market value prices, typically based on historical multiyear market price averages, to determine the resultant coproduct revenues they garner for the biorefinery. More information on our TEA methodologies and sensitivity analyses to factors such as coproduct market values and conversion performance metrics may be found in our design reports (https://www.nrel.gov/docs/fy19osti/71949.pdf).

  On the comment about making TEA more flexible for quick-turnaround analysis, we have been building several tools and methods for performing such higher-level/back-of-the-envelope analyses that don't involve as rigorous (but time-consuming) Aspen Plus modeling. These typically are based on exercising

the economic spreadsheet portions of the TEA models to adjust yields, unit operation inclusions/exclusions, input/output costs, etc. (although our TEA team also has built separate quick-turnaround tools for newer/less-established concepts as well), which we leverage frequently to help answer "what-if"-type questions from researchers or BETO managers around process modification opportunities. Given inherently higher uncertainty in such analyses, these are typically used for internal purposes and are not published, and time was not available to cover such approaches in the presentation.

To the comment about screening out infeasible projects, this type of analysis is also applied for that purpose, such as generating order-of-magnitude estimates to establish feasibility for early-stage concepts prior to extensive modeling when economic challenges are anticipated due to factors such as high-severity operating conditions, high usage of a solvent, or high costs of a chemical co-feed.

In response to the comment about also focusing on tools and not just reports, we have established several such tools over recent years for use by the public and industry partners. Two examples as mentioned briefly in the presentation include a public TEA sugar model (https://www.nrel.gov/extranet/biorefinery/aspen-models/, second set of files) and an Excel-based TEA tool to estimate the cost of biochemical intermediates over varying inputs for feedstock type, composition, cost, and conversion performance that does not require the use of Aspen (provided to an industry collaborator investigating opportunities for excess pulp mill capacity). We are also working to evaluate opportunities in the context of today's existing industry resources—for example, to understand technology "bolt-on" possibilities to add cellulosic biomass processing capabilities to the front end of a Gen-1 facility (e.g., via DMR processing) and/or opportunities to switch to a new fermentation product with minimal redesign (e.g., 2,3-BDO), recognizing and fully agreeing that the capital expenses for such complex biorefineries as those described in our "design cases" may prevent implementation of such designs in the near term.

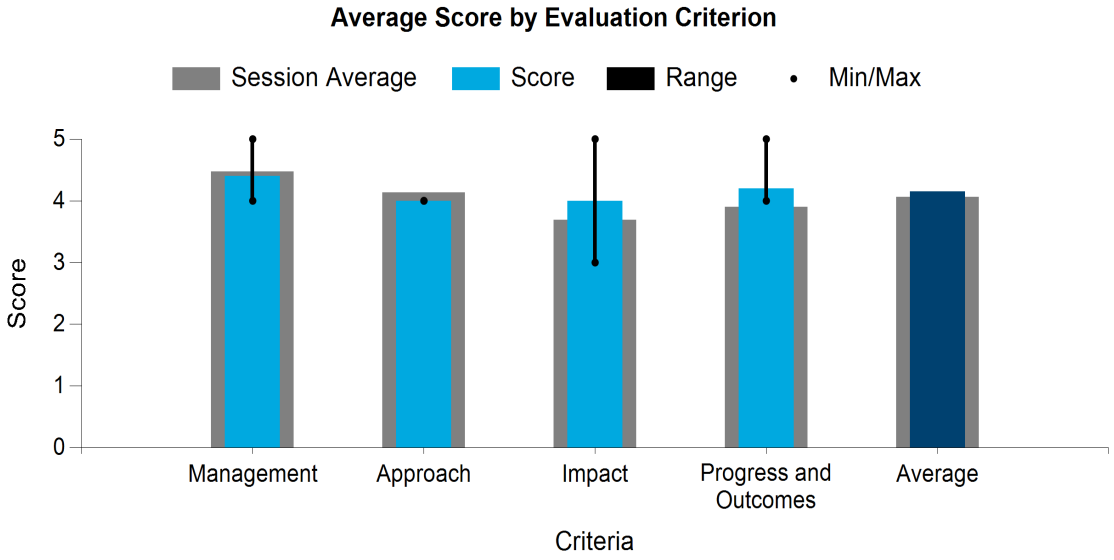
LOW-TEMPERATURE ADVANCED DECONSTRUCTION

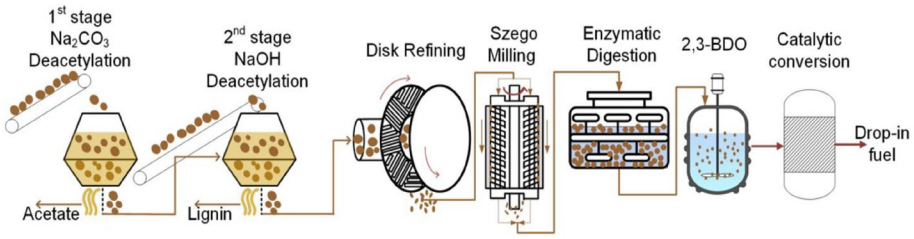
National Renewable Energy Laboratory

PROJECT DESCRIPTION

WBS:	2.2.3.100
Presenter(s):	Adam Bratis; Xiaowen Chen; Zia Abdullah; Courtney Payne; Jessica Krupa
Project Start Date:	10/01/2018
Planned Project End Date:	09/30/2021
Total DOE Funding:	$4,500,000

The Low-Temperature Advanced Deconstruction (LTAD) project continues its mission to develop industry-relevant low-temperature biomass deconstruction/fractionation processes. These processes produce cost-competitive, low-GHG-emitting, clean-sugar syrups and reactive lignin streams from relevant feedstocks to enable economic and environmentally sustainable production of biofuels and bioproducts. Previously, we have demonstrated >80% sugar yields with 12-mg enzyme protein per gram of cellulose using DMR corn stover substrates and achieved over 270-g/L monomeric sugars. To achieve BETO's 2022 target of 90% sugar yields with 10 mg protein/g of cellulose, we have modified the DMR process by using a two-stage Na_2CO_3 and NaOH deacetylation, which allows us to achieve 90% glucose yield and 88% xylose yield. In addition, by reducing NaOH with a lower-cost and lower-GHG-emitting alkali (Na_2CO_3), the modified DMR process reduces MFSP by approximately $1/GGE in the FY 2020 SOT and reduces GHG emissions by about 22% in sugar production. In collaboration with Princeton University, we have developed a novel NaOH recovery system that enables high NaOH recovery, reduces NaOH usage, and produces renewable H_2 by processing the waste organics in deacetylation black liquor. Feedstock variability was investigated in collaboration with the Feedstock-Conversion Interface Consortium using corn stover anatomic fractions to advance fundamental knowledge in the DMR process.





The 2-stage Na_2CO_3 and NaOH deacetylation contributes nearly $1/gge reduction on the Minimum Fuel Selling Price (MFSP) in the FY20 SOT and up to 23% off of GHG emissions.

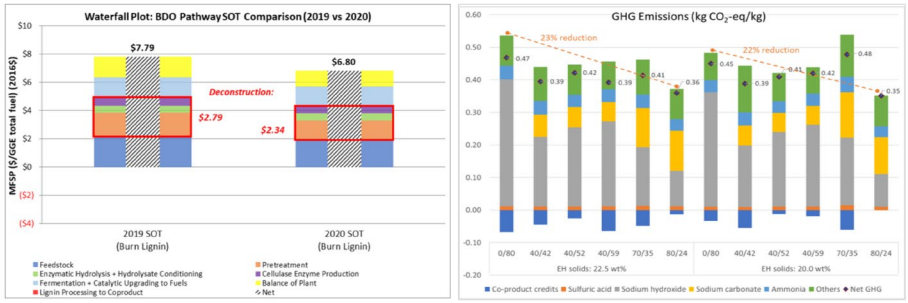


Photo courtesy of NREL

COMMENTS

- Management team, research, and communications plans are adequate. Risk mitigation strategy is in place. Three parallel approaches used to address NaOH uses, as well as NaH recovery and the determination of feedstock variability. Impact is hard to assess, as the challenges identified by TEA/LCA done by the modeling group do not confirm cost-effectiveness and viability for the use of NaOH. Two-stage Na_2CO_3/NaOH or NaOH/ozonolysis and operation can be challenging. TEA does not support the use of NaOH DMR as having a low GHG impact. The two-stage processes are promising but there is additional cost from additional unit operations. RCF looks more promising but comes at a significantly higher cost. The use of existing plant equipment may not be as easy as it sounds, as extra handling, storage, and costs of chemicals need to be factored in for existing ethanol plants. LTAD is a better fit than high-pressure/high-temperature specialized equipment that will require higher maintenance and cannot be operated easily in continuous mode. LTAD trades higher chemical/mechanical energy cost for lower chemical and higher energy cost and CapEx needed for specialized high-pressure reactors. NaOH recovery by microbial electrochemical technology still needs to be demonstrated at scale. This approach also comes at additional operational costs and risks associated. Progress and outcomes seem to be reasonable based on original assumptions made and targets. On the overall, having also reviewed this project in 2019, it seems that the research keeps identifying new hurdles, bottlenecks, and challenges. RCF seems to be a more viable approach to pretreat corn stover, as it has the potential to reduce GHG emissions, but it comes at a higher CapEx. Progress and timeline of this project need to be revisited, and it would be helpful to have a side-by-side of all pretreatment options with pros/cons with cost and material balance provided.

- Strengths: This process is projected to generate a sugar with a minimum selling price of $0.25/lb, while lowering overall pretreatment cost by 30%. These would be significant improvements, as pretreatment is a major cost contributor to the overall price of the final biofuel. Importantly, the team has achieved their 2022 targets early by demonstrating a 90% yield of glucose and an 88% yield of xylose via 10 mg cellulase/g cellulose. This has been translated into a $1/GGE reduction. The new approach also offers significant GHG and energy consumption improvements, specifically by employing the two-stage Na_2CO_3/NaOH treatment, with the best results being obtained by using less-expensive carbonate as the

primary pulping chemical. The team's partnership with Andritz and Novozymes, two heavy hitters in the mechanical and enzymatic world, respectively, is a good addition to the project. On slide 17, the principal investigators (PIs) describe an approach to convert entrained syringaldehyde to dimethoxybenzoquinone, and then use a Diels-Alder reaction to generate naphthoquinones and anthraquinones. This is an interesting concept, but this exact transformation of monomers from lignin was first invented at the Solar Energy Research Institute (now NREL) between about 1989 and 1995, and was the subject of several publications. The goal of the early work was to make anthraquinone derivatives as pulping catalysts, and thus it would be interesting to see if anthraquinone would also improve LTAD, given its similarity to soda pulping. Alternatively, anthraquinone is used in the industrial production of HOOH, offering another opportunity for stakeholder discussions.

Areas for improvement: The project is based on DMR as its baseline source of sugars and lignin. However, there is no evidence that the biorefining industry will adopt this as their front-end technology. This presents a risk to the program of developing a pretreatment process and downstream conversion technology that is internally consistent but not an industrial standard. Further, given the similarities to processes in the pulp and paper industry, it would be valuable to know the applicability of this approach to different lignocellulosic feedstocks, as corn stover may not be the feedstock of choice for the biorefining industry in different parts of the United States. It would be helpful to have a better idea of how deeply the industrial partners are involved. A willingness by the partners to build a demonstration or pilot plant around this technology would go a long way toward establishing DMR as a credible industrial pretreatment. The electrochemical approach for recovery of NaOH is a scientifically interesting and elegant approach for NaOH recycling. However, industry still sees electrochemistry as exotic and expensive. It would be helpful if the PIs could provide more information regarding the possible use of this approach at scale. The economic feasibility of this approach among a network of distributed biorefineries is also questionable, as the CapEx might be high. The team is investigating whether more value can be obtained from black liquor via biological transformation. Recycling of black liquor affords 4 g/L of muconate after a week of treatment, which is a small amount. Mass balances evaluating the volume of black liquor needed at a commercial scale to generate this 4 g/L would be revealing, as it is unlikely that this amount of product would have a significant effect on biorefinery profitability.

- The goal of this project is to develop a low-temperature deconstruction process for producing sugars and reactive lignin. The project is well managed with clear and compelling milestones. Overall, the team is making excellent progress with the DMR process. All milestones have been met or exceeded. It is also great to see the team starting to investigate feedstock variability, which was a concern raised at the previous review. In addition, the project is closely guided by TEA/LCA, as evidenced with the efforts to reduce NaOH usage by replacing it with sodium carbonate and developing a two-stage process. There is some concern regarding the use of microbial fuel cells for NaOH recovery. This project seems premature and will likely require multiple technological advances before it can be utilized in an industrial process. While it is great to see new technologies being explored, it is questionable whether this project provides the best mechanism. In particular, it may be better to focus on DMR such as further developing a continuous process. Lastly, it may help to provide more comparisons with existing deconstruction processes. This will address concerns about impact.

- This project is improving the DMR process, which is a core biomass treatment process in the NREL model cellulosic biorefinery. Their goal is to achieve 90% sugar yield during enzymatic hydrolysis after DMR pretreatment using a new DMR-specific enzyme cocktail formulation at a loading of 10 mg protein/g cellulose. The team reported achieving these milestones, and with these achievements contributing to reducing the MFSP in the model cellulosic biorefinery. This is important progress. A specific emphasis in the project has been the reduction in NaOH utilization, which is viewed as improving the life cycle assessment of the DMR process. A promising approach was separating DMR into two stages, with the first stage using Na_2CO_3 instead of NaOH, and the second stage using a lower amount of NaOH. This was also achieved while maintaining the goals of 90% sugar yield and enzyme

loading of 10 mg protein/g cellulose. This progress is impressive, although from the data provided it is difficult to see how these improvements resulted in an MFSP reduction of almost $1 per gallon (i.e., $2.79 – $2.34 = $0.45 per gallon). A secondary approach to increase sugar yields from the DMR process was the use of ozone to enhance delignification after milling. Although the team reports this approach is effective at increasing sugar yields, it is likely that ozonation would reduce the quality of the lignin that is recovered after fermentation (i.e., more condensation occurring), negatively affecting the yield of lignin that could be converted to adipic acid, which is one of the requirements to make the lignocellulosic biorefinery ultimately achieve the 2030 goal of an MFSP of $2.5/gallon. It is possible that after ozonation, the recovery of products from lignin would need a different approach, such as products for jet fuel. Since the TEA of the lignocellulosic biorefinery is critically dependent on generating revenue from lignin, this project would benefit from a better integration with the Biochemical Platform Analysis project to evaluate whether this alternative to gaining value from lignin is economically feasible. A significant amount of effort has been placed on developing microbial electrochemical technology for the recovery of NaOH for reuse in the DMR process. Interesting progress has been made in this front. This is an emerging technology, and the project would also benefit from insights that a TEA could provide on whether this would be a cost-effective approach when scaled up, since microbial electrochemical processes have had many practical limitations for scale-up. The project would also benefit from insights from industry on whether electrochemical technology could be embraced as an essential component of the lignocellulosic biorefinery. Finally, the project uses a different metric than other projects for reporting TEAs. Instead of the analysis being based on the MFSP (in $/GGE), the main metric is the MSSP (in $/lb). The use of a different metric makes it difficult to make direct comparisons. Is there a direct correlation of the MFSP to the MSSP for a 2,3-BDO-producing biorefinery? Is there a target for the MSSP for sugar production that would lead to the 2030 target MFSP? Furthermore, it is indicated that the reported MSSP does not include CapEx. It would be important to improve the analysis to include CapEx, particularly for evaluating the contribution of scaled-up microbial electrochemical technology to the overall economic predictions.

- Your project appears on track, and successful execution, progress, and outcome advancements have been substantial. The team has been successful at achieving GHG reductions, and by all visible measures the catalyst switch did not negatively impact the process. The project's commercial partners are top-notch and speak to the project's relevance. I see great inter-project collaborations within BETO and team diversity of fields. The track record for patents and publications resulting from this work is applauded. Direct engagement of the 2019 Peer Review comments is well received. I am happy to see the refiner plate redesign, and the feedstock anatomic fractionation could potentially lead to a major process breakthrough; this will be interesting work in due time. Similarly, the ozone efforts and theory regarding applications with renewable electricity and more recalcitrant biomass is interesting, but adding another $0.20 to the MSSP quickly blows the $0.25 target stated early in the presentation. The need for catalyst recovery looked to inspire the novel microbial electrochemical technology, although I didn't see the argument where NaOH recovery was even necessary from a GHG emissions standpoint when using the two-chemical catalyst system. More precise justification would be helpful. Carbon-negative sugar production from only agricultural wastes will limit potential scope significantly. Purpose-grown feedstock inclusion could positively broaden project impact. The upside to decreased catalyst and enzyme usage is fairly straightforward with regards to economics; however, these reductions could significantly change the rheology and perhaps the process design in terms of pumping, mixing, heat transfer, etc. If possible, the downside to catalyst/enzyme reductions should be reflected in the CapEx, or at a minimum discussed to some degree. Small loading changes can be significant and hopefully are not being overlooked. The loading changes might be simple in the pilot in a noncontinuous batch system, but at scale in an integrated process, the minor decreases could spell complex challenges. Ideally this would be solvable at small scale during an integrated campaign, reducing the risk and cost of having to do this at scale. A table showing the technology baseline vs. state-of-the-art data to clearly illustrate improvements in the areas of natural gas (Btu/lb sugar), electricity (kWh/lb sugar), connected

horsepower, enzymes (mg protein/lb sugar), process chemicals ($/lb sugar), and water (gal/lb sugar) would be helpful. Good job!

PI RESPONSE TO REVIEWER COMMENTS

- We sincerely appreciate all the valuable reviewer comments and suggestions. Over the last 2 years, the LTAD project has focused on improving sugar yields while reducing GHG emissions of the DMR process. As pointed out by the reviewers, the LTAD project has achieved multiple significant improvements, especially on achieving BETO's 2022 sugar yield targets at 90% with an enzyme loading of 10 mg protein/g of cellulose. We will continue working on reducing GHG emissions of the DMR process while optimizing the process with higher sugar yields, titers, and quality. The DMR process is a new cutting-edge technology that still needs additional research and development. It was developed to solve multiple issues encountered by pioneer second-generation ethanol plants. The older high-temperature dilute-acid-pretreatment-based cellulosic ethanol processes caused the pioneer plants to struggle with several operational challenges that are related to feeding lignocellulosic biomass across high-pressure boundaries, char formation clogging reactors, low sugar fermentability of the hydrolysates, and high capital costs incurred by using corrosion-resistant materials of construction. The DMR process mitigates most of those issues by using an atmospheric pressure dilute alkali pretreatment, which has demonstrated greater sugar yields (>90%), higher sugar concentrations (>230 g/L), and higher sugar utilization in fermentations (>90% sugar utilization in ethanol and BDO fermentations). However, high GHG emissions caused by NaOH usage is a major challenge for the DMR process. Therefore, we developed the two-stage Na_2CO_3 and NaOH deacetylation option to reduce the usage of NaOH, which allowed us to reduce GHG emissions by 20% or more. We will continue exploring this new method. We will also optimize the mechanical refining process to reduce its energy usage. Reducing GHG emissions is critical for the DMR process to be successfully commercialized. The reviewer comment on applying the DMR process to other feedstocks besides corn stover is well received. We agree that corn stover may not be available in certain area of the United States. However, we presented our results of single-stage (NaOH only) DMR pretreatment using switchgrass and sorghum as feedstocks in our previous 2019 Peer Review of the LTAD project. In that presentation we showed that higher NaOH loadings were required using switchgrass, which could further increase GHG emissions. Sorghum was less recalcitrant to DMR pretreatment, requiring lower NaOH loadings. We plan to test the two-stage Na_2CO_3 and NaOH deacetylation and mechanical refining pretreatment on other feedstocks, including switchgrass, sorghum, and wheat straw, in the future. We use both MSSP and MFSP as metrics for analysis with the TEA group, as a reduction in MSSP will correlate with MFSP assuming all else equal downstream of enzymatic hydrolysis. MSSP provides a means of more rapid, less intensive TEA modeling to evaluate trends when focused only on biomass deconstruction steps (for example, identifying an optimal Na_2CO_3/NaOH loading scenario out of multiple cases via MSSP, and then applying that scenario in the integrated SOT models to evaluate overall MFSP impacts). We showed that the two-stage deacetylation could reduce MFSP by nearly $1/GGE because of cost reduction on NaOH/enzyme usage, as well as improvements in sugar yields. The $0.45/GGE cost reduction indicated in the figure of interest reflects mainly the cost reductions associated with NaOH usage and enzyme loading improvements, while the remaining $0.54 is attributable to deconstruction yield improvements (also enabled by the move to the two-stage DMR system), which cascade into GGE fuel yield increases and thus a larger denominator in $/GGE overall MFSP, which in turn reduces all of the bars accordingly. The MSSP analysis does include CapEx, as does MFSP, based on standard NREL TEA methodologies (the preliminary TEA on ozonolysis-aided DMR did not include the capital cost for the ozone generator as we do not currently have a reliable source for that unit cost). We do not have a formal MSSP target in 2030; however, the 2030 target performance parameters would translate to approximately $0.18/lb sugars as reflected in NREL's stand-alone sugar model available publicly (https://www.nrel.gov/extranet/biorefinery/aspen-models/). In the future, we will also include a detailed cost breakdown on energy usage as suggested by the reviewers. The high-temperature/high-pressure RCF and atmospheric pressure DMR processes are targeting different products from biomass. RCF is targeting to produce lignin oil, while the DMR process

is more focused on producing fermentable sugars, with reactive lignin waste streams a potential source of target chemicals such as high-energy-density sustainable aviation fuels. RCF also targets highly recalcitrant woody biomass, while the DMR process focuses on herbaceous crops such as corn stover, switchgrass, sorghum, etc., where more ash is present in these agricultural feedstocks, which is notorious for deactivating catalysts. Both processes are facing chemical/solvent/catalyst recovery challenges with potentially high energy usage, either directly in the process (RCF) or indirectly through sourced chemical usage (DMR). To the team's best knowledge, the GHG analysis on RCF will be highly dependent on the use of solvent and the solvent recovery process. We have not yet compared RCF to DMR on a GHG emissions basis, which will require more research effort. The microbial electrochemical technology being investigated in the LTAD project utilizes the breakdown of waste organics in the DMR black liquor to generate renewable H_2 while recovering NaOH, all of which are tangible benefits for DMR operation. The microbial electrochemical technology investigated in LTAD is different from microbial fuel cells, which are used primarily to generate electricity, while our technology generates electrical energy to desalinate our waste streams to recover the NaOH and produce H_2. Our preliminary studies have verified such benefits and capabilities. We are working on improving the microbial electrochemical technology system performance and using those results to conduct TEAs and LCAs to quantify such impacts. We do acknowledge our microbial electrochemical technology investigations are still in an early TRL stage compared to other processes, but we believe our results show the potential for application to the DMR operation, and we will work on advancing the technology. We are working with the Lignin Utilization project led by Gregg Beckham on lignin conversion to adipic acid. The Biological Lignin Valorization project is working with the Separations Consortium to develop nanofiltration strategies to separate and further concentrate low-molecular-weight components from black liquor (among them monomeric aromatic compounds, which are the precursors of muconate). The deacetylation unit operation in DMR solubilizes primarily ferulic and p-coumaric acids, which we have shown can be concentrated to useful levels by recycling the black liquor back into deacetylation. The Lignin Utilization project has shown that the ferulic and p-coumaric acids from deacetylation black liquor recycled five times can be converted to muconic acid in high yields, as shown by the Biological Lignin Valorization project. That way, we can develop bioprocesses to enhance muconate concentration in bioreactors (as shown in the presentation of the Biological Lignin Valorization project).

LIGNIN-FIRST BIOREFINERY DEVELOPMENT

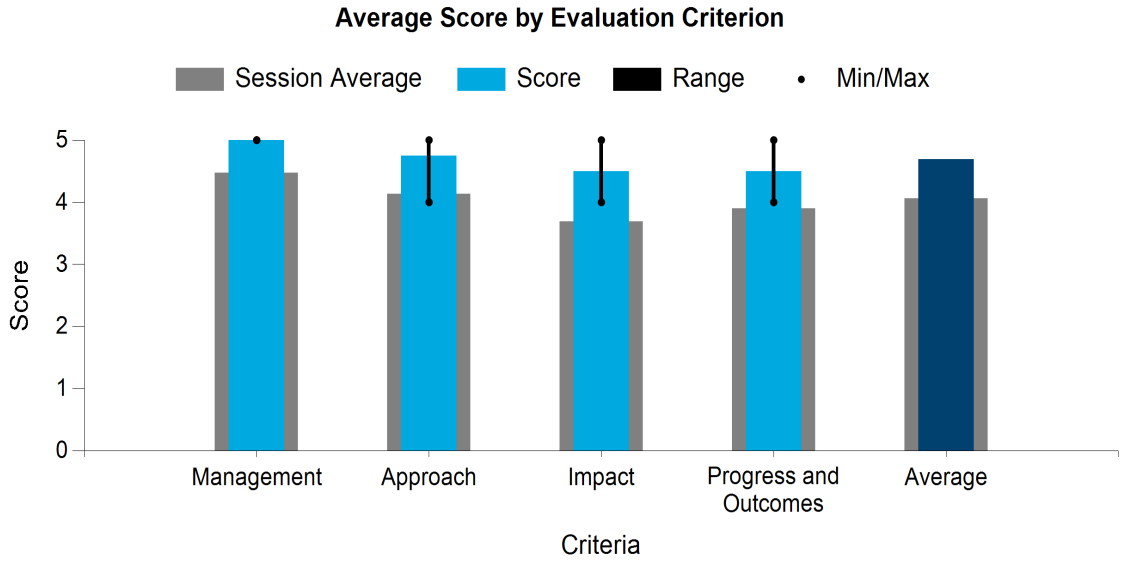
National Renewable Energy Laboratory

PROJECT DESCRIPTION

WBS:	2.2.3.106
Presenter(s):	Adam Bratis; Gregg Beckham; Michelle Nolker; Zia Abdullah; Courtney Payne
Project Start Date:	10/01/2020
Planned Project End Date:	09/30/2023
Total DOE Funding:	$2,100,000

Cost-effective biomass fractionation is an enabling, grand challenge for biorefining, especially when both carbohydrates and lignin are targeted for valorization. The advent of reductive catalytic fractionation—an active stabilization approach that solubilizes lignin from biomass and catalytically depolymerizes it into a narrow slate of monomers and oligomers—represents a potential step forward for this important goal. In the Lignin-First Biorefinery Development project, we are employing TEA and LCA to guide bench-scale R&D efforts towards cost-effective RCF-based biorefining. This project is critical to lignin valorization efforts and could enable the use of woody feedstocks in a traditional biochemical conversion context.

To date, we have conducted the first rigorous TEA and LCA study of the RCF process, developed a flow-through system to separate biomass and the chemical catalyst in RCF chemistry, developed models for solvolysis chemistry and transport phenomena for poplar, and co-led an international, authoritative perspective on guidelines for the research community on how to best practice lignin-first biorefining. The primary challenges for the lignin-first RCF process going forward are catalyst stability; the need to utilize, recover, and recycle high-boiling-point solvents to lower RCF reactor pressure; and the challenge of operating RCF continuously—all of which are being directly tackled by this project.



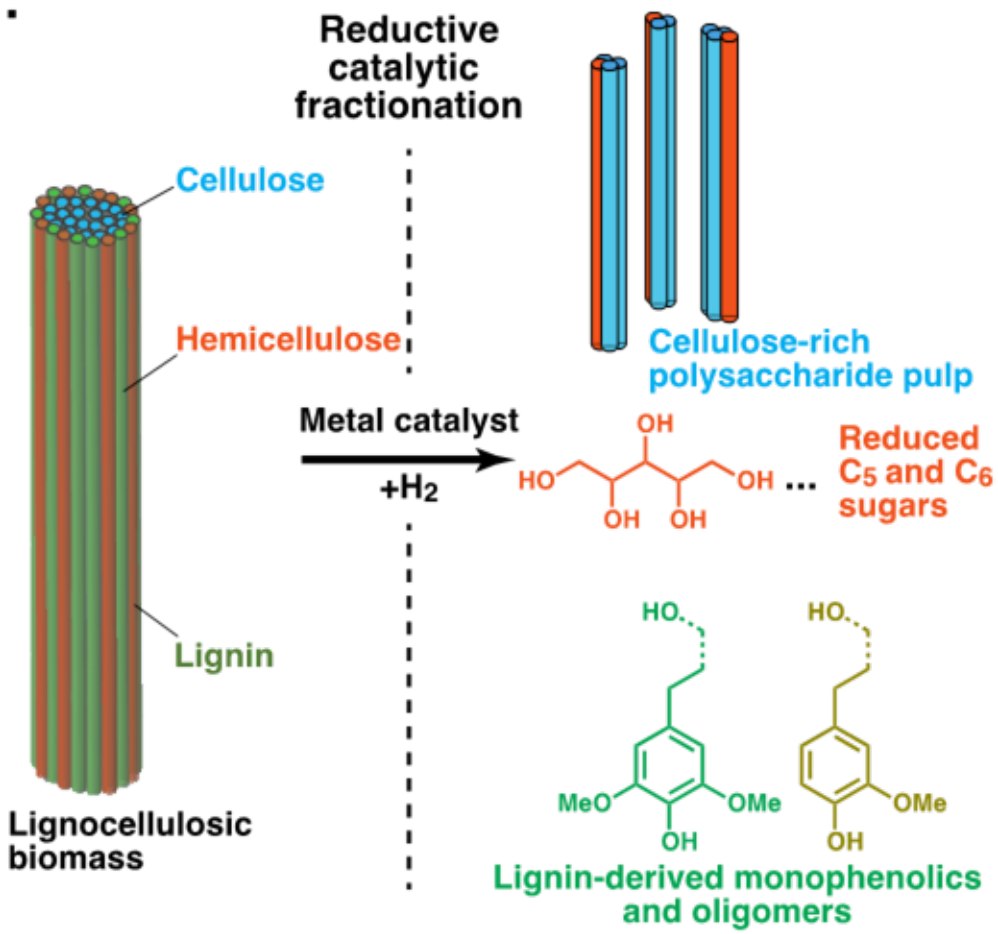


Photo courtesy of NREL

COMMENTS

- Another methodical, systematic, thoughtful, and organized presentation—this project is being well managed and is successfully on track; well done. In principle, the project foundation is perhaps actually novel. The collaboration efforts are substantial (LU, SepCon, Biochem Analysis) and reflect the need for cooperation and the knowledge leveraged to other projects. Similarly, direct industry interactions that inform and help guide the project are a necessary utility. I am very happy to see the addition of a detailed block flow diagram and TEA; thank you. Overall, carbon conversion amplification with RCF will benefit the entire biochemical conversion platform, and once developed could have a broad impact. With RCF, in this particular instance, I do like to see the process focus on edging the product. Again, I see feedstock agnosticism being mentioned, but honestly to what end? How variable (dirt-laden, moisture-ridden) and how many feedstocks have been legitimately evaluated to assert this? I am glad to see membranes mentioned as an energy reduction alternative to distillation, but no CapEx justification or modified MFSP were presented. It is noted that the RCF process is 57% of the total capital and also quite energy-intensive as practiced in the lab. Some predictions or estimations for capital and energy approaching demonstration viability would be helpful to understand. Great project!

- Management of project is good with university collaboration and leveraging with other groups. Risks are identified. Since the inception of this project, the overall approaches are reasonable for a first-of-a-kind

lignin biorefinery. Fine-tuning of the approaches is made possible by having in place good analytics. This is good project for BETO's focus on lignin valorization to offset feedstock cost and enable cost-effective liquid biofuels. Impact will be realized from quantitative recovery of solvent and good fractionation of biomass to upgradeable higher-value products as an alternate to lignin burning. Progress and outcomes from a detailed TEA and LCA for first implementation of the RCF process are helpful in defining better costs and impacts of RCF with solvents used. A continuous system was identified as a best practice. Difficulty with continuous systems is getting timely material balance as compared with a batch reactor. Over time, buildup of byproducts can result in reduced productivity and yield with increased reactor cycle. On the overall, the target of 50% conversion of lignin is hard to achieve with high solids loading and reduced solvents. Particle size can contribute to reduced reactive surface area and impede channeling unless the mixing system is robust, thereby increasing reactor energy requirement. Reactor design, solids loading, mixing, particle size, catalysts used, solvent use, and recycle and solvent type need to be optimal to achieve good yield and conversion. Need additional information on material balance.

- Strengths: This project is possibly the program's most promising current approach for obtaining value from biorefinery lignin. By using RCF and making lignin removal from lignocellulosics the primary goal, the PIs can: (1) generate a lignin with higher reactivity and more opportunity for productive conversion to high-value products; (2) improve selectivity by generating a much lower number of structures than other lignin deconstruction processes; (3) apply the methodology to a wide range of biomass feedstocks, including softwoods, hardwoods, and agricultural residues, offering opportunities beyond the current DMR baseline operating scenario; and (4) still generate a stream of fermentable sugars for fuel production. These features suggest that RCF offers a reasonable alternative approach to the biorefinery front end that stands in contrast to conventional pretreatment. The ability to generate a smaller number of monomeric compounds from the lignin in good yield (~35%) is particularly important. RCF for wood was invented in the 1940s as a method for lignin analysis. In the context of generating chemical products from lignin, these early experiments showed that lignin could be converted into a small number of structures by initial treatment of wood with H_2 and a catalyst. The catalytic expertise available to this project within the larger BETO program offers a good opportunity to improve this process for the biorefinery. With a much smaller number of lignin fragments, the chances for generating high-value products in reasonable yields is increased over other lignin conversion approaches. The close structural similarity between these compounds also suggests that the RCF oil can undergo further structural simplification with relatively straightforward catalytic transformations. The science in this project is impeccable, and also affords a practical solution for industrial stakeholders. If the PIs are able to work through the challenges, this approach has some of the best potential to serve as an economically and commercially viable means to use lignin as a chemical feedstock. The PIs have incorporated a number of efforts to overcome these challenges, including catalyst lifetime improvement, transition to continuous RCF, membrane solvent recovery, and the use of different solvent. While results from this additional work are still limited, the approach is targeting those experiments identified as most critical from TEA/LCA evaluation. The solvent studies are notable, as ethylene glycol gives a simpler product mixture. Overall, the project offers a great scientific concept, an opportunity to significantly change a bioprocessing model that's been in place for years, good initial results, and a cross-disciplinary team possessing all the skills necessary to bring it to fruition.

  Areas for improvement: It would be useful to know the nature of the inter-unit linkages that are more resistant to hydrogenolysis. It would also be interesting to know whether the project is limited to a small number of robust heterogeneous catalysts by economics, and the kind of new reactivity that can be built into these systems.

- The goal of this project is to develop a lignin-first biorefinery for simultaneous biomass fractionation and lignin deconstruction. The core technology is based on reductive catalytic fractionation. The work is strongly guided by TEA/LCA, which is considered a strength. They are also partnering with

ExxonMobil, which demonstrates potential economic viability. Overall, this is a strong project that fits well within the BETO portfolio. The transition to continuous processing is very promising, as is the work on finding better solvents. Another notable deliverable was the publication of standards to lignin biorefining, which will enable more meaningful comparison between different process.

PI RESPONSE TO REVIEWER COMMENTS

- We thank the reviewers overall for the positive and constructive feedback on the Lignin-First Biorefinery Development project. We completely agree with the potential for RCF-based processes to replace the conventional thermochemical pretreatment strategies that focus solely on carbohydrate valorization. This is certainly a potentially promising attribute of the lignin-first approach. In terms of the inter-unit linkages present in the lignin-derived compounds that are resistant to hydrogenolysis—as noted during the Lignin Utilization presentations—these compounds are mostly carbon-carbon-bond-linked dimers and oligomers. Hydrogenolysis as practiced in the RCF process will not be able to cleave these linkages, and accordingly, cleavage of these C–C-linked compounds is a major effort in the Lignin Utilization project to add value to this important fraction of the RCF-derived products. In terms of the catalyst design components and building in new reactivity, this is an excellent comment, and something that we did not discuss during the Peer Review presentation, but which we are actively pursuing. If C–C linkages could also be cleaved by the catalytic system, that could potentially substantially increase the valuable monomer yield from RCF processes. The literature shows a wide range of performance for catalysts outside of the metal/carbon group that depends on lignin source, solvent properties, and support functionality. Clarifying the effects of these parameters is a primary effort in FY 2021 on this project. In terms of mass balance, we fully agree with the reviewer that this is indeed a critical component of process development, and given that, we are focused on achieving good mass balances in the batch, flow-through, and continuous systems for the RCF process that we are developing. For example, we have achieved >90% mass balance in a continuous extruder system to date. We fully agree that particle size is an important factor, and this is why we embarked on a study to examine the impact of particle on solvolysis of lignin from poplar. As presented in that component of the presentation, it is predicted that mass transfer limitations will dominate, unsurprisingly, in biomass particles of industrial-relevant sizes (above 2 mm). Moreover, we fully agree that reactor design, solids loading, mixing, particle size, catalysts, and solvent use and recycle are all critical components, as identified by the TEA and LCA that was presented during Peer Review—this is why we are quite focused on these critical variables in our process development efforts. Our initial results indicate that at 10% solids loading, lignin monomer yields are not impacted through three reaction cycles with the same solvent, indicating that byproduct buildup does not reduce the productivity by impacting the lignin. We are currently working to determine the impact of byproduct buildup on the catalyst performance. In terms of the guidelines for lignin biorefining, we are hopeful that this perspective (published in 2020 in *Energy & Environmental Science*) will be a useful resource for both the research and industrial communities in the scale-up and improvement of RCF processes. In terms of the product portfolio from RCF, this is something that we are working with the Performance-Advantaged Bioproducts projects in the BETO portfolio to define. We agree that this is an important component overall of the RCF process, and this is something that we anticipate focusing on more in out-years, after challenges with the RCF process development are achieved, as noted by the reviewer. In terms of feedstock evaluations, this is something that we are doing for the RCF process in other projects (industrially and BETO-funded) outside of the Lignin-First Biorefinery Development project, and these projects all inform one another. To date, hardwoods, softwoods, agricultural residues, and grasses have been evaluated in the peer-reviewed literature (by us and others). Moisture is not a major challenge for the RCF process, at least as determined to date. The impact of dirt and ash is being investigated now in the Feedstock-Conversion Interface Consortium in the BETO portfolio. In terms of the use of membranes as an alternative to distillation and a mention of the resulting CapEx and MFSP changes, this has been done, but was not presented for the sake of time. We are working with industrial partners now with experience in membrane technologies and the BETO-funded Separations Consortium to ascertain the right type of membrane for fractionating RCF oils. More

broadly, indeed, the way that RCF is commonly practiced in the laboratory now was predicted to be quite expensive and energy-intensive. In terms of predicting capital costs and energy use approaching viability, salient points to these ends were mentioned during the presentation, and these data directly inform the technical directions of the project that were presented in the latter half of the presentation (e.g., continuous operation, higher boiling point solvents, lower solvent loadings).

ALKALINE-OXIDATIVE PRETREATMENT OF WOODY BIOMASS FOR OPTIMAL COPRODUCT

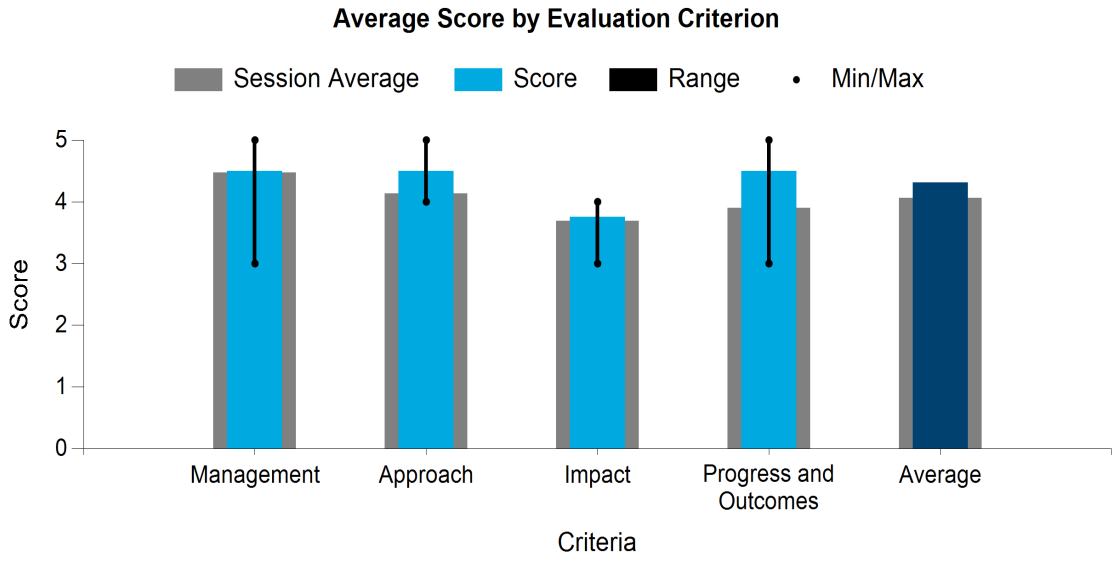
Michigan State University

PROJECT DESCRIPTION

WBS:	2.2.3.601
Presenter(s):	Eric Hegg
Project Start Date:	10/01/2017
Planned Project End Date:	12/31/2021
Total DOE Funding:	$1,800,000

Woody biomass represents a vast source of carbohydrates and aromatics, and it is envisioned as a key feedstock for the sustainable production of biofuels and bioproducts. The goals of this project are to (1) optimize our two-stage alkaline-oxidative deconstruction approach, (2) understand how pretreatment conditions impact lignin properties, (3) employ techno-economic and life cycle analyses to inform the experimental work, and (4) identify strategies to decrease the MFSP.

Our results showed that employing both oxygen (O_2) and hydrogen peroxide (H_2O_2) as co-oxidants during the two-stage catalytic alkaline-oxidative pretreatment process resulted in a substantial improvement relative to using H_2O_2 alone, leading to high sugar yields (>90% glucose and ~100% xylose), even at 2% H_2O_2 loadings (w/w of original biomass). Lignin isolated from our two-stage alkaline-oxidative process was amenable to depolymerization using our newly developed process, resulting in up to 30% aromatic monomer yields. In addition, the lignin is also a promising substrate for polyurethane production. Importantly, the high sugar yields and lignin quality were preserved when we scaled this process from 5 g to 1.2 kg. Combined, the lower processing costs, high sugar yields, and high-quality lignin amenable to both depolymerization and polyurethane applications could potentially reduce the MFSP to as low as $2.71 per gasoline gallon (40% lower than using H_2O_2 only).



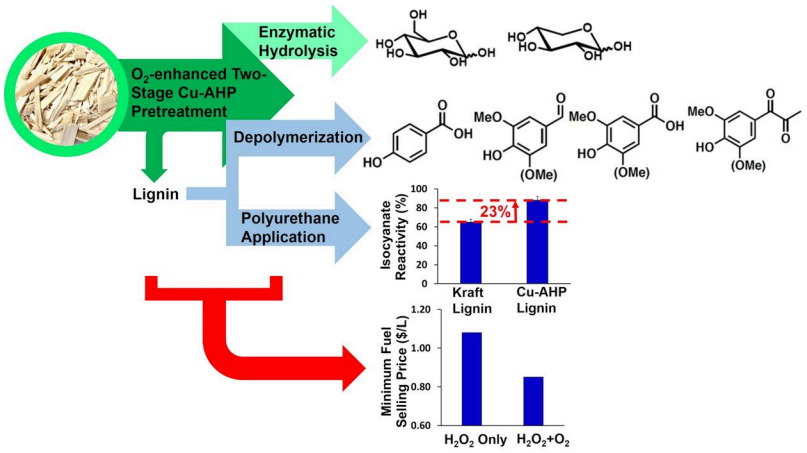


Photo courtesy of Michigan State University

COMMENTS

- Management and tasks are good, taking advantage of several skilled researchers. Risks are identified and addressed. The overall approach seeks to lower chemical inputs while improving yields from lignin conversion to 30% monomers from depolymerized lignin using a two-stage catalytic alkaline-oxidative treatment. The combined approach results in improvement of lignin monomer yield when compared to alkaline pretreatment alone. The project is relevant to BETO's focus on increased value from lignin to offset feedstock costs of lignocellulosics. It also provides an alternate to lignin burning for fuel as currently practiced. TEA indicates that the new approach results in a significant reduction in production costs. LCA has not been performed. Progress and outcome-process has been scaled to 20-liter reactor vessels. This is significant, as it allows for better validation of the technology. Good lignin monomer and sugar yields. LCA is missing and is a critical piece, but the TEA sensitivity analysis confirms that the lower pretreatment chemical inputs, derived increased lignin value, and high sugar yields are key to favorable economics of this process. On the overall, this is a good project with tangibles and deliverables by a good team of experienced researchers.

- Strengths: The PIs have developed a potentially valuable pretreatment able to generate (1) high yields (>90%) of glucose and (2) a reactive lignin fraction (up to 80% of the contained lignin in biomass) that is susceptible to subsequent oxidative depolymerization, affording up to 30% lignin monomer. The remaining lignin offers two additional fractions of differing molecular weight for product applications. The science behind this project is excellent, and the results have been published in high-impact journals. More generally, the concept and approach are interesting, especially with its ability to give separate lignin and sugar streams under quite mild conditions. The current work has completed an in-depth study of reaction conditions for optimization of glucose yield along with the production of reactive lignin and its conversion to monomers. Overall, the project has achieved its goals and milestones, offering an interesting alternative process for supplying glucose for fermentation and a useful amount of lignin monomers for conversion to chemicals. The ability to isolate >90% sugar is notable, and important for BETO's overall operating scenario for the biorefinery. Further, the team has scaled the process to a 1.2-kg scale, demonstrating a first step toward potential industrial validation. The various correlation studies, connecting conditions to lignin properties and yields of extracted or isolable fractions, is particularly valuable and provides considerable insight into the control of the process. A valuable improvement to the process is the incorporation of aerobic oxygen as a co-oxidant, allowing reduction of the amount of HOOH used, and thus also reducing the cost of the process. The copper-catalyzed alkaline hydrogen peroxide lignin is generated, and then treated with more Cu, base, and high-pressure air (~400 psi) to generate a mixture of monomers in about 30% yield.

Areas for improvement: Understanding the impact is complicated by a lack of economic information in the presentation. The primary high-value product from this work is polyurethane for coating applications. The presentation would be strengthened if the assumptions made for the economics for this application were included—for example, a comparison of the production cost of the lignin-based polyurethane to the closest polyurethane analog made industrially. There is an assumption (slide 27) that the lignin monomers as a polyol replacement can be valued at $0.80/kg, but the cost of the comparative polyol is not mentioned. The scenario that induces a customer to buy a mixture (which may vary with feedstock changes) in preference to a pure polyol that is predictable and well understood is not obvious. Similarly, the value of $2.00/kg for monomer production seems high for a mixture of seven materials. There needs to be a better justification for these values. Assessing the impact of this work would be easier with a better definition of the weight percent yield of each stream generated from the process, as well as its composition. It is significant that the process affords access to most of the sugar in the biomass (a strength) and 30% monomers, which is roughly equivalent to the yield of monomers in the NREL RCF project. However, the complexity of this monomer fraction is much higher than the RCF material, which complicates its utility in high-value applications.

- The goal of this process is to develop an economic oxidative-alkaline pretreatment process focused on woody biomass. In addition, the team is using acid-soluble lignin to make polyurethane resins. Overall, the team is making good progress and meeting their milestones. It was somewhat unclear what the advantages of the oxidative-alkaline pretreatment process are relative to other pretreatment methods. Directly addressing this question would help improve the perceived impact of the work. Another concern is the high amount NaOH required. Clearly, this will need to be recycled. The team is also starting to scale up their process with promising results. Lastly, the assumptions regarding the economics of the process were unclear, making some question whether this process will ever be feasible. In summary, the project is going well. The team is also doing good science and achieving the milestones. However, more expansive TEA would be helpful.

- The project appears to be on schedule and well situated for achievement of defined metrics. Milestone completion and task progress are both reported to be on track. Substantial developments have occurred related to tasks 1–5, leading to a reduction in MFSP by ~40% relative to the base case. It was mentioned that the process is tunable, which understandably changes operating expenses. I think this is a great technical facet, and I congratulate the team on that as it is useful to have levers to pull when market conditions change. If possible, a simplified mass and energy balance should be available allowing reviewers to technically assess the project more thoroughly. The simplified block flow is quite handy; however, this is a complicated process that could benefit from more detail. Process complexity at scale can be difficult to conceptualize and predict, but some discussion, thought, or framework related to this could be insightful. The sheer number of unit operations within the process looks to be staggering: two-stage pretreatment, enzymatic hydrolysis, four intermediate groups requiring additional processing/conversion, and five discrete final product groups with unique storage and loadout. I would like to better understand the proposed total capital investment per annual gallon gasoline equivalent.

PI RESPONSE TO REVIEWER COMMENTS

- It is challenging to fit all the assumptions used in economic modeling into a single 20-minute presentation. However, the reviewer is correct in noting that the assumption on selling price of lignin is critical for the overall economics. The assumption of $0.80/kg is estimated to be approximately one-half the selling price of polyol. The selling price of polyol was confirmed to be around $1.60/kg from multiple sources, including literature, Alibaba.com, and market surveys. The lower value (50% of the value) takes into account the cost of isolating the lignin and the lower value compared to pure polyol. However, as noted in the presentation, we have already demonstrated that the recovered crude lignin has value as a polyol substitute. Regarding the monomers, the value of $2.00/kg is more ambitious. We considered three possibilities: 4-hydroxybenzoic acid, vanillin, and syringic acid, which accounted for five monomers (we assumed the aldehyde and carboxylic acid forms of vanillin and syringic acid could

be interchangeable). The market research suggested potential selling prices of 4-hydroxybenzoic acid and vanillin of approximately $5/kg and $9/kg, respectively. Syringic acid did not have a developed market, but there are several potential high-value applications, suggesting its value could be equal to that of vanillin. As more processing is required to obtain the monomers than the crude lignin, a value ~25% of the selling price of the monomers was used. This was approximated as $2/kg, although the individual monomers could be split into individual prices for a more detailed analysis.

Because the focus of the BETO project was to reduce the cost of the biofuel, the modeling section of the presentation focused on the TEA in the interest of time. However, the LCA has been performed. We estimate that the process produces 11.9 kg CO_2/gal gasoline equivalent biofuel compared to 13.4 kg/CO_2 at our initial state prior to the start of the project. While an improvement was made, we acknowledge that the intermediate LCA value is still quite high. It should be noted that a significant reason for this is the model used for the process. The scope of the project only considers the pretreatment and subsequent sugar (and lignin) yields, not any further upgrading to biofuel. To model the overall process, therefore, we chose to adapt the NREL 2015 Davis et al. economic model to our pretreatment. This model uses purchased, nonrenewable H_2 to produce the final drop-in fuel. In our model, nearly 50% of the CO_2 emissions can be attributed to the purchased H_2. It is entirely possible to use a different model and reduce these emissions, although this would also impact the minimum fuel selling price. If renewable H_2 is used, the CO_2 emissions are reduced to approximately 6 kg/gal. The other major portion of the CO_2 emissions is the sodium hydroxide usage. We investigated various options to recycle the NaOH, but these options had approximately the same carbon footprint as the purchased hydroxide. Thus, while it is undoubtedly preferable from an overall waste perspective, this approach will not improve the CO_2 emissions, and thus the main alternative is to reduce NaOH loading.

We agree that a high amount of NaOH is used. As stated above, this has a significant impact in the LCA, although surprisingly not as large of an impact in the TEA due to the relatively low price of NaOH. Fortunately, there are several known commercial processes to recycle NaOH, such as the Solvay process or electrolysis. While not explicitly taken into account for the TEA, one of these options would likely be used.

Due to multiple streams containing valuable sugar or lignin, it can be very confusing when discussing yields with this project. To assist in this endeavor, we provide some key processing data. (Note: all values are expressed as the percentage of initial component from the raw, untreated poplar. The initial poplar contained 45.5% glucan, 15.8% xylan, and 22.3% lignin.) During alkaline pre-extraction, the solubilized individual component of glucose, xylose, and lignin were 6.2%, 35.2%, and 25.7%, respectively. The precipitated lignin from the alkaline pre-extraction liquor was 20.3%. During copper-catalyzed alkaline hydrogen peroxide pretreatment, the solubilized individual component of glucose, xylose, and lignin were 9.6%, 25.8%, and 49.9%, respectively, while the precipitated lignin was 16.0%. After enzymatic hydrolysis of the two-stage pretreated solid poplar, the yields of glucose and xylose were 81.1% and 40.7%, respectively. For example, the untreated poplar contained 15.8% xylan. The 40.7% yield of xylan in hydrolysis is 40.7% of the 15.8%, or 6.43 g xylan converted to soluble sugar per 100 g dry initial poplar. Also, note that the precipitated lignin is also as a percentage of the initial component, and the soluble lignin yield is as a percent of the initial lignin prior to precipitation. For example, 79% of the soluble lignin precipitated in the pre-extraction step (20.3% yield of precipitated lignin divided by 25.7% yield of soluble lignin). Thus, the total precipitated lignin was 20.3% for alkaline pre-extraction liquid stream plus 16.0% for the copper-catalyzed alkaline hydrogen peroxide pretreatment liquid stream, which was 36.3% of the initial lignin (22.3%), or 8.09 g precipitated lignin per 100 g dry initial poplar. Note that because the xylose recovered is slightly higher than 100% due to several different measurements, a value of approximately 99.5% was used in the TEA. In the future we will try to use a Sankey diagram during the presentation to make the mass balance easier to follow.

The total capital investment per annual GGE is $6.45/gal. Note that this capital cost only includes the cost of obtaining crude lignin; it does not take into account upgrading to polyols or monomers. For a 2,000-tonne/day facility, the major capital costs related to this project are pretreatment ($31.7 million), oxygen production ($9.6 million), and pretreatment liquor treatment ($62.9 million). The last item is key for this process, as the lignin must be precipitated via pH adjustment and the sugars must be concentrated to be used for biofuel production.

UPGRADING LIGNIN-CONTAINING BIOREFINERY RESIDUES FOR BIOPLASTICS

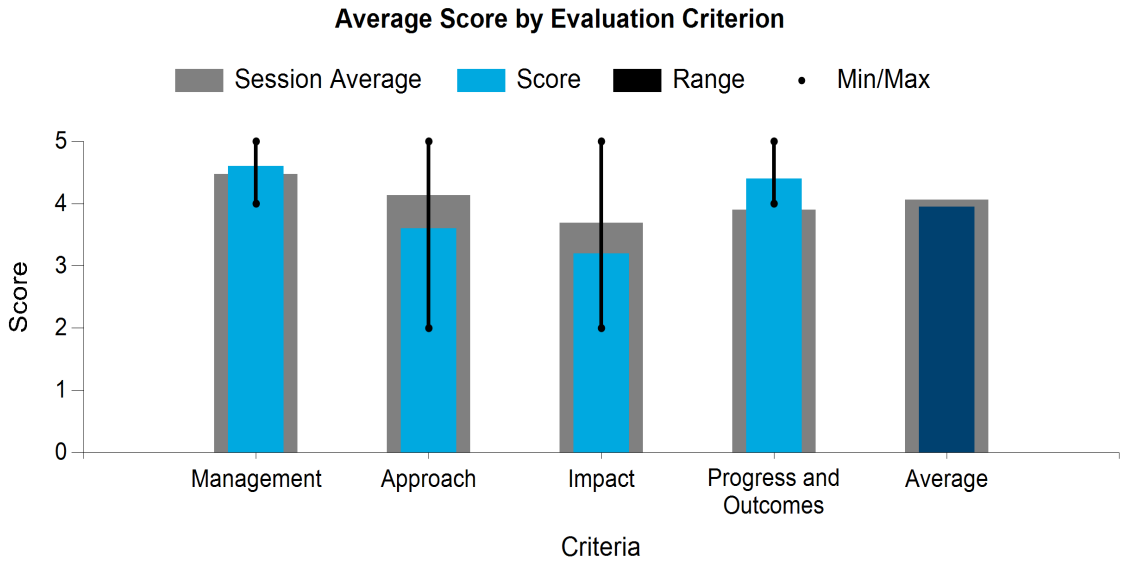
Texas A&M

PROJECT DESCRIPTION

WBS:	2.3.1.206
Presenter(s):	Joshua Yuan
Project Start Date:	04/15/2016
Planned Project End Date:	06/30/2021
Total DOE Funding:	$2,499,993

The proposed research integrated the latest advances from a multidisciplinary academic-industrial coalition to address one of the most challenging issues in lignocellulosic biofuel production: the use of lignin-containing biorefinery residues for cost-effective bioproduct production. The success of a modern biorefinery heavily depends on the diverse product streams and the utilization of all fractions of input material. Even though extensive progress has been made to process cellulose and hemicellulose into advanced biofuels, the utilization of lignin for fungible biofuels and bioproducts has not been achieved. Lignin is much more under-researched as compared to cellulose, which has become a setback for the efficiency, cost-effectiveness, and sustainability of lignocellulosic biorefinery. Essentially all current bioconversion platforms lead to a lignin-containing waste stream that needs to be further processed into valuable products. Although a certain amount of lignin (~30%–40%) is needed for the thermal requirements of biofuel production, a modern cellulosic processing plant will have ~60% excess lignin. The utilization of this excess lignin-containing residue as feedstock for renewable fuels and chemicals offers a significant opportunity to enhance the operational efficiency, lower the overall biofuel cost, reduce the net carbon footprint, and improve the replacement of petroleum. The proposed research will translate the latest technology breakthroughs into a transformative lignin-to-polyhydroxyalkanoates (PHAs) route.
This project uniquely addresses the mission and goals of BETO through process development and optimization of a single-unit operation for the upgrading of chemically or biologically derived intermediates to fuels and products with the following three objectives: (1) process enablement by engineering and optimizing microorganisms to convert biorefinery waste streams to PHA for bioplastics; (2) process development by characterizing biorefinery residues, optimizing lignin treatment and fermentation, and designing the novel bioprocess; and (3) process integration and optimization by biorefinery on-site scale-up and techno-economic and life cycle analysis for the lignin-to-PHA upgrading process. The project has an integrated, rigorous milestone-based management plan with broad support from industry.

To date, the project has met all milestones at the 1-L level, with PHA production titer >10 g/L, conversion efficiency >40%, and price <$2.5/kg. The performance has the potential to lower ethanol price to ~$2.4/GGE. Scientifically, the project has led to 44 publications with a cumulative impact factor of 270. Besides the impact on biorefinery, the process will also address the plastics crisis and maximize replacement of petroleum products, as bioplastics have a rapidly increasing market and represents a sustainable way to replace nondegradable petroleum-based plastics. This novel platform complements the current technologies supported by BETO to enable the utilization of all structural components of cell wall for better cost-effectiveness, overall carbon efficiency, and sustainability for biofuels. The proposed initiative is well supported by the biorefinery industry in multiple ways.



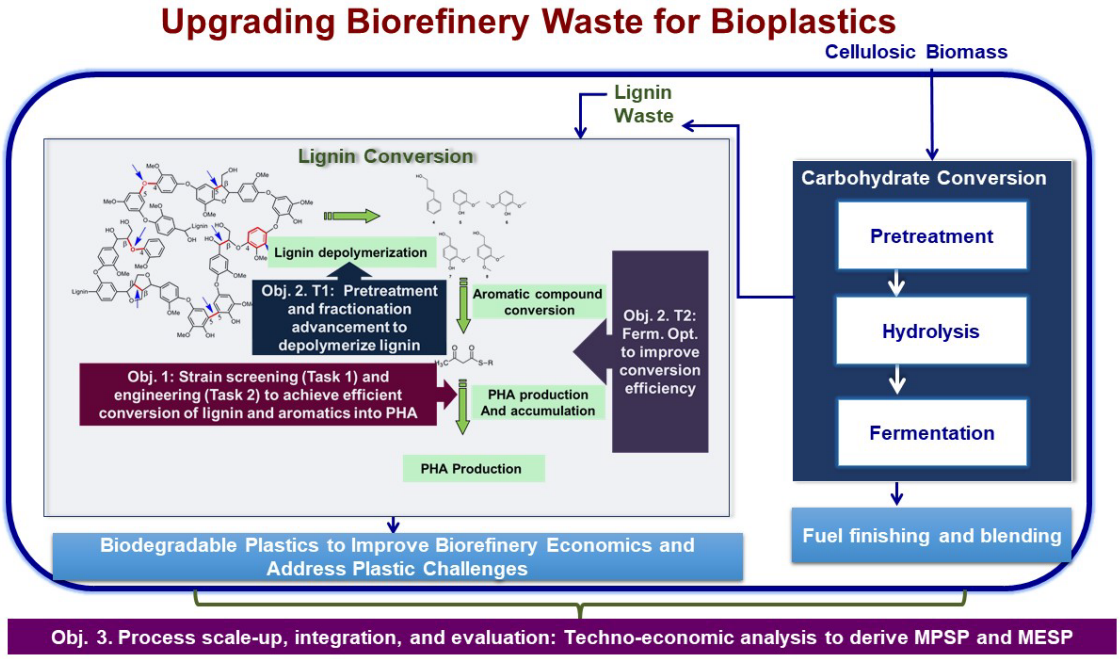


Photo courtesy of Texas A&M

COMMENTS

- Management of project and partnership looks adequate. Risks are provided and addressed. Approaches taken involve microbial engineering, improving process for fractionation of biomass fermentation improvement and deployment of TEA. The project is within BETO's mission to enable a viable biorefinery by capturing more value from lignin. The potential impact of producing PHA by engineered strains looks promising, but I'm not sure that an aerobic process can be cost-effective. Cost-effective recovery of intracellular PHA cannot be scaled up commercially for commodity-type products, so robust TEA and LCA are needed. Progress and outcomes follow targets set, and the use of Aspen Plus and preliminary TEA have helped guide the overall strategy. My overall impression is that strain and

fermentation development were good, but data on recovery of PHA and testing of fermentation-derived material post extraction in application work are missing. This is an area the team can benefit from addressing before the project ends. While the team should be congratulated on their efforts, PHA may not be a good target given that commercial production and costs of PHA have faced market resistance. ADM's experience with the production of PHA from starch-derived glucose illustrates these challenges well.

- Strengths: This is another effort within the BETO portfolio that attempts to convert biorefinery waste streams in the biorefinery into value. The PI presents a clear goal of converting biorefinery waste to bioplastics at less than $5/kg, or about $2.50/lb. The PIs have made progress in each of the three focus areas of the project, including the development of new engineered organisms and an evaluation of the effect of several pretreatment scenarios on PHA production. Each approach was subjected to TEA, and the comparative results are shown on slide 18. The PIs present clear and well-laid-out TEA evaluations for the various processes in their program (e.g., slide 13 presents the results of economic analysis for several different scenarios). A real strength is the inclusion of production costs in the form of a unit cost for a given operation. The only improvement to the slide would be a column with current costs for conventional PHA production, and the assumptions behind the numbers. What is the yield from lignin? How much of the lignin is converted?

  Areas for improvement: The target polymers in this project are PHAs, which is surprising because of the known issues that PHAs have with cost and performance—issues that have stymied their acceptance for years. The presentation would be strengthened with a clearer justification of this material as the target. The description of the approach would be improved if there was more information on the exact biorefinery waste streams being targeted, their concentration, their composition, etc. This is information critical to determining whether waste stream conversion makes sense. Some of the assumptions behind the TEA are not well defined. Information on current costs for conventional PHA production and the assumptions behind the numbers, such as the required yield from lignin and the amount of lignin converted, would be valuable.

- The goal of this project is to convert lignin into PHA, a bioplastic, using *Pseudomonas putida*. Overall, the talk was very confusing and difficult to follow. The pace was too fast and the slides too dense. It would greatly help the review process if concepts were carefully explained. Otherwise, it is difficult to evaluate the technical component of the work. Overall, the team is making good progress and exceeding their milestones. The project also fits well in the BETO portfolio. However, PHA is not a very compelling product. Also, the cost estimates are questionable. It would greatly help if more details regarding the assumptions in the TEA were provided.

- This is an interesting project that uses an integrated approach to evaluate the viability of converting lignin into medium-chain-length PHAs. TEA and Aspen modeling shows that the selling price of the produced PHA could bring down the MFSP in a lignocellulosic biorefinery to below $3 per gallon of gasoline equivalent. The project has met all the milestones at the 1-liter scale. Current efforts are toward scaling up to the 50-liter scale. At such scale, the project would produce sufficient PHA materials to potentially explore extraction and purification of the PHA and its usability in the production of biodegradable plastics. Such studies could help refine the TEA to provide better estimates of the PHA production cost and marketability, which would ultimately determine the possible selling price for the product.

- This project has assembled a really nice team consisting of industry and academia. Unfortunately, due to the project metrics being developed in 2016, the goals have not withstood the test of time. Despite appropriately assessed PHA cost targets, the PHA titers and conversion rates, although achieved, are not commercially viable, as alternative technologies have far surpassed this pathway. Completion of all milestone is a great achievement, and I congratulate the team on that success!

PI RESPONSE TO REVIEWER COMMENTS

- We appreciate the reviewers' acknowledgment of our progress and accomplishment of milestones, along with congratulations on the success. We hereby address all the questions.

  Reviewer 1: Reviewer 1 is "not sure that an aerobic process can be cost-effective." The reviewer's comment is a very fair one, as most of the current biofuel platforms are anaerobic. Aerobic platforms for sugar to fatty acid or terpene (hydrocarbon) faces scrutiny for carbon efficiency, and ultimately cost-effectiveness. However, we need to consider the product and pathway efficiency in evaluating the cost-effectiveness. The product for this project is not fuel, but a higher-value bioplastics product PHA, which has a current market price about 10 times higher than fuel price. Even though we are targeting to produce PHA at a lower price, it is still going to be a much higher price than fuel. Second, the most common substrate for industrial PHA production is a fatty acid or organic acid. The carbon-to-carbon conversion efficiency for organic acid or fatty acid to PHA can be as high as 80%, which is highly consistent with our data from lignin. In fact, from a chemistry perspective, aromatic compounds turn into organic acids after ring opening. Overall, the lignin-to-PHA conversion will have a much higher carbon-to-carbon conversion efficiency than sugar to fuel under aerobic conditions. Considering the relatively higher price and conversion efficiency, the concerns over cost-effectiveness for aerobic conversion in conventional biofuel production do not apply to lignin-to-PHA route.

  "Cost-effective recovery of intracellular PHA cannot be scaled up commercially for commodity-type products, so robust TEA and LCA are needed. … My overall impression is that strain and fermentation development were good, but data on recovery of PHA and testing of fermentation-derived material post extraction in application work are missing." We fully agree with the reviewer's comment that the integration PHA recovery cost is needed to fully evaluate a scaled-up platform. However, such effort is beyond the scope of work as defined by the statement of project objectives and contract. The project is ending with limited funding, focusing on scale-up to 50 liters. In fact, we have been working with an industrial collaborator with proprietary PHA recovery and separation technologies to submit proposals for scaling up the current technology with an entire supply chain to better evaluate the TEA, LCA, and market impact. We certainly agree with the reviewer that integrating with PHA recovery is an important part of such analysis, and hope to get a chance to carry out a more comprehensive scale-up to enable commercialization if we were given such an opportunity.

  "PHA may not be a good target given that commercial production and costs of PHA have faced market resistance. ADM's experience with the production of PHA from starch-derived glucose illustrates these challenges well." First of all, glucose or sucrose might not be a good substrate for PHA production, in particular for *P. putida*-based platforms, due to the inefficient pathways and low conversion rate. Fatty acids and organic acids are common substrates for industrial PHA production. Our previous study highlighted that lignin conversion to PHA also goes through the fatty acid beta-oxidation pathway. The project highlighted that channeling carbon from fatty acid beta-oxidation to PHA biosynthesis significantly improves the lignin-to-PHA conversion efficiency. Therefore, the failure of a glucose-based platform does not necessarily inform the PHA marketability. In particular, the United States is currently lagging significantly behind Asian and European countries in PHA market share. The United States is estimated to manufacture about 18% PHA products, while East Asian countries capture 44% and Europe captures 25%. That being said, the current market resistance in the United States does not reflect the global market potential, but rather that our industrial technology is lagging significantly behind other parts of the world. Further scale-up and supply chain integration of this project will allow us to catch up.

  Reviewer 2: "The talk was very confusing and difficult to follow. The pace was too fast and the slides too dense. It would greatly help the review process if concepts were carefully explained. Otherwise, it is difficult to evaluate the technical component of the work." We apologize for being fast, as the project renders 44 publications and two patents, leapfrogging the technology and the state of the art. We have chosen only to present part of the progress, yet the time is really too short to cover the breadth of the

activities. We also presented fast to leave enough time for questions and answers, yet only one question was raised during the session. We have included significant details in TEA in the additional slides, and they were never given the opportunity to be presented, as no question was raised about the TEA.

"PHA is not a very compelling product. Also, the cost estimates are questionable. It would greatly help if more details regarding the assumptions in the TEA were provided." Again, the United States is lagging significantly behind Europe and Asia on PHA manufacturing and market share. However, this does not mean that PHA is not a product with market potential. In fact, the global bioplastics market was valued at $10 billion in 2020 and is projected at $28 billion by 2025. The rapid growth of the entire bioplastics market is partially driven by the rising demand for PHA for packaging, food services, biomedical, agriculture, and other applications. PHA is not only compostable but also biodegradable. It represents one of the tangible near-term solutions for plastics challenges. We indeed prepared a lot of details for TEA assumptions and models in the attachment slides but were never able to present them as only one question was raised after the presentation.

Reviewer 3: "The PHA titers and conversion rates, although achieved, are not commercially viable, as alternative technologies have far surpassed this pathway." The reviewer is right in that bioplastics, in particular PHA manufacturing technology, is rapidly growing. The current consensus on market value is $5/kg. Asian companies are claiming a lower manufacturing price, yet such a lower price was never substantiated. As compared to the current market value, our PHA production price from lignin is estimated to be around $2/kg, a significant advancement over the current state of the art.

Reviewer 4: "At such scale, the project would produce sufficient PHA materials to potentially explore extraction and purification of the PHA and its usability in the production of biodegradable plastics. Such studies could help refine the TEA to provide better estimates of the PHA production cost and marketability, which would ultimately determine the possible selling price for the product." We agree with the reviewer that the entire supply chain, including PHA extraction, needs to be evaluated in estimating TEA. Such an effort is beyond the current scope as defined by the statement of project objectives, and no funding is available for this effort. As aforementioned, we are making efforts to obtain more funding to achieve the reviewer's suggested goal.

Reviewer 5: "The only improvement to the slide would be a column with current costs for conventional PHA production, and the assumptions behind the numbers. What is the yield from lignin? How much of the lignin is converted?" We appreciate the reviewer's question. The current price for PHA production is $5/kg. The yield of lignin to PHA is between 40% and 75% depending on the strain and substrate used. The utilization of lignin depends on the pretreatment and dissolution technology. About 20%–30% of total lignin can be used.

"The target polymers in this project are PHAs, which is surprising because of the known issues that PHAs have with cost and performance…issues that have stymied their acceptance for years." Please find the previous discussion regarding the rapid increase of the PHA market and the position of the United States in this market. The project has reduced the production cost. We do agree with the reviewer that the performance of PHA-based plastics is an issue. We have developed technologies beyond this project scope to improve the performance to the range that can be used to manufacture packaging material. We are proposing to scale up the technologies with our commercial partner.

"The description of the approach would be improved if there was more information on the exact biorefinery waste streams being targeted, their concentration, their composition, etc. This is information critical to determining whether waste stream conversion makes sense. Some of the assumptions behind the TEA are not well defined. Information on current costs for conventional PHA production and the assumptions behind the numbers, such as the required yield from lignin and the amount of lignin converted, would be valuable." We actually prepared a complete Aspen model. The model has contained

the waste stream and relevant information. The detailed information was attached in the supplementary slides, but no questions were raised in these details and we never got a chance to present them.

LIGNIN FRACTIONATION AND VALORIZATION: FOCUSING ON BOTH VALUE AND QUALITY

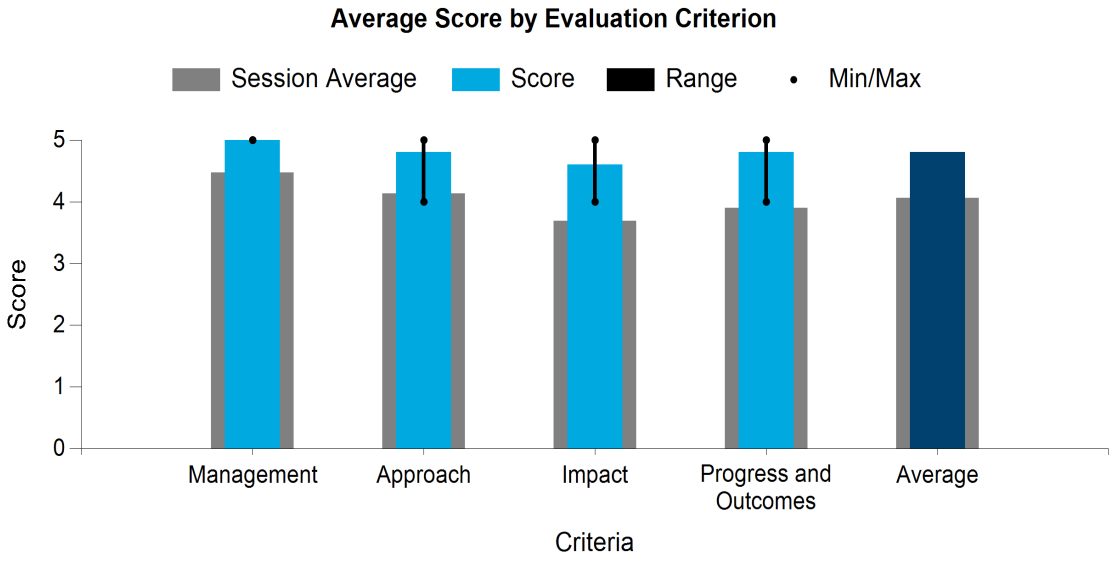
Clemson University

PROJECT DESCRIPTION

WBS:	2.3.1.211
Presenter(s):	Mark Thies
Project Start Date:	10/01/2018
Planned Project End Date:	03/31/2022
Total DOE Funding:	$1,795,216

By exploiting the novel liquid–liquid equilibrium that exists between lignin and hot, one-phase solutions of aqueous renewable solvents, crude bulk lignins can be simultaneously fractionated, cleaned, and solvated for conversion to high-value, high-quality bioproducts. This unusual phase behavior forms the basis for a fractionation process that we refer to as Aqueous Lignin Purification with Hot Agents (ALPHA). With this recently patented technology, control of both the purity and molecular weight of lignin becomes possible. Furthermore, the technology has also been developed for continuous operation, so it can be commercially scaled. Finally, ALPHA has the added advantage of using renewable solvents that are produced within the biorefinery, including aqueous solutions of acetic acid or ethanol.

Lignin is like any other polymer in that the molecular weight can have a dramatic impact on its suitability for a given application. Furthermore, polymer purity can be an equally important factor if the desired materials properties are to be achieved. Three large and growing markets have been identified for ALPHA lignin use: (1) high-performance carbon fibers for automotive applications, (2) rigid polyurethane foams for spray insulation for buildings, and (3) activated carbon for food and pharmaceutical use. For such higher-value applications, we hypothesize that today's commercially available bulk lignins have too broad a molecular weight distribution and are too low in purity to give acceptable performance.



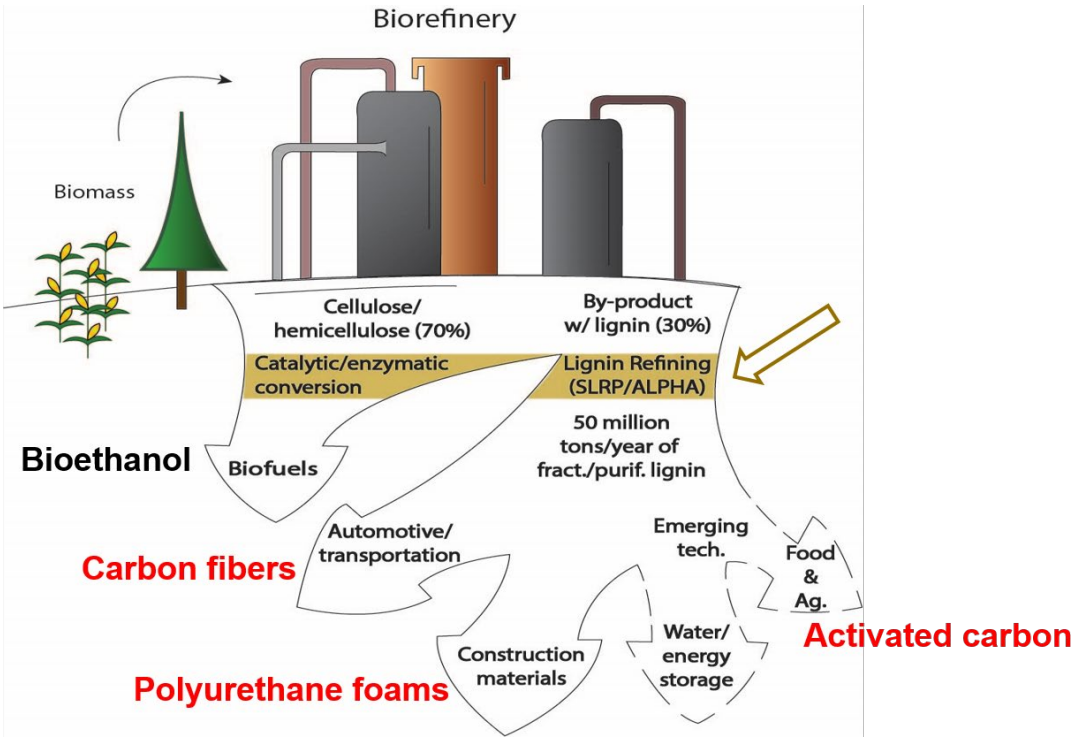


Photo courtesy of Clemson University

COMMENTS

- This is a good, diverse academic team with well-defined areas of expertise and roles. Risks and risk mitigation strategy are outlined. The team combines several approaches for the pretreatment and fractionation of lignin derived from poplar and/or corn stover. These approaches are consistent with BETO's focus on enabling the processing of woody biomass and crop residues to liquid fuels and other products that can be derived from the lignin fraction. This project can have a significant impact on valorization of lignin-derived products provided that cost of production and yields are confirmed by TEA. Markets for the target products exist already for polyurethane foam, activated carbon, and carbon fibers. Clean fraction of lignin using the ALPHA process makes a compelling argument and has potential commercial value. Good progress has been made so far with the recovery and fractionation of lignin from ALPHA and byproduct from black liquor residue. Looking over the biorefinery structure with products and coproducts and the deployment of TEA and LCA would be useful in assessing commercial viability and environmental footprint for the proposed biorefinery. My overall impression is that the approaches taken by the team and deliverables warrant more close engagement with paper pulp wood processors that maybe interested in value addition and alternates to burning lignin provided. LCA/TEA can demonstrate economic and environmental benefits over the current widely used kraft process. On the plus side, markets already exist for the products they are making from lignin fractions, which should enable commercial development in short term to midterm. The reviewer believes that this project is a better fit for a first-generation lignin biorefinery.

- Strengths: The PIs are pursuing a project with a high level of practicality and pragmatism, using new technology for lignin isolation from kraft black liquor. Interestingly, this approach could be considered "lignin last," but interesting results have been obtained. This project fractionates lignin with a hot aqueous EtOH wash to make low-, medium-, and high-molecular-weight lignin fractions for products that are not exotic (carbon fibers, polyurethane, activated carbon), but which could have a known place in the chemical market, and importantly, have the chance of providing value to the biorefinery in the short term. As such, the work could serve as a bridge while challenges with more sophisticated

technology are worked out. This project is complementary to efforts tackling the barrier of converting lignin to low-molecular-weight products in high yield and selectivity. The impact of this project is good. By choosing carbon fiber, polyurethane foams, and activated carbon as products, the need to generate, isolate, and convert individual low-molecular-weight monomers is not necessary. Instead, these final products need only compete on reproducibility and performance. The impact of the process is also enhanced by its simplicity. ALPHA treats bulk lignin with hot aqueous solvent mixtures. The resulting material undergoes a phase separation, fractionating the lignin by molecular weight. The PIs have made some impressive demonstrations showing that their product targets can be made from the different ALPHA fractions. For example (slide 13), lignin was spun from solution and carbonized to fibers. Slide 14 shows that the team is able to make polyurethane foams from their lignin by taking advantage of the higher OH content in this lower-molecular-weight fraction, which makes them usable as the polyol component of polyurethane with isocyanates. It looks like anywhere from 30%–60% of the polyol can be replaced by this lignin fraction. The PIs have also done a good job of describing the use of one of their lignin fractions for activated carbon production. Overall, this is a nicely practical project and a good addition to the BETO portfolio. The PIs have directly addressed the BETO goal of deriving value from lignin in the form of coproducts, and have a straightforward process for generating starting lignin streams that are applicable to a number of different higher-value (if not high-value) compounds.

Areas for improvement: The presentation would be improved by information describing the distribution of lignin among the three phases, as that will have an impact on process economics. Further, the use of sequential liquid-lignin recovery and purification to recover lignin fractions suggests that kraft black liquor could also be used directly. Information on the use of ALPHA with kraft lignin (Indulin?) would be useful. It would help if the PIs included more information on how the measured properties of lignin-based carbon fibers compare with polyacrylonitrile fibers. Greater clarity in the TEA would be helpful. For example, on slide 24, ALPHA lignin is estimated to cost $0.21/lb, but it is not clear if this is a production or sales cost. Further, the relative production cost of each of the three individual lignin fractions isn't given.

- The goal of this project is to produce carbon fiber, polyurethane foams, and activated carbon from lignin. The core technology involves a process that separates lignin based on its molecular weight. Different fractions are then targeted to specific products. Overall, the project is making excellent progress and has a compelling vision. It fits well within the BETO portfolio. The team is also strong with diverse and complementary expertise. No concerns were noted.

- This project offers a simple, elegant, and efficient alternative to gaining value from lignin in the context of the lignocellulosic biorefinery. The goal is to fractionate the lignin into three products based on molecular weight using a patented process for separation of the lignin components. The low-molecular-weight fraction is used for production of polyurethane foams, the high-molecular-weight fraction is used for making carbon fibers, and the medium-molecular-weight fraction is used for producing activated carbon. The simplicity of separations results in favorable TEA, with low production costs for the different proposed products. The team has made progress characterizing the properties of the different products. It would be interesting to see economic predictions from integrating the outputs of this project into a lignocellulosic biorefinery model, such as the ones produced by the Biochemical Platform Analysis project for production of 2,3-BDO. Would this alternative use of lignin allow reaching the 2030 goal for the MFSP of $2.5/GGE?

- This was a great presentation, and I very much enjoyed the content and project concept. This group, while appearing to be primarily academic, is keeping a strong grasp on the industrial pulse and the challenges of scale and commercial markets. The range of feedstock substrates and the variable qualities of the substrates have led the team to develop a robust system. Utilization of model substrates and defined media from bench to large scale encourages identifying major problems at the highest possible costs. This has been avoided by the team's thoughtful and comprehensive approach. I infer CapEx to be substantial due to system complexity. A brief indication of the envisioned or modeled capital would be helpful to see. Great project, well done!

SELECTIVE PROCESS FOR EFFICIENT REMOVAL OF LIGNIN AND UPGRADING (SPERLU)

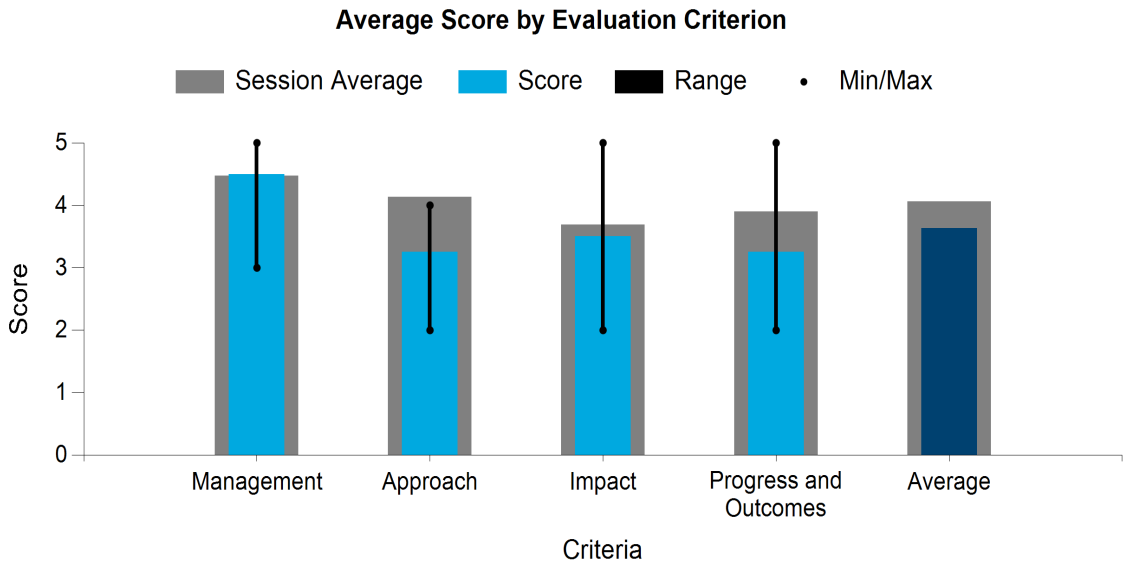
Spero Renewables

PROJECT DESCRIPTION

WBS:	2.3.1.212
Presenter(s):	Ian Klein
Project Start Date:	10/01/2018
Planned Project End Date:	09/30/2022
Total DOE Funding:	$1,613,457

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 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 to produce less than 10 bio-phenol products in a liquid stream. The SPERLU process has been demonstrated and vetted on a lab scale to convert intact lignin from lignocellulosic biomass in excellent yields of 50% (based on lignin) to bio-phenols while coproducing delignified solid carbohydrates. The phenolic products can be separated and further upgraded through catalytic or biological means.

Spero has perform a detailed kinetic study of the SPERLU process and used the resulting kinetic data to design and construct a mini-pilot-scale reactor, which will be used for production of large amounts of phenolic products for biological and catalytic upgrading. Collaboration with NREL is being used to further upgrade the SPERLU products through biological means into valuable chemicals. Spero will also investigate the use of the lignin-derived bio-phenols as replacements for bisphenol A in thermoset synthesis. A comprehensive life cycle analysis of the SPERLU process and monomer upgrading is underway. At the conclusion of this project, Spero expects to have sufficient fundamental data on the SPERLU process and biological monomer upgrading to be ready for large-scale pilot testing in preparation for a commercial launch. It is anticipated that commercialization of the SPERLU process would lead to significant increases in the profitability of the emerging biorefinery industry through valorization of lignin.



SPERLU™ Project Overview

Project Goals:

- Convert 50% of lignin carbon into bio-phenols.
- Make polymers from lignin bio-phenols.
- Bioprocess lignin bio-phenols into chemicals.
- Establish technoeconomic analysis (TEA) and life cycle analysis (LCA) for SPERLU™.

Photo courtesy of Spero Energy, Inc.

COMMENTS

- Management and collaborations are in place. It is not clear what the risk mitigation plan is. Approaches taken to produce bio-phenol with good yield, as well as the bioconversion of bio-phenol to chemicals/polymers derived, is technically feasible but so far not commercially viable. TEA and LCA work is being carried out in parallel. On the overall, the project is consistent with BETO's support to enable a lignin biomass biorefinery. There is significant impact from valorization of lignin fraction that can enable cellulosic ethanol production with better economics than burning lignin, as has been past/current practice in kraft pulping. Good progress has been indicated in this project, with current focus on pilot plant scale to produce 200 g of bio-phenol. My overall impression is this is a well-integrated project that will enable valorization of lignin. TEA and LCA will help in addressing economic and environmental viability of SPERLU. Based on current yields and cost of raw material (>$100/ton), it is hard to see how bio-phenol can be cost-competitive with existing processes. There are no details provided on the SPERLU process, and that would be needed to confirm technical feasibility. A complete material balance needs to be provided that accounts for the different fractions.

- Project interactions and collaborations appear to be strong and cross-functional. Industry engagement, catalyst suppliers, separations companies, and cellulosic ethanol developer utilization has better positioned this project for a successful outcome. The TEA industrial relevance justification is logical, although 3-hour reactor retention times at the 2-kg/day scale does not, however, appear commercially relevant. The project approach and milestones appear coherent and logical; however, the project is mid-stride in the development cycle and makes the review task slightly challenging. Again, I see feedstock agnosticism being mentioned and promoted, but honestly to what end? How variable (dirt-laden, moisture-ridden) and how many feedstocks have been legitimately evaluated to assert this notion? The presentation cites only one hardwood species. A product push based on estimated target market pricing rather than production costs is, I suppose, possible, although the notion seems risky. In the end, with limited information being presented, to complete a comprehensive review assessment was difficult.

- Strengths: Spero's project is closely related to the broader RCF work being carried out at NREL, and as such, is a potentially important alternative to conventional pretreatment technology. By going after the

lignin first, a more reactive and tractable feedstock is obtained as a smaller number of low-molecular-weight monomers is available for conversion to high-value products. Advantages of the approach include the ability to use a wide range of biomass feedstocks (i.e., success does not depend on DMR pretreatment). The project is targeting, among other things, the production of lignin-based bisphenol A replacements via conversion of a targeted 200 g/day bio-phenols, which is a large volume product of the chemical industry.

Areas for improvement: The potential impact of this approach could be substantial, but it is almost impossible to gauge from the very minimal presentation given. There is a lot of discussion about potential advantages and a description of the "what," but no "how" or "how much." This is a serious omission. Examples: (1) no information about catalyst or reaction conditions is given; (2) Spero projects a $1–$2 reduction in GGE cost with this technology, but the basis for this estimate is missing (i.e., a credible production cost needs to be assigned to the final products for this assessment); (3) the post-presentation discussion claims that 50% of lignin leads to a $1–$2 reduction in GGE, but that has to be based on the missing production costs and yield of the target products, so the credibility of the estimate cannot be determined; and (4) slide 19 shows that flavoring chemicals have been made in high yield, but product composition and potential separation requirements are missing. There is also no indication of how the process translates to value. The PIs give a very brief overview of polymer production leading to a material with properties similar to bisphenol A. The TEA will be critical here. If they assign a market price to their materials based on conventional material, then it is hard to believe if the Spero product is a mixture, as implied. A customer is unlikely to buy a blend with potentially variable properties when they can get pure bisphenol A whose performance is assured. On slide 21, the PIs allude to initial TEA for bio-phenol production, but no details are given. It is important to understand the comparative processes and the relative production costs (e.g., the composition of the mixture of phenols that they are making and the values that are attached to this mixture). The PIs claim that their product value is 2.5 times larger, but no standard of comparison is given. Unfortunately, the information in this presentation was insufficient to give a credible evaluation of impact and progress. The need to protect proprietary information is understandable—perhaps by sending more sensitive information to the Review Panel alone under secrecy agreements, stronger support could be given. Without this information, the panel scores for Spero suffer.

- The goal of this project is to replace bisphenol A from lignin. The basic strategy involves the catalytic deconstructing lignin into less than 10 chemicals and then upgrading these chemicals to bio-phenols. Due to the proprietary nature of the work, few technical details were provided. However, a clear vision was logically presented that fits well within the BETO portfolio. In addition, clear milestones and compelling were provided. The team is also making good progress. No concerns were raised.

PI RESPONSE TO REVIEWER COMMENTS

- Spero thanks the reviewers for their time in reviewing this project. We understand that further technical details would be useful for review of the project, but unfortunately, we cannot disclose confidential information in a public meeting. It should be noted that all project milestones are on track for timely completion per the schedule in the Statement of Project Objectives.

GAS-PHASE SELECTIVE PARTIAL OXIDATION OF LIGNIN FOR COPRODUCTS

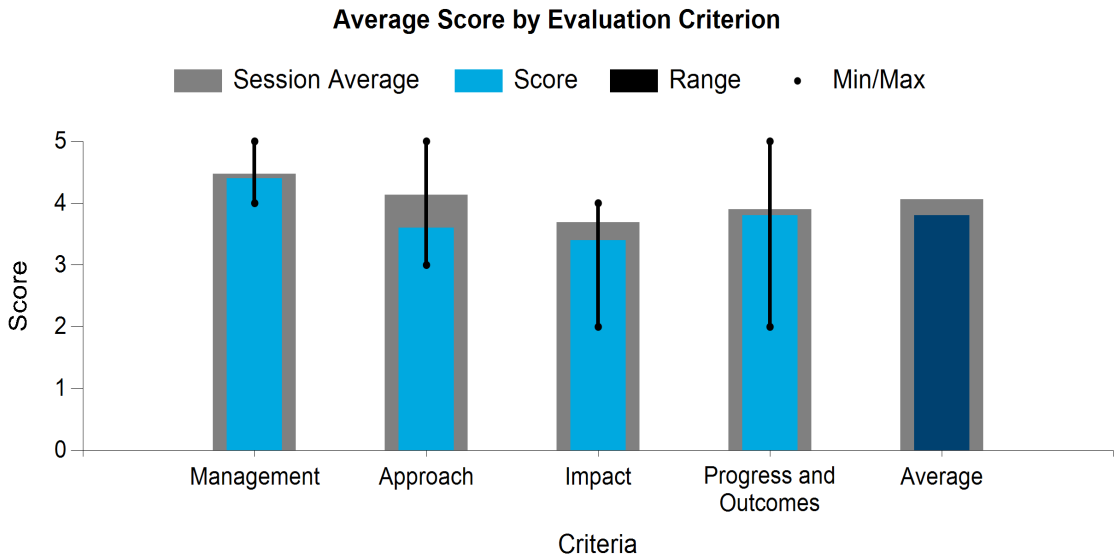
National Renewable Energy Laboratory

PROJECT DESCRIPTION

WBS:	2.3.1.501
Presenter(s):	Zia Abdullah; Courtney Payne; Matthew Yung
Project Start Date:	10/1/2017
Planned Project End Date:	9/30/2020
Total DOE Funding:	$1,200,000

This project seeks to develop a catalyst and process that can convert low-value lignin streams from biorefineries into valuable phenolic coproducts by the gas-phase catalytic partial oxidation of lignin pyrolysis vapors. The technology will enable a diversified revenue stream for an integrated biorefinery in which the cellulose and hemicellulose biomass fractions are primarily used to produce a suite of fuels and coproducts, creating valuable phenolic compounds from lignin. The focus on producing simple phenols (phenols that do not contain methoxy functional groups) is due to their high value and market sizes (~$1,100/tonne and 11,400 kT/y for phenol, $3,800/tonne and 3,400 kT/y for p-cresol, and $6,000/tonne and 45 kT/y for catechol).

The approach for catalyst development focuses on design of catalysts by varying two parameters known to be important in selective oxidation catalysis: metal-oxygen bond strength and active site density. The activities that were performed and will be discussed include: (1) establishing benchmarks on yield/composition for various types of lignin pyrolysis vapors, (2) establishing a benchmark with a commercial catalyst for phenol yield from partial oxidation of lignin pyrolysis vapors, (3) computational guidance on materials selection, (4) synthesis and characterization of catalyst materials, (5) reaction testing of catalysts using both model compounds and the whole vapor stream from lignin pyrolysis, and (6) integration of techno-economic analysis with experiment results. Recent catalyst and process development accomplishments are highlighted by improvements in the carbon yield to the desired phenolic compounds, and the resulting estimated impact on the reduction in modeled minimum fuel selling price will be presented.



COMMENTS

- Management and plans are good with tasks and subtasks highlighted, as well as risks and mitigation strategy. Approach is reasonable given the need to monetize lignin fraction from DMR with specific increased in yields and the reduction of lower-value char. The impact of cost reduction is consistent with BETO's mission in enabling the production of biofuels and coproducts from biomass. Progress and outcomes indicate the developed catalysts have a 2x improvement in phenol yield over commercial benchmark catalyst. The overall estimated MFSP reduction is estimated at $0.60/GGE since 2017. The current yield of 15% phenol is low to have a significant impact on GGE, so the target of 30% phenolic yield from enzymatic hydrolysis DMR would be an important milestone to achieve by the end of this project. On the overall, the project goal of increasing phenolic yields will result in cost-effective aromatics from a renewable feedstock. The current most economic route for production of phenol involves reacting petroleum-derived benzene and propylene to form cumene, which is subsequently oxidized to the hydroperoxide, followed by acid-catalyzed cleavage to yield phenol and acetone. Acetone is a valuable byproduct of that reaction. Therefore, the focus on lignin enzymatic hydrolysis DMR is interesting, as the average price for biochar in the United States was reported at $1.29 per pound, or $2,580 per ton. In the near term, due to price point, biochar will likely only be used in high-end specialty markets, but there is growing interest in developing biochar into new products that take advantage of its unique chemical properties and its potential use as a catalyst, as fertilizer, and to amend soils. Phenol yields and improved catalyst performance and longevity, as well as current price of phenol and coproduct value, are critical to successful deployment of this approach.

- Strengths: As thermochemical approaches go, the approach is reasonable and complements the several projects trying extract coproduct value from lignin. The scientific approach is reasonable, with the evaluation of catalysts based on metal-oxygen strength being an interesting approach to the problem. A further strength is the use of computational modeling of the pyrolysis catalyst, as it has provided useful insight to the catalytic transformations on surfaces. It would be helpful to know whether any of this modeling has been translated into accurate prediction of pyrolysis outcomes, or been demonstrated to improve catalyst activity (i.e., validation of modeling through experiment). The PIs have done a good job in understanding the challenges to operation of their pyrolysis reactor, and are pursuing some interesting solutions for increasing its efficiency. The testing of multiple lignin sources as feedstock is a strength. The results show that kraft lignin may be an ideal source, as it gives the highest level of phenolics. The amount of char also drops significantly at higher pyrolysis temperatures.

  Areas for improvement: What is missing from the approach is a credible description of the primary advantages over other pyrolytic conversions of biomass/lignin to phenolics. It is not clear what characteristics of this research will make it economically and industrially feasible where others are not. A primary concern with this project is that it is unclear whether this approach to lignin upgrading will have a significant impact. This concern has largely to do with the complexity of the starting pyrolysis oil, the separation of the various fractions, and the considerable loss of mass over multiple steps. For example, the PIs wish to generate a phenolics stream in small batches at individual 2,000-ton-per-day biorefineries. The value chain would be 2,000 tons/day biomass? 400 tons usable lignin? 60 tons/day mixed phenols? Smaller amounts of individual phenols, say, 20 tons each, all spread over 200 biorefineries? Collection and centralization of this material would be difficult, making economies of scale hard to achieve. The presentation uses phenolics and phenol almost interchangeably, which causes confusion (example, slide 14: the milestone calls for 20% yield of phenolics, but the slide indicates a 15% yield of phenol, which is not what is actually isolated). This needs to be cleaned up so that a stakeholder knows exactly what is being made, and exactly on what the economics are based. The TEA results are also questionable, as they use sales/market costs of pure phenols rather than production costs. Moreover, these projections are based on the market value of the individual phenols themselves, and not the mixture generated after lignin pyrolysis. This is not a credible way to position the work, as there is really no way that a mixture will command such prices when pure materials are available. Even if the

materials are separated, their production cost would be too high to make them competitive. Showing the production costs of each isolated phenolic is critical. Finally, it would strengthen the presentation if the PIs could project the amount of phenol necessary to realize the projected impact on $/GGE. It's hard to believe that the small amount of phenol added to the production available from the chemical industry will make a difference. A better description of the PIs' proposed operating scenario would be helpful.

- The goal of this project is to convert lignin into phenolic compounds by partial oxidation of lignin pyrolysis vapor, which fits well within the BETO portfolio. Overall, the approach appears technically sound. The team is also making excellent progress. They have successfully achieved their milestones and the project is effectively complete. A key strength is the tight connection with TEA. No concerns or weaknesses are noted.

- The goal of this project is to create catalysts to convert aromatics in lignin pyrolysis vapors to simple phenolics, such as phenol, cresol, and catechol. The project demonstrated production of phenol at 15% yield from DMR-derived lignin and with newly designed catalysts. TEA suggests that in order to contribute to the target 2030 MFSP of 2,3-BDO, a product shift from phenol and alkyl phenols to the more valuable cresols is needed. However, it is not clear how this shift would be implemented or whether it is even possible given the structures of the main aromatics found in the vapors from lignin pyrolysis.

- The presentation layout and format were excellent. The presentation had a nice, logical, and insightful flow. Good progress on DMR lignin and improvements over commercial catalysts have been demonstrated. The ability to produce catalysts internally has the project well integrated. The claim of process-agnostic technology seems validated by utilization of multiple industrially sourced lignin substrates and the discovery that residual carbohydrate content improves operability. The thoughtful focus on high-value, large-market products will definitely enhance industrial bio-adoption. Similarly, the project has targeted industrial lignin producers and phenol consumers directly, which is great. The list of industrial partners is long, robust, and credible. The technical accomplishments, patenting, publications, and external presentations are significant. The economic sensitivity tornado is not legible and would be useful to understand. On the surface, char and coke production ranging from 25–60 wt % of the feedstock appears to be a significant issue. More detail relating byproduct TEA impact and sensitivity would be helpful. More explanation for the sample expansion during testing, which appeared to be a problem, and preventative or future process solutions rectifying that would be informative.

PI RESPONSE TO REVIEWER COMMENTS

- We thank the reviewers for their comments and are pleased to know that they found numerous strengths to the project in terms of its progress and missions—specifically in that the project is well managed with a tight connection to TEA and aligned with BETO's broader goals, while maintaining a logical flow to both the research and presentation. The positive comments about studying a process-agnostic technology that could be applied to multiple lignin feedstocks was also well received by our team, as we tried to evaluate the technology and real streams as opposed to focusing on single-compound model studies.

  In terms of weaknesses/concerns, the valuation of the product stream came into question. Our TEA team made assumptions based on the selling price of phenol, cresols, and catechols and incorporated separations (distillation processes) in order to achieve high-purity fractions of the products. Additional refinement of the TEA model has been ongoing, and recent sensitivity analysis shows which factors are most important: the overall yield and selling price(s) of product(s) are the key drivers. In order to address concerns of the catalyst cost, we are exploring multiple types of catalysts, including non-vanadium catalysts (e.g., perovskites and industrially supplied catalysts from partners) and will also use a BETO-developed tool (CatCost) to estimate the scaled-up cost of our catalyst to see if the catalyst cost is a driver and look for opportunities to reduce this cost. The end-of-project goal is to demonstrate a 10% yield to simple phenols on the bench scale, and while this has been demonstrated on the lab scale,

improving the overall economics will require additional improvements in the yield to simple phenols, which is why we will continue process/catalyst development throughout the project. We are actively working on improving our TEA model to reflect the experimental data and understand the separations costs/requirements. There are existing markets and values for phenol ($1.1/kg), o-cresol ($2.4/kg), m-cresol ($3.9/kg), p-cresol ($4.0/kg), and mixed m-/p-cresol ($2.8/kg). The lower cost of the mixed cresols has the separations costs "built in." There is ongoing work at NREL within BETO projects focused on separation methods, which has shown the ability to produce >97% purity fractions of phenol, o-cresol, and m-/p-cresol from the condensed catalytic fast pyrolysis broth from whole biomass. The price of phenol (and all commodities) fluctuates, so we have used a phenol price band in our TEA, which is representative of both fluctuations in market prices and fluctuations in the product distribution (i.e., lower-value phenol vs. higher-value cresols). Our condensed simple phenols consist of 40% cresols, 30% phenol, and 30% alkyl phenols. We are still determining the appropriate markets/prices for alkyl phenols, but using a weighted average of phenol and mixed cresols values gives us estimated market prices ($2.1/kg) in line with what we used in the analysis ($1.9/kg).

BIOLOGICAL LIGNIN VALORIZATION

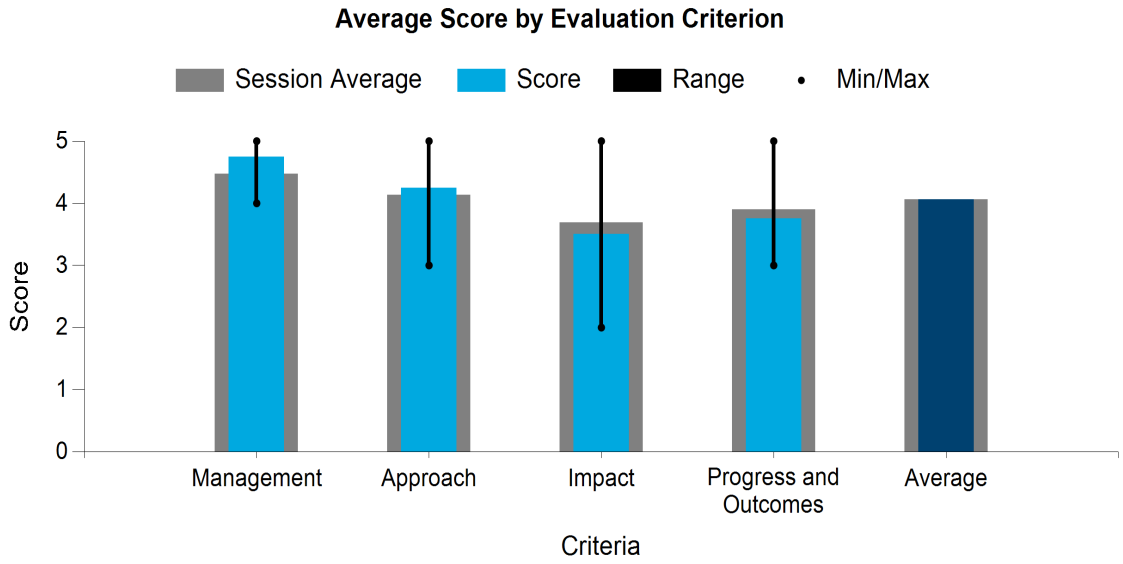
National Renewable Energy Laboratory

PROJECT DESCRIPTION

WBS:	2.3.2.100
Presenter(s):	Adam Bratis; Davinia Salvachua; Michelle Nolker; Zia Abdullah; Courtney Payne
Project Start Date:	10/01/2020
Planned Project End Date:	09/30/2023
Total DOE Funding:	$2,100,000

Given lignin's heterogeneity, catalytic depolymerization results in aromatic compound mixtures, and conversion of this complex substrate to a single product is challenging. To that end, the Biological Lignin Valorization (BLV) project is pursuing biological funneling, wherein aromatic catabolic microbes are engineered to convert a mixture of lignin-derived compounds to a single product. Namely, we employ *Pseudomonas putida* and pursue atom-efficient products such as muconic acid, which can be further converted to direct replacements or used in performance-advantaged bioproducts. Overall, biological lignin conversion can make major contributions to reduce the minimum fuel selling price of the integrated biorefinery. Early industrial efforts in this area are also leading to value-added products, including in collaboration with the BLV project.

Primary challenges associated with BLV efforts include accessing bioavailable monomers from lignin (with the Lignin Utilization project); enabling commercial titers, rates, and yields of bioproducts from lignin-derived compounds; and overcoming substrate and product toxicity. To date, we have (1) demonstrated 49 g/L of muconate from aromatic compounds and 4 g/L of muconate from lignin, (2) improved the toxicity tolerance of *P. putida* to key aromatic substrates, (3) debottlenecked biological funneling for higher rates, and (4) engineered *P. putida* to convert S-, G-, and H-type lignin-derived compounds to a single product.



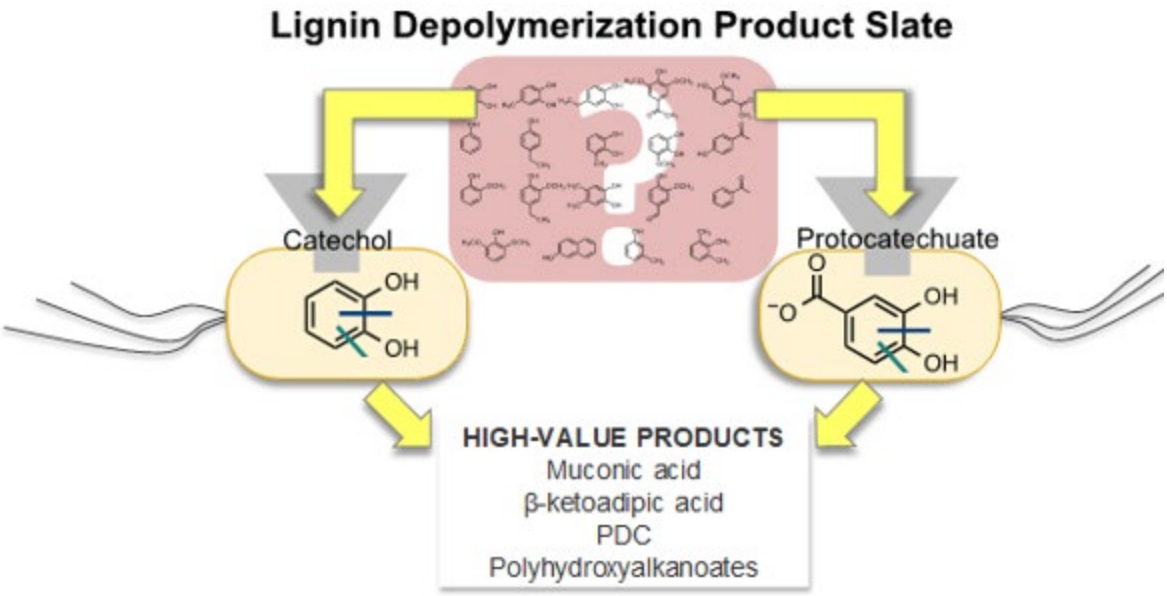


Photo courtesy of NREL

COMMENTS

- Good management team in place with university/other DOE lab collaborators. Risks are identified. Approach uses good analytics with strain engineering and adaptation with model compounds to products of interest. These represent sound technical approaches consistent with BETO's focus on lignin valorization. Once demonstrated with crude lignin fraction from pretreated biomass and the process validated, this development can have a positive impact on the bioconversion of aromatic phenolic compounds to higher value-added product provided titer, rate, and yield (TRY) are in the range of what is reported with model compounds. Progress and outcomes demonstrate that *P. putida* strains can be engineered and adapted to use aromatic phenolic compounds that are derived from lignin. The major challenge will be to demonstrate TRY with paper and pulp kraft lignin, as well as enzymatic hydrolysis DMR prepared from poplars/corn stover. Material balances need to be provided to account for all inputs, products, and coproducts.

- Project interactions and collaborations are strong. The project approach and milestones appear coherent and logical; however, the project is very early in the development cycle and makes the review task slightly challenging. Overcoming substrate toxicity and increased tolerance work look promising, and the project appears to be on track by all measures.

- Strengths: This project offers a scientifically interesting approach to lignin conversion using biological routes to transform low-molecular-weight lignin into chemical products. Project milestones set a goal of 60 g/L of muconate from lignin monomers and 10 g/L from lignin residue. Muconate is positioned as a precursor to adipic acid, offering an interface with a large, recognized chemical market. The project incorporates genetic modification of *Pseudomonas*, which is an area of strength for the NREL team and a good choice given its previously demonstrated tolerance to the wide range of different structures from lignin deconstruction. In addition, the project has developed a standard analytical procedure (high-performance liquid chromatography) for rapid assay of muconate levels resulting from biotransformation. The impact of this project in a scientific context is clear. There is really no question about the value of the fundamental knowledge that has been acquired through this program, which has been used as the basis of a number of peer-reviewed publications. The team has managed to eliminate some nonproductive pathways for muconate production and has improved the organism's tolerance to

substrate toxicity. The presentation does an excellent job of presenting a systematic identification of several production bottlenecks in the bioprocess and improvements that have been made to overcome the bottlenecks, highlighting the extensive experience at NREL in genetic and metabolic engineering.

- Areas for improvement: It is not clear whether this approach will be useful in providing value to a biorefinery operation. A few examples are illustrative. Slide 3 highlights the bioavailability of lignin as a risk. This is really a critical issue, as it is important to know how much lignin is actually convertible via these processes. Since the only substrates that seem to undergo conversion are coumarate and ferulate, there is a concern with diminishing returns and mass loss as one goes from biomass → lignin → lignin monomers (coumarate and ferulate) → muconate → adipic, β-ketoadipic acid, etc. The presentation mentions that corn stover contains a maximum of 28% ferulate and coumarate as a mixture, which is a relatively low amount of material on which to base an industrial process. Further, this effort focuses on the DMR process. But if DMR is not chosen as a pretreatment by a given biorefinery, do other lignin sources contain sufficient amounts of coumarate and ferulate to be useful? A potential risk to the program exists if these biochemical conversions work only with the process streams from DMR, and DMR is not chosen by industry. Muconate is positioned as a precursor to adipic acid and as a plasticizer. Although its properties as a plasticizer are improved compared to other materials, the amount that could be made and isolated from lignin conversion seems limited, especially when scattered across several biorefineries. Further, since it is it a mixture whose composition could change as a function of starting lignin or lignin source, a customer would be less likely to buy a muconate mixture if they could get a pure compound. Related is the team's work to produce β-ketoadipic acid as a replacement for adipic in nylon, but the scenario that would get a nylon producer to walk away from adipic is unclear. A major polymer manufacturer would not move out of their comfort zone, except for the production of a new, low-volume niche polymer with unique properties. And once one moves into niche materials, it becomes questionable whether the amount of β-ketoadipic acid polymer produced would really make a difference in the GGE goals at BETO. The same questions are pertinent for the production of 2-pyrone-4,6-dicarboxylic acid. The chemical industry is always on the lookout for cheap, structurally well-defined low-molecular-weight monomers, but it would be helpful to have an idea of their utility areas and why a customer might adopt them over known starting materials. Finally, there is little or no TEA data for any of these approaches, in particular a comparative evaluation of the production (not sales) cost of conventional adipic vs. lignin-derived adipic. This is a critical number, as it will determine whether a stakeholder could be induced to switch to a bio-based material over a conventionally sourced product. These would not be difficult calculations, as the research has considerable TRY data available for their systems.

- The goal of the project is to develop fermentation processes using *Pseudomonas putida* to upgrade lignin monomers to a diverse range of value-added products. The project fits well within the BETO portfolio and leverages NREL's strength in *P. putida* metabolic engineering. The science is outstanding, and the team is making excellent progress with model lignin compounds. The results concerning outer membrane vesicles is a remarkable finding. Overall, the team is making outstanding progress. The transition to real lignin streams will greatly improve the impact of the work. The only question concerns the choice of products. This was not clearly explained. That said, this is an exciting project that is clearly heading in the right direction.

PI RESPONSE TO REVIEWER COMMENTS

- We thank the reviewers overall for their positive feedback and constructive comments. In terms of the overall mass conversion of lignin to exemplary products like muconate, the BLV project alone cannot address the question of bioavailability of lignin. As presented in the Lignin Utilization project in the same session, oxidation chemistry is being applied to produce high yields of bioavailable molecules such as 4-hydroxybenzoate, vanillate, and syringate from lignin to far exceed the 28% of corn stover lignin that is ferulate and p-coumarate. We also stress that the BLV project does not focus solely on a DMR process—that is merely a process concept being developed in BETO at large that we interface with. The

intention in the BLV project is to generate chassis strains and associated bioprocesses that are able to take streams of bioavailable lignin and convert them to value-added products in a cost-effective and energy-efficient manner. Indeed, the key collaboration with the Lignin Utilization project does not solely focus on DMR, but rather is attempting to take lignin from the kraft process, the DMR process, and many others to oxidize lignin catalytically to produce monomers useful for biological funneling. Thus, the feasibility and ultimate success of the BLV project is not tied to the DMR process. We are unsure of what the reviewer means in terms of producing muconate mixtures. In all cases we showed, we are able to make muconic acid as a single product. In terms of beta-keto adipic acid, as presented in the "Synthesis and Analysis of Performance-Advantaged Bioproducts" project (2.3.4.501), this molecule offers multiple performance-advantaged properties relative to adipic acid, including in nylon-6,6. Similarly for 2-pyrone-4,6-dicarboxylic acid, applications thereof are not being pursued directly in the BLV project, as our focus here is on enabling the bioprocesses, but applications are being pursued in the "Synthesis and Analysis of Performance-Advantaged Bioproducts" project (2.3.4.501), especially focused on high-volume, high-value applications.

In terms of the TEA and LCA, we included these data and analyses in the presentation. We have conducted rigorous TEA and LCA of muconic acid and conventional adipic acid production cases and have been publishing those for several years now. These were also referenced during the presentation. We have shown that these bio-based processes from lignin offer both a cost and environmental impacts advantage relative to petroleum-based adipic acid. We totally agree with the reviewer that the major challenge (and opportunity) is to demonstrate high TRY for bioproducts with realistic lignin streams. This is a major effort ongoing now between the BLV and Lignin Utilization projects, which we will have updates on for the next BETO Peer Review. The choice of products is informed by products that (1) are atom-efficient from lignin (namely those that can retain much of the carbon and oxygen from the substrate) and (2) could potentially offer high-volume, high-value products for both direct replacements (e.g., adipic acid) and performance-advantaged bioproducts. To the latter point, this is why the BLV project collaborates closely with the "Synthesis and Analysis of Performance-Advantaged Bioproducts" project (2.3.4.501) in the BETO portfolio as well.

Lastly, we agree that the project is early in the development cycle, but we emphasize that the concept of using engineered microbes for lignin valorization was only originally proposed as proof of concept 7–8 years ago. Thus, the demonstration of >40-g/L titers of products already is an important advance, relative to the many decades of work across a huge number of research groups that has gone into metabolic engineering for valorization of carbohydrates.

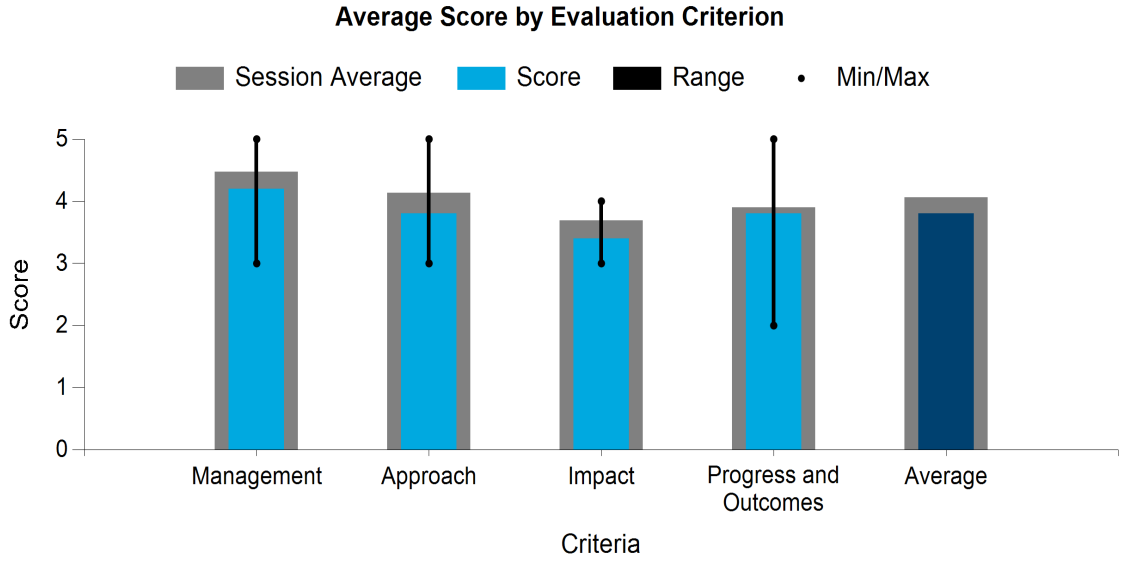
SYNTHETIC METABOLIC PATHWAYS FOR BIOCONVERSION OF LIGNIN DERIVATIVES TO BIOFUELS

Oak Ridge National Laboratory

PROJECT DESCRIPTION

WBS:	2.3.2.104
Presenter(s):	Adam Guss; Jim Parks; Tim Theiss; Missy Miller
Project Start Date:	10/01/2018
Planned Project End Date:	09/30/2021
Total DOE Funding:	$1,050,000

Lignin valorization will be critical for economic production of sugar-based biofuels in a biorefinery. Therefore, technologies are needed to convert lignin into more valuable products. Because chemical markets are small compared to fuel markets, the ability to produce a range of products from lignin would increase the flexibility of biorefineries to meet market demands. One attractive approach for lignin valorization is to engineer microorganisms for the bioconversion of lignin-derived aromatic compounds into products of interest. *Pseudomonas putida* KT2440 is a highly robust bacterium capable of efficiently utilizing a variety of carbon sources, including aromatic compounds derived from lignin, and it has recently been engineered to convert lignin streams from plant biomass into muconic and adipic acids by deleting genes involved in aromatic catabolism, preventing the carbon from getting into central metabolism. In this project, our goal is to engineer *P. putida* to convert lignin into products derived from other parts of cellular metabolism such as fatty acid metabolism and the tricarboxylic acid (TCA) cycle, thus demonstrating that we can diversify the portfolio of products that can be made from lignin. One initial example was medium-chain-length PHAs, which are natural carbon storage compounds produced by *P. putida* that can also be used as bioplastics. We have leveraged progress on this front to also produce medium-chain-length alcohols from aromatic compounds, since both biosynthetic pathways are offshoots of fatty acid biosynthesis. To further expand the number of compounds that can be made from lignin, we are also targeting production of molecules derived from the TCA cycle such as itaconic acid, which we have engineered *P. putida* to produce at gram/liter titers and high yield. Together, this work is a critical step forward in expanding microbial approaches for lignin valorization to enable sustainable biorefineries.



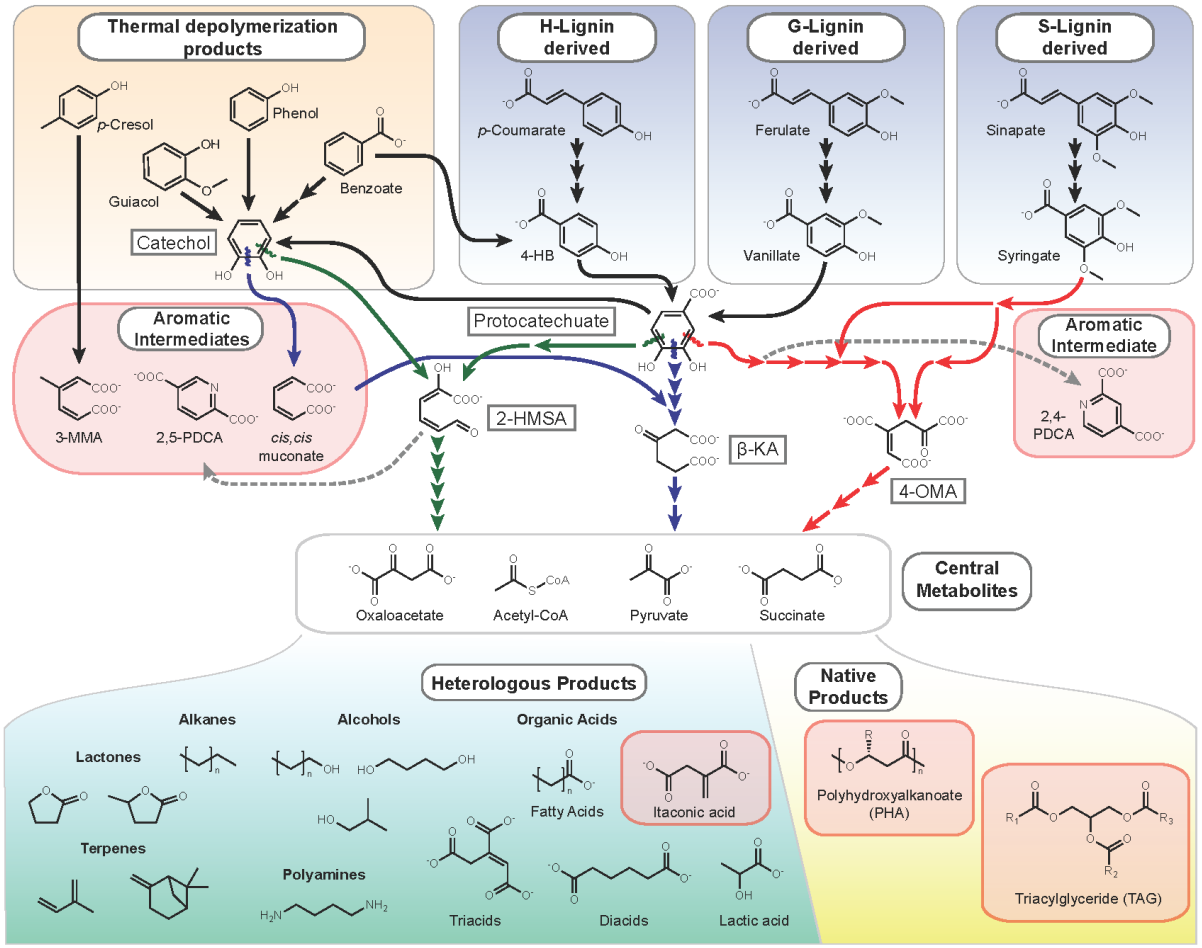


Photo courtesy of Oak Ridge National Laboratory

COMMENTS

- Management is adequate and a risk mitigation plan is in place. Approaches focus on using engineered strains expressing heterologous proteins in *Pseudomonas putida* to derive products from fatty acid biosynthesis (e.g., PHAs and alcohols) and the TCA cycle (e.g., itaconic acid). The impact consists of producing a range of products that can be derived from breakdown of lignin that feed into biosynthetic pathways, thereby increasing the value of these aromatic compounds. TEA has shown that lignin valorization is key to producing fuel and chemicals from biomass feedstocks such as DMR corn stover. Progress consists of demonstrating that a range of products can be derived from DMR lignin breakdown products, providing proof of concept. On the overall, sufficient progress has been made since 2018 that indicates that the overall approach is viable. Itaconic acid (other TCA organic acid) and PHA may not be the best targets, but terpenes, diacids, and higher alcohols maybe worth pursuing. It would be good to provide additional information on target products and a technical/economic/market side-by-side analysis.

- Strengths: The PIs present a reasonable approach that complements the other synthetic biology projects in the portfolio. Further, the scientific background and concept of using central metabolism as the source of new lignin derivatives is interesting. A useful organizing concept for the work is the plan to produce a diversity of products so that the chemical market for a single product isn't saturated by a small number of biorefineries. The team targets the production of PHAs as a demonstration of principle, and medium-chain-length alcohols and itaconic acid as subsequent targets. Itaconic receives the greatest attention, and its production targets 5 g/L in model systems and 2 g/L from DMR lignin residue. In 2020, the PIs report

that they have achieved this go/no-go point and are ready to move to 20 g/L of itaconic in model systems and 5 g/L from actual depolymerized lignin.

Areas for improvement: Although the PIs report that they have achieved their target titers, 5 g/L in model systems and 2 g/L from DMR lignin residue is low, unlikely to lead to a commercially viable process or to have a significant impact on $/GGE goals. Overall, this project seems less productive than comparative efforts at NREL. The targets remain the same, but the productivity levels are still low, even when compared to 2017 reports. The concern about saturating the market is commonly heard for the production of chemicals, but it would be helpful if it were more clearly described. The concern fails to account for increased markets and access to new families of derivatives as the price of the chemical drops. The approach used for TEA is confusing. Preliminary TEA led to the evaluation of medium-chain alcohols as their target molecules, based on similar economics for adipic acid. But production costs are not mentioned for bio-based alcohols when compared to their production from petrochemicals. If the production costs are higher (and they undoubtedly will be), the bioprocess will not be able to make the transition to industry. It is further unclear why a chemical price of $1.20/lb is given for all of the alcohols, including mixtures. There is no way that a mixture of materials will command the same price as a pure compound. Further, they get only 100 mg/L of product as a mixture of four alcohols. This titer and lack of selectivity would not seem sufficient for commercial lignin conversion, regardless of what the market value of the alcohols would be. Slide 9 indicates that itaconic predicted market prices are similar to or higher than adipic acid. This sounds backwards, in that it implies that higher sales costs are better because they will improve the effect on $/GGE (i.e., itaconic has to sell for a *minimum* of $1,750/ton). What is really needed is a significant reduction in itaconic cost to enable interest in the use of a new, cheap performance-advantaged bioproduct in previously unavailable markets. More clarity around the TEA assumptions and planned operating and market scenarios would help the presentation. Process optimization produces itaconic from coumarate at a 60% molar yield. The significance of this result is unclear, as it depends directly on the availability of coumarate from other lignin utilization projects. The key unknown is the maximum amount of coumarate available from lignin conversion processes. The presentation would be strengthened with a simple mass balance showing the transition from 100 g biomass → 20 g lignin → *X* g coumarate → *X* g itaconic. There would appear to be too much mass loss and not enough product to justify its isolation and use as a chemical product.

- The goal of this project is to produce itaconic acid from lignin monomers. This goal is well aligned with BETO objectives. Overall, the technical approach seems sound and the milestones are compelling. The team is making excellent progress with near-stoichiometric production of itaconic acid during the production phase. The work is also strongly motivated by TEA, which is considered a strength. Also, the use of both model and real lignin monomers appears to be a sound approach. This work is still in the proof-of-concept phase. As a consequence, it is not clear whether it will yield a feasible process. However, the science is compelling and the team is making excellent progress towards reaching their goals. Overall, this is an excellent project with no significant weaknesses.

- This project aims to expand the types of products that can be made from lignin. The research initially focused on engineering microbial strains for production of medium-chain-length alcohols and polyhydroxyalkanoates, and more recently on itaconic acid (2-methylenesuccinic acid) production. With itaconic acid, the project reached its milestone of achieving a 5-g/L titer from an aromatic and 2 g/L from real depolymerized lignin. This shows that there has been good progress on the engineering of the microorganism for itaconic acid production. The rationale for this project appears to be the predicted need to make valuable products from lignin to achieve the 2030 goals for the MFSP. The project goal is to diversify the portfolio of products that could be made from lignin because markets for potential products are small. There is some logic in trying to diversify the portfolio of products that a lignocellulosic biorefinery could produce. However, from a techno-economic perspective, it is not clear how using lignin to make products that could more easily be made from the carbohydrate-rich hydrolysates, such as TCA cycle derivatives, is a good approach for the biorefinery. A useful analysis to

answer this question would be a TEA comparison of using a small percent of the hydrolysate to produce the valuable itaconic acid, the rest to produce the fuel, and all the lignin burned to produce the energy needed to operate the biorefinery plus selling the excess energy as electricity. Would such analysis predict that the best MFSP be achieved when 100% of the hydrolysate is used for fuel production, or is there room for optimization of how much fuel is produced versus how much itaconic acid is produced, both from hydrolysate?

- Uncoupling the growth and production phase utilizing different metabolic nodes is noteworthy and hopefully can be leveraged to other organisms and product pathways. The project's product portfolio diversification will be a great outcome, although it is not explicitly clear that the final product (itaconic acid) is more valuable than the process intermediates. As the microbes appear to utilize carbon from a variety of substrates, do the economics on glucose as a feedstock from a Gen1 ethanol refinery work? Greater detail or illustration of the TEA could be helpful. Industrial challenges with the current benchmarks appear evident, microbial (*Pseudomonas*) biocatalysts, aerobic nitrogen-starved process, and limited productivity. As the presentation suggested, a couple of large biorefineries would saturate the chemical market; therefore, is the juice worth the squeeze?

PI RESPONSE TO REVIEWER COMMENTS

- We thank the reviewers for the constructive feedback. In regard to TEA, we are working toward higher titer, rate, and yield of molecules such as itaconic acid, but as a reviewer noted, we are still in the proof-of-principle stage. Therefore, instead of modeling the current state of the art, we have used TEA to ask the initial question: "Could a plausibly mature bioconversion process be economically viable?" This allowed us to determine whether the process is worth the R&D effort to develop. We have taken a similar approach for other molecule targets. We agree that additional TEA will be critical in further evaluation of progress, and we plan to continue to engage with the NREL TEA team to further explore this. We also agree that other products may also be promising, and we will continue to use TEA to help inform our choices of which molecules to pursue as we work to expand the portfolio of products that can be made from lignin. The reviewer makes a good point about some molecules being easier to make from sugars. However, aromatics feed directly into the TCA cycle, making TCA cycle intermediates relatively easy to make from aromatics as well. In separately funded work, we have demonstrated simultaneous co-utilization of glucose, xylose, arabinose, acetic acid, and p-coumarate. An interesting potential future direction could be conversion of mixed sugar and aromatic feedstocks for the production of itaconic acid and other products derived from central metabolism. It is important to note that our intermediate titer goals are 5 g/L (achieved) and 20 g/L (future), but we ultimately aspire to much higher titers (>100 g/L). A major challenge in reaching this goal is getting sufficient carbon into central metabolism, especially since the solubility of aromatics is low, which will likely require advances in lignin deconstruction and processing projects.

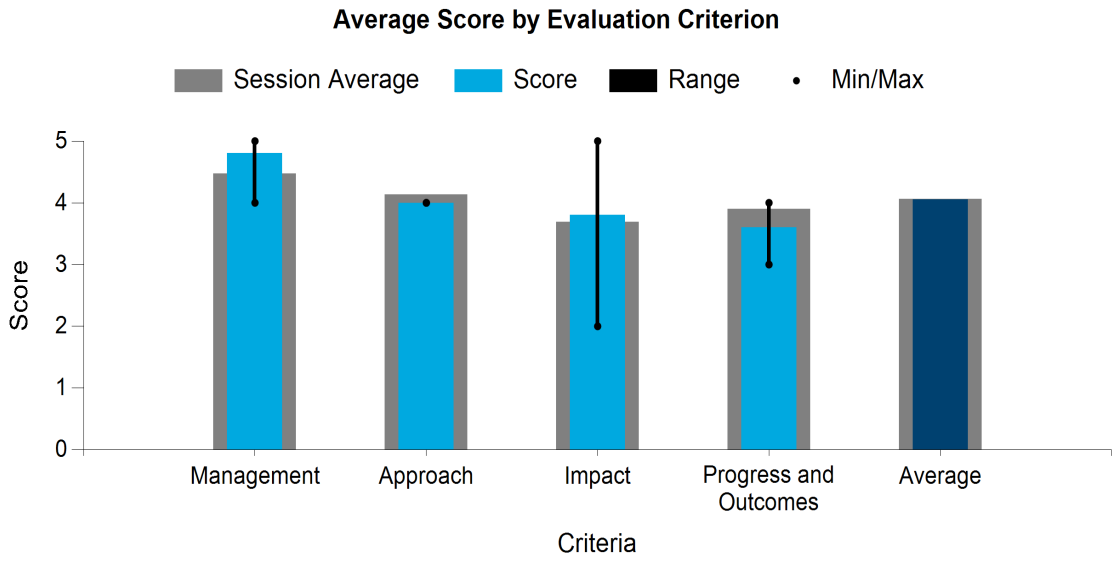
BIOLOGICAL UPGRADING OF SUGARS

National Renewable Energy Laboratory

PROJECT DESCRIPTION

WBS:	2.3.2.105
Presenter(s):	Adam Bratis; Jeff Linger; Michelle Nolker; Zia Abdullah; Courtney Payne
Project Start Date:	10/01/2020
Planned Project End Date:	09/30/2023
Total DOE Funding:	$950,000

The Biological Upgrading of Sugars project directly targets the anaerobic conversion of lignocellulosic feedstocks into intermediate molecules readily upgradeable to fuel precursors. Recent efforts on the project have a particular emphasis on the biological production of butyric acid, an intermediate that can be readily upgraded to sustainable aviation fuel, diesel blendstocks, and high-value chemicals. The project approaches this direction through a combination of strain engineering, fermentation process engineering, development of novel separations technologies, and the design and build of pilot-scale systems. Our ultimate project goal is to develop an integrated cost-effective process at pilot scale to achieve DOE's Multi-Year Project Plan targets of $2.50/GGE. The major thrust of the project over the last project cycle was on the development of integrated processes surrounding the anaerobic production of carboxylic acids using diverse *Clostridium* species. We developed and expanded genetic tools for several *Clostridium* species and rewired microbial metabolism in an attempt to maximize substrate utilization and flux towards butyric acid. We designed and built novel bioreactors with an in situ product recovery system enabling the biological production and recovery of highly purified acids. We leveraged this system to generate hundreds of grams of acid from corn stover hydrolysate. In this presentation, we highlight data surrounding our proposed process and accompanying results from techno-economic and life cycle analyses of our integrated process. Finally, we detail plans of our pilot-scale reactor system that is in process and discuss our future routes towards achieving economically viable and sustainable diesel and jet blendstocks.



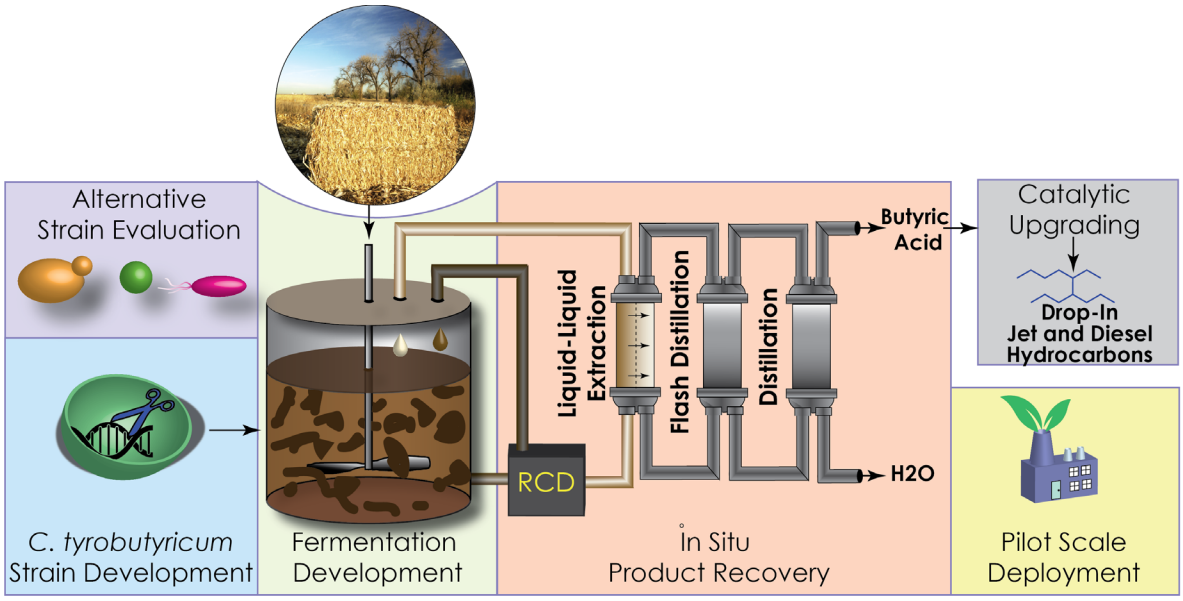


Photo courtesy of NREL

COMMENTS

- Strengths: The PIs present a reasonable approach for improved butyrate production. In particular, the planned move to pilot scale is important and will generate data necessary to attract (or keep) potential partners on board. The project has identified and demonstrated conversion of the butyric acid intermediate to derivatives that will be drop-in hydrocarbons for jet fuel. Work in the planned research areas is showing progress. The team has identified and prioritized organisms showing high butyrate specificity while also being able to ferment both C5 and C6 sugars, which will be required for effective use of the carbohydrates from lignocellulose saccharification with production approaching 50 g/L in about 100 hours. This can lead to purified butyric acid via a hybrid extraction/distillation, in situ product recovery approach, which affords a good lab-scale demonstration of the planned process. The team has constructed a hybrid extraction-distillation in situ product recovery system that can run continuously, and has been used for butyric production for 300 hours. This is a strength of the project, as it provides proof of concept at a scale that will be of interest to commercial stakeholders. An additional promising achievement is the engineering of an organism with improved xylose utilization, countering xylose's ability to restrict the active lifetime of *C. tyrobutyricum* during fermentation.

  Areas for improvement: Greater clarity in the envisioned operating scenarios would be helpful. In this presentation (and others), the production of a single chemical is being pursued that has a known value in the chemical marketplace. The butyric acid market is reasonably large (slide 10), along with its utility as a precursor to specialty chemicals and materials. These would seem to be better biorefinery targets, to be added to the portfolio of developing lignin coproducts. Thus, it is not clear why butyric is ultimately being down-valued to fuel components or precursors, and how the economics hang together. Specifically, production of butyric from petrochemicals offers significant competition based on production costs. To that point, the PIs indicate a goal of reducing dependence on petroleum-derived butyric, but this will not happen unless the production cost is lower than conventional production (oxo reaction of propylene followed by oxidation of the resulting butyraldehyde). Slide 20 indicates that total projected expense is $0.84/kg, with current butyric sales price of $1.80/kg. More clarity around these numbers and the assumptions behind them would be helpful. Later discussion with the PIs indicated that total projected expense is actually a production cost. But since none of the numbers in the bar chart add to $0.84/kg, it is unclear what this means. In any case, the best and most transparent economic

comparison would be on production cost of butyric via conventional means vs. this new approach. It is not clear how well the hybrid extraction-distillation in situ product recovery approach would scale to commercially useful levels, and accordingly, more information about whether this technology is currently practiced in industry, and at what scales, would be helpful.

- The goal of this project is to produce butyric acid and hydrogen from lignocellulosic sugars. The hydrogen will then be used to upgrade the butyric acid to jet and diesel fuels. Overall, the objectives are clear. However, the milestones are not aggressive and would fail to substantively push the state of the art. It would help to better justify these objectives. That said, the team has made solid progress, so they on track to achieve their stated goals. No concerns were raised regarding the management of the project. In addition, the target of butyric acid is well justified. However, it is not clear whether upgrading butyric acid makes the most economic sense. The reviewer is also somewhat skeptical about developing co-utilizing C5/C6 strains. Significant effort has been devoted to developing co-utilizing strains of yeast without much success. Recent work, in fact, suggest that co-utilization comes at a cost due to the limited resources and capacity of the cell. In other words, you need to downregulate C6 metabolism in order to enable C5 metabolism. Lastly, it was unclear what the relative advantages of liquid–liquid extraction versus extractive distillation were. It would help to provide some cost figures. That said, it was refreshing to see separations being considering in this project.

- The project appears well organized, successfully executed, and running well. The track record for patent filings, publications, invention disclosures, and public presentations resulting from this work is quite good considering the development cycle. The anaerobic platform for blendstock production from sugar is a highly versatile and useful pathway. It is my opinion that the project pathway will have rapid impact if the end-of-project milestones are achieved during development. To meet the outlined milestones will be a demanding feat. Novel in situ product recovery is a substantial achievement, and the project team should be proud of the accomplishment. The internal collaboration team is excellent, and a highly diverse and strong resource pool is found on this project. Large-scale anaerobic bacterial fermentations are formidable; having the organism/process conditions self-select the fermentation pH in a desirable space in terms of infections is good luck. The effort to leverage organism improvement strategies through metabolic engineering, rational engineering, and culture evolution to optimize the strain rather than optimize the fermentation conditions to match the strain will be of greater impact. Your employed strategy is smart and aggressive. The FY 2021 design and build process versatility will be a great asset and utility for BETO. It is not clear how the current state of the art compares to the end-of-project milestones. The figure of 78 g/L of butyrate on corn stover hydrolysate and 300 hours of continuous operation is impressive. The engineered strain was reported to produce only 18 g/L butyrate in 120 hours, however, so further clarity would be helpful. Good job!

- This project focuses on producing butyric acid from the carbohydrates in lignocellulosic hydrolysates. Butyric acid would be a precursor for further upgrading to drop-in jet and diesel hydrocarbons, and it is central to one of the process pathways investigated for production of hydrocarbon fuels in the TEA performed in the biochemical platform analysis project. The project has many collaborations with other projects in the BETO portfolio. Good progress has been reported on using *Clostridium tyrobutyricum* as the key organism for production of butyric acid from glucose and xylose with induced cells and in fed-batch systems. The progress made with the development of a hybrid extraction-distillation in situ product recovery system is worth highlighting, where the project is reporting higher titer, rates, and yields compared to experiments without in situ product recovery. There are abundant examples in the literature of lab-scale research into the potential benefits of in situ product recovery from bioreactors producing a variety of short-chain and medium-chain organic acids. In most cases, in situ product recovery uses liquid–liquid extraction and pertractive membrane systems. While most of the literature has examples at the small bench scale, this project has the opportunity for making a great impact on analyzing the benefits and challenges of in situ product recovery in a scaled-up pilot system. Equally important is the opportunity for helping define what key metrics should be considered and how they should be compared

to systems that do not have in situ product recovery. For example, the metric of butyrate production (i.e., titer in g/L) has a different meaning in batch or fed-batch reactors compared to reactors with in situ product recovery, since with in situ product recovery the reactor would be expected to experience lower accumulation of product. How should this metric be calculated, and what is a proper comparison with systems without in situ product recovery? This comparison is made in the report from this project, but it is not clear to this reviewer how the production was calculated. Is this a hypothetical concentration, or is this an actual measured concentration inside the bioreactor? The higher yields of butyrate when using corn stover hydrolysate compared to when glucose is the only substrate are curious (e.g., 0.53–0.59 g/g versus 0.44 g/g). Perhaps this is a result related to how this metric is defined, and some clarification would be useful. Is the metric not taking into account other carbohydrates that may be present on the hydrolysate and only based on the measurements of glucose and xylose concentrations? Lastly, one of the potential benefits of in situ product recovery in bioreactors producing organic acids is that pH control will require the use of lower amounts of base to keep the pH in the desired range. This was not highlighted in the techno-economic analysis. Is it not a significant factor on cost, or was it just not included in the analysis?

- Well-balanced team with expertise in strain development, fermentation process development, end-product process recovery, and chemical catalytic conversion. Risk mitigation is not outlined. Communication with collaborators is addressed. Approach rests on improving sugar utilization by the production strain and in situ end-product recovery by integration with the fermentation process. It is not clear what the method of pretreatment of corn stover used was. The PI is aware of some of the challenges for low-pH butyric acid production, C5 sugar utilization, and microbial toxicity from solvent use. The use of NaOH to neutralize acid and subsequent generation of free acid form contribute a significant cost to the production of butyric acid. The team reports a titer of 78 g/L with low productivity and a yield that estimates final cost of $0.84/kg, which is relatively high for a commodity-type chemical. The impact from a successful development of a continuous process for butyric acid that competes with fossil-fuel-derived material is worth undertaking. C14 targeted as the drop-in fuel is interesting. These still need to be demonstrated at scale. Progress and outcomes described are reasonable, but meeting a favorable cost for production at pilot scale has not been demonstrated and will be challenging. On the overall, the project is worthwhile, but additional strain engineering selection and adaptation, as well as improved cost-effective recovery in continuous mode, are needed for a successful outcome. A side-by-side comparison of the production of butyric acid with 2,3-BDO by BETO will be helpful in assessing long-term strategy with pros and cons.

PI RESPONSE TO REVIEWER COMMENTS

- We thank the reviewers for the thoughtful responses that will certainly help improve the future impact of the Biological Upgrading of Sugars project. We appreciate the many supportive comments from the panel, but will focus our response here on clarifying some selected points that may not have been sufficiently explained during the oral presentation through answering some questions laid out in the reviewer responses.

  Q1: It is not clear what the method of pretreatment of corn stover used was.

  The experiments we presented utilized deacetylated, mechanically refined and enzymatically hydrolyzed corn stover. However, this organism has also been shown to efficiently utilize hydrolysates from dilute acid pretreatment.

  Q2: The team reports a titer of 78 g/L with low productivity and a yield that estimates final cost of $0.84/kg, which is relatively high for a commodity-type chemical.

  Total projected expense is $0.84/kg, with current butyric sales price of $1.80/kg. More clarity around these numbers and the assumptions behind them would be helpful.

First, we anticipate that the use of a less-toxic extractant in the hybrid extraction-distillation in situ product recovery will increase our productivity and yields, thus reducing our modeled costs. However, productivity is not a major cost driver in this anaerobic process. The detailed techno-economics will be available in an upcoming manuscript. Briefly, the total projected expense is a summation of CapEx and operating expenses, including corn stover handling, pretreatment, enzymatic hydrolysis, fermentation, and downstream processing. If variable operating expenses and taxes are incorporated, the minimum product selling price for butyric acid from the hybrid extraction-distillation in situ product recovery setup is modeled at $0.99/kg. The $1.80 butyric acid selling price that was referenced is a published market price of petroleum-derived butyric acid. It is not uncommon for bio-derived products containing oxygen or other heteroatoms to have a cost advantage relative to petrochemicals that start with a non-oxygenated feedstock.

Q3: The reviewer is also somewhat skeptical about developing co-utilizing C5/C6 strains.

We apologize for the lack of clarity in our description of *C. tyrobutyricum* metabolism. *C. tyrobutyricum* is a native utilizer of xylose, and through our development of a high-xylose-containing seed culture, as well as a fed-batch operation, we can achieve efficient co-utilization of glucose and xylose. Enhanced glucose/xylose co-utilization in *C. tyrobutyricum* has also been demonstrated via genetic engineering approaches, and we have replicated similar approaches in our lab. The initial co-utilization of sugars in the batch phase is poor. To overcome this problem, we initiate the batch phase with diluted hydrolysates. However, this dilution would add an additional cost to the process. Thus, if we improve the co-utilization, fermentations could be initiated at higher initial sugar concentrations.

Q4: How should this metric be calculated and what is a proper comparison with systems without in situ product recovery? This comparison is made in the report from this project, but it is not clear to this reviewer how the production was calculated. Is this a hypothetical concentration, or is this an actual measured concentration inside the bioreactor?

We measure actual concentrations within the bioreactor as a means to understand pertraction efficiency and compare it to traditional fed-batch processes without extraction. Our stated titers represent the total amount of acid recovered via pertraction (g) divided by the final volume of the fermentation broth at the end of the cultivations.

Q5: Lastly, one of the potential benefits of in situ product recovery in bioreactors producing organic acids is that pH control will require the use of lower amounts of base to keep the pH in the desired range. This was not highlighted in the techno-economic analysis. Is it not a significant factor on cost, or was it just not included in the analysis?

We apologize that this was not sufficiently explained. You are correct, this represents a drastic cost savings provided by the in situ product recovery system, and we fully take this into account. In fact, during the fermentations, the pertraction of butyric acid is able to auto-control the fermenter pH so that no expensive base addition is required.

Q6: [The hybrid extraction-distillation in situ product recovery system] is a strength of the project, as it provides proof of concept at a scale that will be of interest to commercial stakeholders.

We could not agree more, and that is why this is the central focus of our project during this research period of performance.

Q7: The butyric acid market is reasonably large (slide 10), along with its utility as a precursor to specialty chemicals and materials. These would seem to be better biorefinery targets, to be added to the portfolio of developing lignin coproducts. Thus, it is not clear why butyric is ultimately being down-valued to fuel components or precursors, and how the economics hang together.

This is a fantastic and central point to our project philosophy and warrants further discussion. The primary objective of this project is to identify economically and environmentally feasible routes towards

making biofuels. However, it should be noted that while we are modeling the production of a biofuel from butyric acid, we fully support using it as an intermediate for alternative bio-derived chemicals and materials. The entire research portfolio of this project is focused on the production of butyric acid as an intermediate, and we are end-product-agnostic. However, we work with other BETO-funded projects (e.g., CUBI and the Biochemical Platform Analysis projects) to develop and model butyric acid as an intermediate to performance-advantaged, drop-in biofuels. We do not believe that these directions need to be mutually exclusive, and our singular focus during this 3-year period is on demonstrating cost-effective, highly pure, renewable butyric acid at pilot scale that can be utilized by myriad downstream processes to make diverse fuels and chemicals.

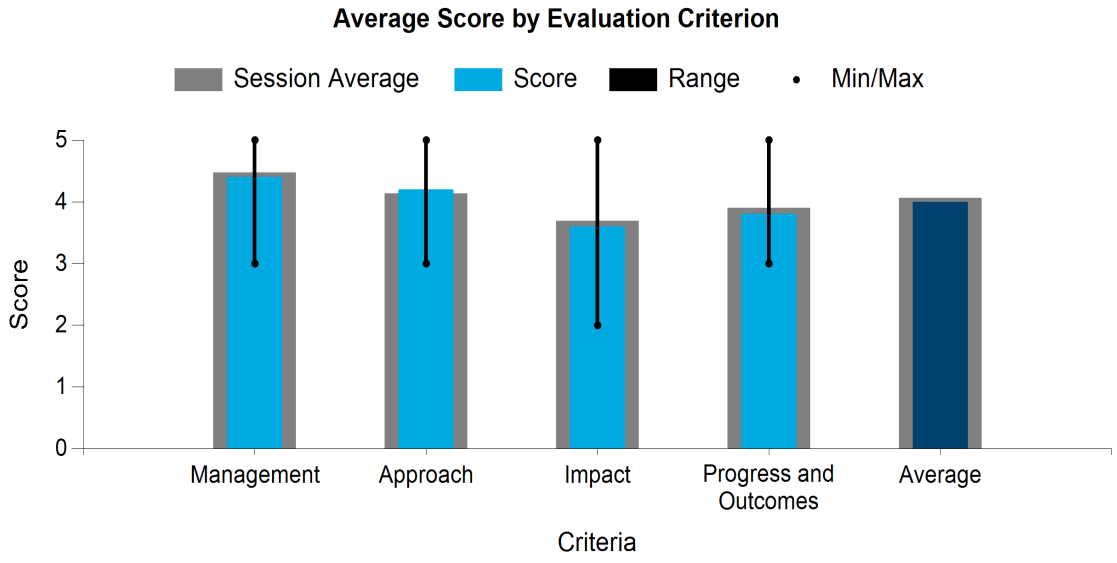
IMPROVING TOLERANCE OF YEAST TO LIGNOCELLULOSE-DERIVED FEEDSTOCKS AND PRODUCTS

Massachusetts Institute of Technology

PROJECT DESCRIPTION

WBS:	2.3.2.208
Presenter(s):	Greg Stephanopoulos
Project Start Date:	10/01/2016
Planned Project End Date:	03/31/2021
Total DOE Funding:	$1,500,000

Combined substrate-product toxicity remains one of the main obstacles hampering the scale-up and cost-effectiveness of bioprocesses harnessing lignocellulosic feedstocks, the most abundant, renewable terrestrial resource. Hydrolytic pretreatments release numerous inhibitors impinging on cell viability: the three most acute to yeast (the industry-dominant biocatalyst)—and universal to all plant sources—are furfural, hydroxymethylfurfural, and acetic acid. Likewise, desired fermentation products, such as fuel ethanol or commodity organic acids, are toxic to microbes, typically via unknown biological mechanisms. Here, we have engineered both hydrolysate and end-product tolerance in yeast by combining previously shown alcohol protective modifications with evolved genetic activities targeting the major pretreatment inhibitors. When tested on a wide sampling of genuine lignocellulosic feedstocks, ethanol production increased by >30% on average to titers >100 g/L, achieving parity with clean-sugar equivalents where conditions permit. Furthermore, we designed the tolerance capability to be fully transferable to preexisting metabolic chassis strains. As such, we "drop in" hydrolysate competence into one producing lactic acid, demonstrating the first-ever production of a cellulosic plastic at industrial titers. Our advances thus renew the potential of cellulosic biomass utilization for sustainable fuel and non-fuel products at scale.



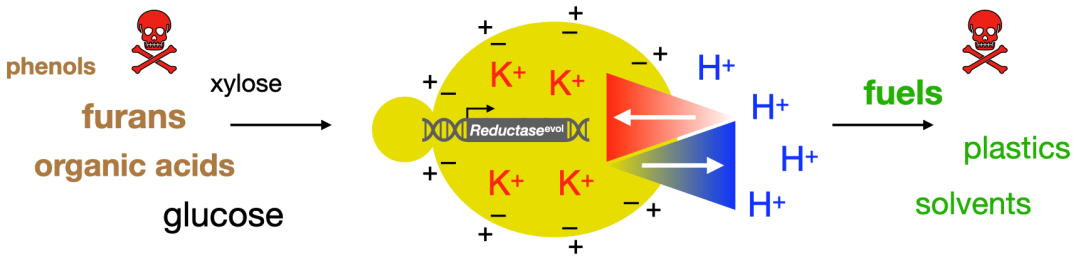


Photo courtesy of Massachusetts Institute of Technology

COMMENTS

- I am very glad to see the focus on yeast as opposed to bacteria within the project for obvious pragmatic reasons. The approach to biologically detoxify the diverse hydrolysates by building catalyst tolerance, toxicity physiology, and toxicity genes is actually pretty exciting, perhaps even novel. The end-of-project milestone of 100 g/L EtOH on real hydrolysates was achieved. The reductase evolution was also successful in boosting aldehydes to aldehyde alcohols. The breadth of the substrates and pretreatments processes tested enhances my opinion of the project and confidence with the methodology, so I am very glad that was pursued. The industrial relevance of the project does not appear to be substantial with respect to titers by contrasting commercially available strains; however, the project's learnings are of value.

- Management consist primarily of MIT team collaborators. Structure, tasks, and subtasks were provided. Some innovation was outlined in approaches. Risks are not described or addressed but may have been provided as challenges. Three primary approaches were taken to characterize toxic compounds—impart tolerance to an engineered strain, and adapt strains to another product (lactic acid). Improving yeast tolerance to biomass hydrolysates is within BETO's focus areas. Much is already known and published about inhibitors and mechanism of inhibition and detoxification. The listed approaches, as such, are not as innovative, but combining multiple approaches can be. Impact is hard to gauge to industry as there is no comparison to current industrial strains that have been engineered, selected, and adapted for C5/C6. Lactic acid production at low pH is already commercially viable with robust strains from C6 sugars, but commercial production from C5 sugars has not been reported. Progress and impacts is narrow and limited to demonstration of production of monoethylene glycol, but no data were obtained with hydrolysate due to the pandemic. My overall impression is that it is a good, successful project but may not have a major impact. The identity of the gene involved in potassium-ion transport and reductase is not disclosed but maybe subject to intellectual property filing. Reduced toxicity to acetate above pH 4.5 has been reported and is well described in literature. Engineered strains with improved reductase has also been reported. Based on that, the best reductase and potassium transporters involved, if characterized, represent new findings. The PIs need to be congratulated on testing hydrolysates past 2 years.

Transferring the reductase and potassium transporter to industrial strains will prove further validation of the approaches taken once demonstrated.

- Strengths: The high-level goals of this project are potentially useful. The PIs wish to introduce both substrate and product tolerance into yeast, initially focusing on cellulosic EtOH production. In parallel with BETO's move beyond EtOH, this approach has been further adapted for the production of lactic acid and monoethylene glycol from lignocellulosics. Overall, the project offers the potential of tailoring organisms for production of a range of different chemical products from sugars. The processes also work with real biomass hydrolysates, addressing comments from earlier peer reviews. The research has made a number of useful observations. The source of toxicity in hydrolysate was linked to acetic acid, furfural, and hydroxymethylfurfural. By operating at a lower pH and increasing the concentration of potassium ion, acetic acid is neutralized, while furfural and hydroxymethylfurfural are converted to the corresponding alcohols, whose toxicity is not observed at higher potassium concentration. Accordingly, reductases have been engineered into their organism to boost further production of the nontoxic alcohols, leading to improved EtOH production. At the same time, they have also shown that their reductase-modified yeast, operated at high potassium and low pH, also can generate 50–60 g/L of lactic acid from real cellulosic hydrolysates. This is a nice combination of solutions to some primary problems in bioconversion.

  Areas for improvement: The presentation fails to include any TEA or comparative production costs, so a side-by-side evaluation of this approach to conventional EtOH production from either starch or cellulose can't be made. While the PIs appear to have developed an interesting solution, it will not be adopted unless there is a compelling economic justification. Overall, the technology is interesting, but the actual impact is unclear because there were no benchmarks given. This approach has been used with xylose, and was ported to the production of monoethylene glycol at 4 g/L. This value is low, but listed as the highest for this particular compound from yeast. The PIs indicated that they simply wanted to demonstrate that a non-native product could be made using their technology, but it would have been useful if the rationale behind the choice of monoethylene glycol was clearer.

- The goal of this project is to improve the tolerance of yeast to hydrolysates. A key finding was that elevated potassium concentration and pH increases tolerance to inhibitors within hydrolysates. In addition, the team screened a number of reductases and found ones that further increase tolerance. The most significant advance was the use of real hydrolysates, a concern raised during a previous review. The team should be commended for listening the reviewers, especially since they achieved strong results. While the initial focus was on cellulosic ethanol, the team then expanded the product portfolio to lactic acid and monoethylene glycol and achieved strong results. Overall, this has been a successful project that has made great strides since the last review.

- The project goal is to engineer yeast strains with tolerance to inhibitors in hydrolysates. The team developed a strategy for cultivation at elevated potassium concentrations and higher pH, which helped prevent inhibition of fermentation from acetic acid. Then, the team identified that conferring tolerance to the alcohols of furfural and 5-hydroxy-methylfurfural was another successful strategy to prevent inhibition. They screened for reductases that conferred tolerance to furfural and hydroxymethylfurfural alcohols and subjected a selected reductase to evolution experiments under increasing concentrations of furfural, hydroxymethylfurfural, and acetate. In the end, the strain with the evolved reductase was shown to have superior performance in ethanol production when grown in several real hydrolysates. These are impressive results. The team also demonstrated that the strategy could be transferred to strains that produce lactic acid, and are now working on using the same strategy for developing strains to produce monoethylene glycol from lignocellulosic hydrolysates. Overall, a well-conducted project with promising results for industrial application.

PI RESPONSE TO REVIEWER COMMENTS

- With the project now sunsetting, many thanks to our reviewers past and present for their feedback. As noted in the current assessment, a particularly fruitful recommendation from a prior Peer Review was to pursue validation on genuine biomass hydrolysates. Although surprisingly nontrivial to source, that we were able to secure and test on a range of feedstocks (representing a variety of pretreatments and crops) ultimately enhanced the impact of our results considerably. Regarding the lack of a TEA, we agree with this deficit and are aiming to gain clarity on the potential cost advantages conferred by this work through an upcoming collaboration. Finally, due to the COVID pandemic, our original plans for cellulosic monoethylene glycol—i.e., demonstration of a non-ethanol cellulosic product—had fallen into jeopardy as a result of lengthy laboratory shutdowns. Fortunately, upon restart, we pivoted quickly to cellulosic lactic acid using a prior-engineered chassis and were able to demonstrate likewise the ease and high degree of tolerance portability. With functional orthogonality established, we look forward to exploring the reach of cellulosic products through additional pathway engineering, as well as that of organisms beyond *S. cerevisiae*.

BIODIESEL AND HIGHER-VALUE PRODUCTS FROM STILLAGE FIBER

Xylome Corporation

PROJECT DESCRIPTION

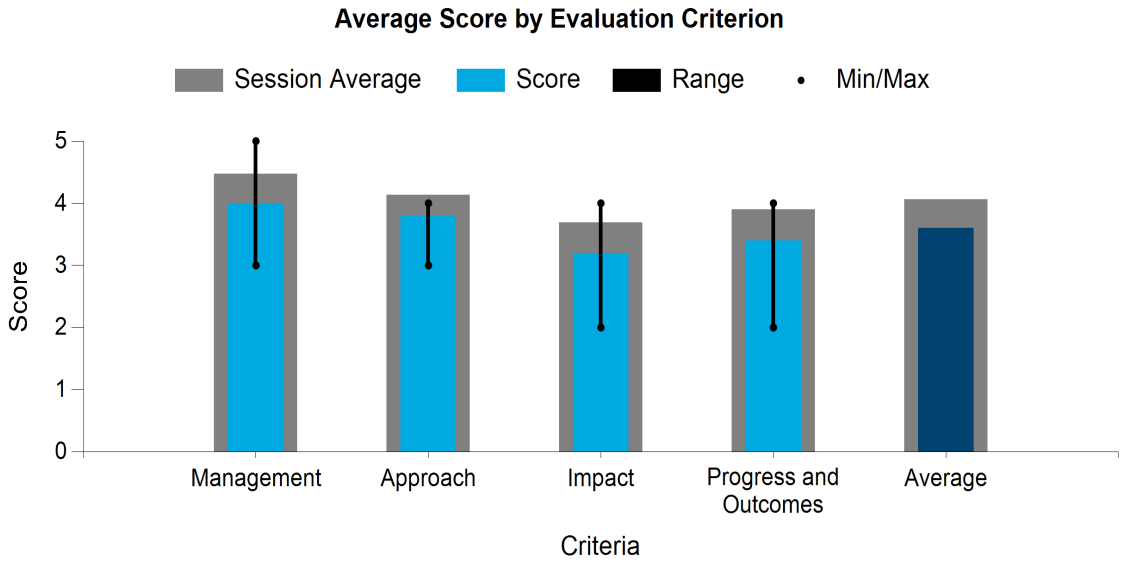
The goal of this project is to make biodiesel precursors from dissolved organics and corn fiber in grain ethanol stillage. We will accomplish this by engineering hyper-lipogenic strains of *Lipomyces starkeyi* to express and secrete cellulases and xylanases. At the end of our project, we expect to demonstrate fermentation with our top strain on stillage fiber in a commercial pilot plant.

WBS:	2.3.2.223
Presenter(s):	Thomas Jeffries
Project Start Date:	10/01/2018
Planned Project End Date:	10/31/2022
Total DOE Funding:	$1,999,333

Our technology draws on newer processes that separate and recover protein from stillage fiber. It is also capable of using pretreated corn stover to make biodiesel precursors. If our project is successful, we will save disposal costs for stillage while moving grain ethanol plants to cellulosic biodiesel. Our goals are to (1) identify useful cellulases and xylanases that are active in *Lipomyces*, (2) obtain transformants that show high activities, (3) mate and screen the strains against model substrates, (4) achieve enzymatic digestion of and growth on commercial substrates, and (5) scale up the process in a pilot plant.

Thus far, we have cloned, transformed, and expressed 14 cellulolytic and xylanolytic enzymes, and we have exceeded our go/no-go Phase 2 by solubilizing 15% of a pretreated corn stover. We have also demonstrated a 33% increase in lipid accumulation by our genetically engineered strains growing on stillage fiber.

In the coming months, we will transform our best strains with more cellulolytic and xylanolytic enzymes, scale up in 3-L bioreactors, and begin plans for commercialization.



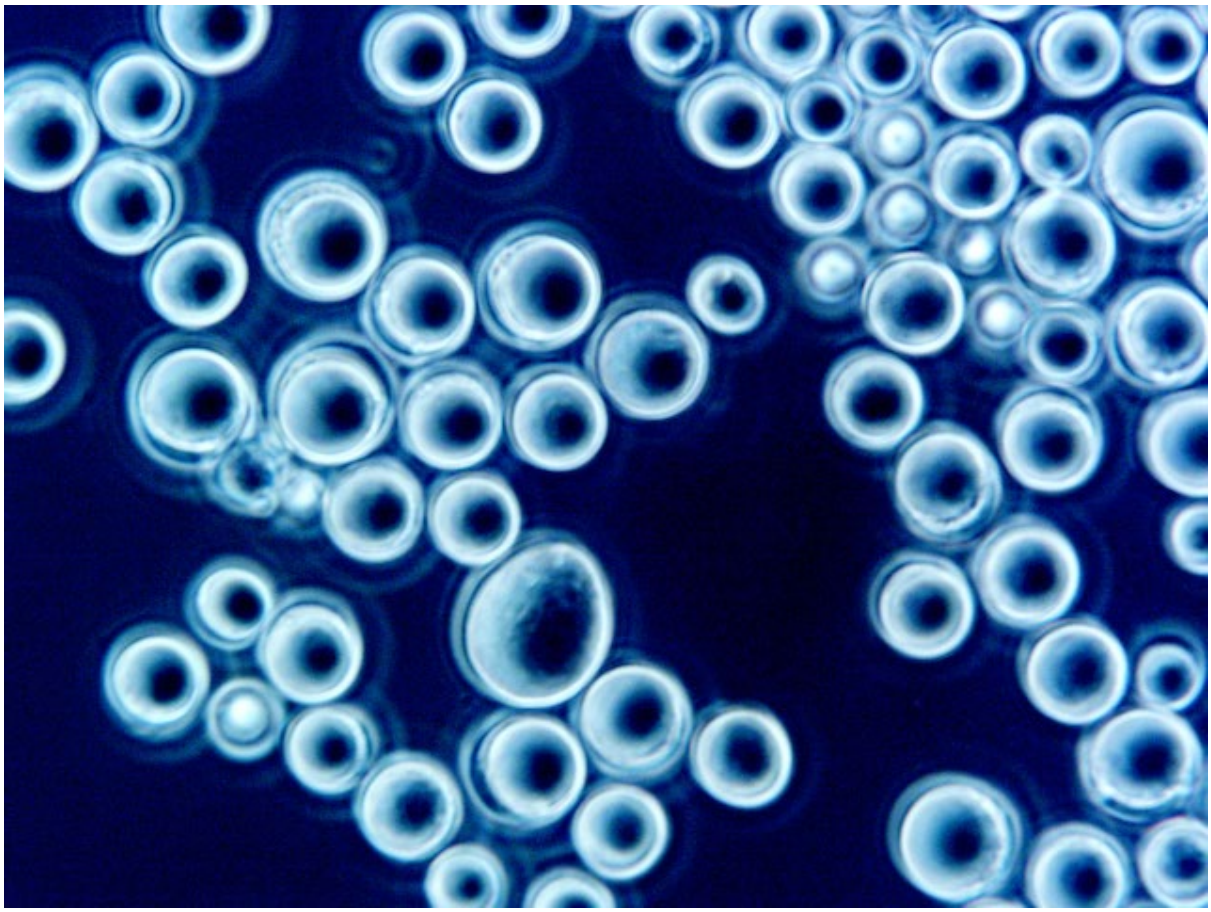


Photo courtesy of Xylome Corporation

COMMENTS

- Management plan looks adequate. There is a reference to two industrial partners, but it is not clear if they are actively involved in the work and their contributions. Risk and risk mitigation were not addressed. The overall approach to engineering *Lipomyces starkeyi* to use fiber cellulose is interesting. I'm not sure why cloning xylanases is needed, as it is not clear if the wild-type strain can utilize pentoses, as not all strains of *Lipomyces* can utilize xylose. It is also not clear if this strain is tolerant to organic acids that are present in stillage fraction, and what is the pH used and other conditions used in the fermentation (aerobic, microaerophilic, anaerobic)? This project, if successful, can serve as a transition from 1st Gen to 2nd Gen, so is within the scope of BETO's mission. The overall impact of this project is limited to dry mill corn ethanol plants. A TEA and LCA is needed to establish economic and environmental footprint from producing biodiesel from cell biomass. There is no process work to indicate how oil is extracted, yield from whole-cell extraction, or extractant used. TRY data are not provided. Two potential industry partners are mentioned, indicating some interest in this process. Progress looks good as outlined in goals and tasks. Outcomes are hard to establish with limited information provided on economics. The overall impression is that it is a good project to establish the feasibility of using stillage to generate lipid-containing cellular biomass. It would be good to have more quantitative information to assess the economic viability of the project. Material balance is critical for a proper TEA/LCA. It would have been good to have more information on lipid yield from cell mass and more information on the fermentation process that is most likely aerobic, so the cost of production would be relatively high. Earlier work done at NREL and the U.S. Department of Agriculture in Peoria has already demonstrated that it is not economical to produce lipid-containing cells. A stand-alone fermentation process would not be commercially viable.

- Strengths: The project is targeting the production of biodiesel precursors through biotransformation of organic species present in ethanol stillage waste streams. The concept integrates nicely with the larger BETO approach, as DMR will be used to convert the insoluble fiber into a feedstock for fermentation. Stillage could be an interesting feedstock, as a typical dry mill produces around 125 million gallons/yr. The presentation describes a well-established approach of engineering, screening, and testing *L. starkeyi* to find organisms with the highest activity in this application. The team has been able to make progress toward their goals. Cellulase activity has been increased (slide 10), and the required cellulase, CBHs, and xylanases have been incorporated into their lead organism. Initial results have been used to generate improvement in second-generation organisms (slide 13), which have been examined for their ability to dissolve filter paper (slide 15). Finally, third-generation organisms have been developed by rolling up bioprocess improvements from the earlier work to identify lead organisms that perform with high efficiency in the team's filter paper tests. In tests on real stillage fiber, the lead organisms display considerable improvement in the production of lipid (slide 18).

  Areas for improvement: The PIs do not report any TEA or potential impact of biodiesel coproduction on the overall economics of a dry mill, which is surprising. Estimates of the biodiesel that an EtOH plant could make, and whether it would be of sufficient volume to affect the much larger biodiesel market, are important. This is a critical omission, as economics are at the heart of any viable fuel production scenario. Information on the concentration of fermentable materials in the thin stillage would be useful so that a sense of the cost for evaporation could be obtained.

- The goal of this project is to capture the residual value in corn by targeting fiber in stillage and dry distillers grains by producing lipid with the oleaginous yeast *Lipomyces*. Overall, the problem is compelling and presents an interesting value proposition. The management plan is strong and clear milestones are provided. The team is meeting their milestones, specifically with regards to engineering a cellulolytic strain of *Lipomyces*. In addition, the project involves some nice science. Going forward, the team is planning to test their strain on stillage in a pilot plant through a contract manufacturer. While the vision is compelling, it is difficult to evaluate whether the process will be economically viable. In particular, the process will require aeration. It is unclear whether lipid from stillage will be able to justify an aerobic fermentation with the associated costs. More detailed TEA would address these concerns.

- The goals of this project are to engineer hyper-lipogenic strains of *Lipomyces starkeyi* to express and secrete cellulases and xylanases and use them to make biodiesel precursors from thin stillage in corn ethanol biorefineries. The team has demonstrated success in cloning and transformation of *L. starkeyi*. Fourteen different enzymes have been expressed in *L. starkeyi*. Good progress.

- The project theory is really great, and I am very excited about lipid production in the Gen1 space. Regarding the presentation, a significant amount of time was dedicated to project leadership and staff duties over the technical features and progress of the project. I would rather have the inverse relationship, emphasizing technical content. There is contention with minor points of the presentation, while composition data appears slightly incorrect, which leads to concern regarding the project's larger points that I hope to clarify. Historically, corn oil has gone to feed markets, biodiesel production, and heating oil. Today, the corn oil market is rapidly changing in terms of value and users, with massive oil diversion to renewable diesel facilities, as new conversion capacity is rapidly coming online. Renewable and biodiesel processes are substantially different, though. My mention of this is of little consequence here; however, a clear understanding of markets past and present (oil, feed, fiber, ethanol) and process requirements or oil quality considerations might be important for project guidance. The de-fatted syrup composition appears fundamentally incorrect; there is no reporting of fat content, and syrup with equal parts glucan and glycerol only exist during process upset conditions. Syrup protein content in standard Gen1 facilities is generally 20% (dry mass basis). These minor homework details lead me to question how syrup impurities affect this process? Basic understanding and discussion of feedstock variability would be helpful. Processing aids, protease usage, decanters on the whole stillage side, and tricanters or

disk stack machines on the oil side will produce highly variable stillage and syrup compositions. My point here is that there will be volatility in the substrates, and any information relating to that would be useful. As the presentation indicates, stillage and fiber are "wet waste products"; however, they are currently selling at $220/dry ton. Are the stillage and fiber feedstocks selected due to their opportunity costs or their composition? If the answer is composition, perhaps utilization of a clarified mash-train, slip-stream, could reduce the proposed biological complexity. I imagine lipids to be of higher value in comparison to ethanol. Addition of a preliminary TEA, mass and energy basis, and clarity on feedstock selection would be helpful.

PI RESPONSE TO REVIEWER COMMENTS

- We greatly appreciate the many positive comments and suggestions, and we welcome the questions because they enable us to think constructively about the work. As noted, our project integrates closely with the larger BETO approach, particularly DMR corn stover, which we see as a major advance in the pretreatment of agricultural residues. By design, Xylome's process integrates with first-generation ethanol facilities, while moving the renewable fuel industry toward biodiesel and high-energy-density fuels from cellulosic feedstocks. Xylome expects existing biofuel facilities to become the cornerstones for advanced second-generation plants because they are optimally situated with respect to existing ag-residue, rail, water, power, and workforce resources. Xylome's native strain consumes amylodextrins and xylose rapidly and glycerol and arabinose slowly.

  With respect to strain development: Through metabolic engineering, Xylome scientists have attained an approximate 300-fold increase in assayable cellulase activity when our first cellulase transformant is compared to our most recent cellulase strains. Our current activity is equivalent to that of a commercial cellulase preparation diluted 1:50, or a loading rate of 2.0%. We observe significant substrate degradation and lipid accumulation when cellulase-engineered cells are cultivated on DMR. With respect to oil recovery, Xylome scientists have developed low-cost and highly energy-efficient processes to recover the oil efficiently and in high yield. One involves homogenization followed by solvent extraction; the other uses an enzymatic process that releases oil bodies from the cell, which then float to the surface. The lipid bodies are separated from the hydrolysate and then homogenized at low pressure and extracted. In the enzymatic process, the hydrolysate can be recycled for cell growth. Both processes result in highly pure oils.

  We completed a TEA in July 2019 as part of our original application but neglected to update it in the review. In a bioreactor trial at Xylome, one of our engineered *L. starkeyi* strains converted a DMR pretreated corn stover hydrolysate (supplied by NREL) into lipid with a yield of 20.5 g of oil per 100 g of DMR syrup (75.5% of standard biochemical yield without reductant balancing). If one assumes that the cost of sugar in the DMR syrup is $0.16/lb and 7.68 lb of oil is necessary to make 1 gallon of biodiesel, this results in a feedstock cost of $6.00 per gallon of biodiesel. In our current economic model, we estimate that the in situ saccharification, DMR pretreatment, and transesterification processes would each add 20% to the cost of the biodiesel. With present oil yields from corn syrup and DMR hydrolysate, we estimate that our lipogenic and cellulolytic strains could produce lipid from pretreated substrate at a level of 20.5 g/g. With an estimated cost of fiber from distiller's dried grains with solubles between $0.036 and $0.044 per pound, the feedstock cost for biodiesel would be between $1.85 and $2.20 per gallon. In the case of stover at $77 per metric ton, the resulting feedstock cost would be $2.51 per gallon. We think that 0.28 g oil/g sugar or 85% of theoretical yield is a realistic goal for our cellulosic *Lipomyces* strain development. The total amount of oil that could be produced by the Xylome process depends on the resource base. Assuming our current yield of 75.5% of theoretical, a 100-million-gallon-per-year ethanol plant generates enough distiller's dried grains with solubles fiber and solubles for the Xylome process to produce 31×10^6 kg of oil per year. This is essentially as much oil as is found in the corn grain, about 4%–5% oil, of which approximately 20%–33% is recovered in current processes. Profitability of dry mill ethanol production remains low, so higher byproduct values are necessary. If 80% of the corn stover used to produce the grain is included as a feedstock for the Xylome process, then

180×10^6 kg of oil could be produced from the stillage, fiber, and stover available from a 100-million-gallon-per-year ethanol plant. More than 90% of U.S. ethanol production of 14×10^9 gal/year comes from dry mill plants, so from about 126 dry mill plants of this size, the Xylome process could produce 22.7 million metric tons of oil. This would be more than twice the U.S. consumption of soybean oil in 2020 (10.43×10^6 metric tons).

BIOLOGICAL CONVERSION OF THERMOCHEMICAL AQUEOUS STREAMS

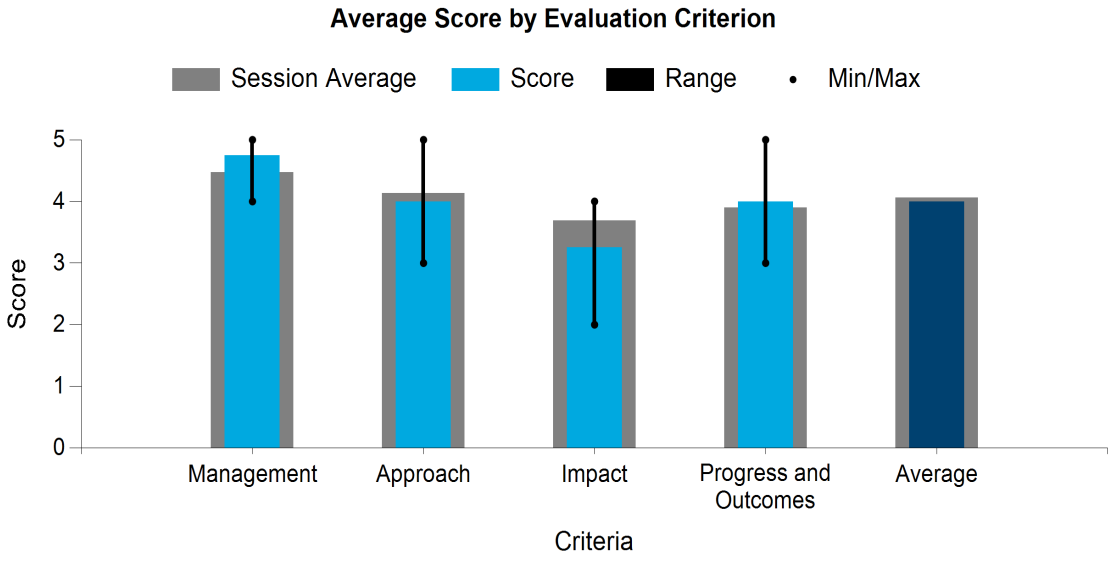
National Renewable Energy Laboratory

PROJECT DESCRIPTION

WBS:	2.3.2.301
Presenter(s):	Adam Bratis; Gregg Beckham; Michelle Nolker; Zia Abdullah; Courtney Payne
Project Start Date:	10/01/2019
Planned Project End Date:	09/30/2022
Total DOE Funding:	$1,200,000

Thermochemical biofuels production via both pyrolysis and hydrothermal liquefaction produces aqueous waste streams, typically with organic compounds at concentrations of ~50–100 g/L. These streams represent a wastewater treatment cost and carbon loss for the thermochemical biorefinery, but the concentration range for these compounds is ideal for bioconversion. To that end, the Bioconversion of Thermochemical Intermediates project is developing advanced analytics and engineered microbes to convert these waste streams to coproducts, with the overall aim of improving the economics and carbon conversion efficiency of thermochemical biorefining.

To date, we have primarily focused on development of advanced analytical chemistry approaches to fully characterize thermochemical aqueous streams and engineering of *Pseudomonas putida* for conversion of nonconventional substrates, including methylated phenolics, cyclic ketones, furans, and C1–C3 light oxygenates, into atom-efficient products. Two primary challenges are the rapid deployment of aqueous-compatible analytics to changing upstream conditions and dealing with the toxicity of the feed streams to engineered microbes. The project efforts have resulted in engineered strains of *P. putida* able to consume 90% of the organic compounds in aqueous waste streams from catalytic fast pyrolysis, more than 300-fold toxicity tolerance improvements in *P. putida*, and carbon closures exceeding 90% for thermochemical wastewater streams across multiple processing technologies.



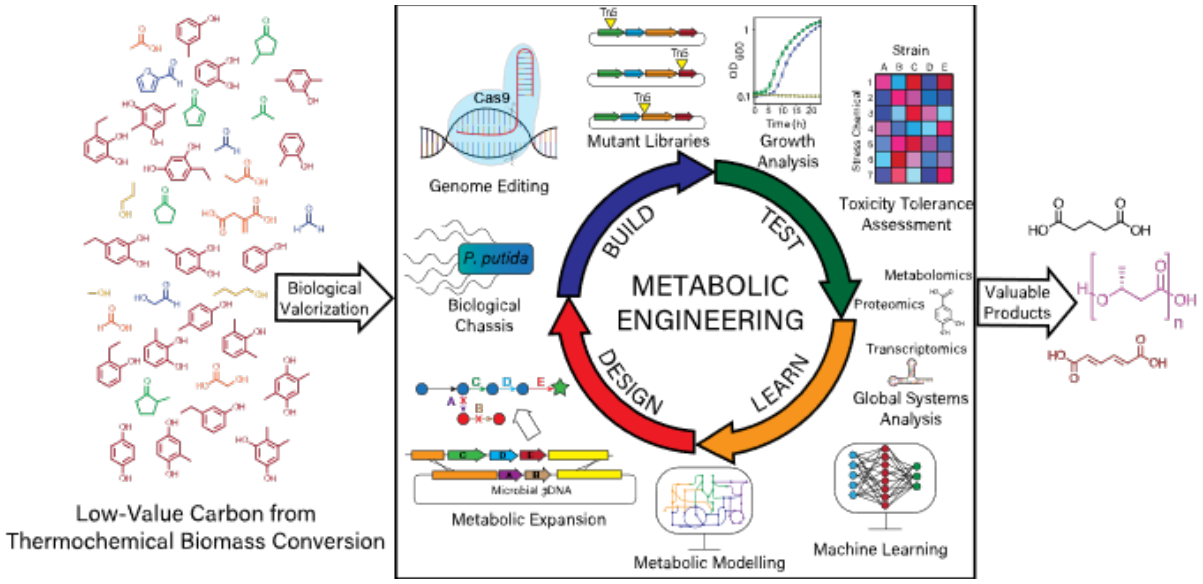


Photo courtesy of NREL

COMMENTS

- Management team is adequate. Tasks and subtasks and risks and mitigation are addressed. The approach is interesting, as it aims to monetize low-value aqueous carbon generated from waste of thermochemical conversion. The approach is also applicable to wastewater at existing treatment facilities. Approach requires timely analytical capabilities and robust *P. putida* strains that are tolerant to toxin. These strains need to have a diverse metabolic repertoire that can convert a range of chemistries. Impact can be far-reaching, as the project aims to create value from toxic compounds in water discharges that would require otherwise added cost to handle and discharge. Material balance and good conversion of waste carbon to desirable end products that were described was not provided. On the overall, the project aims to enable thermochemical conversion by capturing and reducing discharged organic carbon in aqueous waste streams to higher-value end products. A key question for BETO is how technical successes can be deployed at commercial scale and what products and product mixes are competitive based on a thorough TEA/LCA. A review of the current industrial uses of *P. putida* would also be helpful, as it has been used in the past primarily in research on bioremediation of contaminated soils and to address on land and to mitigate damage from aquatic petroleum spills. A compelling TRY, as well as TEA/LCA, would help in determining if this is a commercially viable concept.

- Strengths: The project has identified organics present in the aqueous waste stream of lignin pyrolysis as a potential source of high-value compounds. At a high level, it is a laudable goal to try to take a mixture of dozens of organics in wastewater and convert them to a small number of products. The science in this work is outstanding and includes elegant fundamental strain development targeting conversion of multiple compounds with varying structural and kinetic profiles into a small number of products. The fundamental research also offers potentially practical applications, as engineering has successfully demonstrated high levels of conversion for model mixtures of compounds and the mixture of compounds in the aqueous pyrolysis stream. This is a scientifically interesting result, showing that organisms can be developed that are tolerant to a wide range of structural differences and chemical properties. The team continues to evaluate methods for improving organism toxicity tolerance, and has made useful advances in that area that will have application outside of the specific areas targeted in this project. The research is the basis of multiple publications in high-impact journals. The project's concept of developing robust analytical methods is a real strength, as understanding of the feedstock composition and how it changes with time or pyrolysis conditions is critical for developing organisms insensitive to these changes. The

PI's choice of *Pseudomonas* is appropriate, as the team's previous work has demonstrated that biological funneling accommodates many different materials contained in the feedstock stream.

Areas for improvement: Questions arise with regard to whether this work will be industrially viable. Pyrolysis oil is exceptionally complex by itself, and the isolation of a dilute aqueous stream simply multiplies that complexity. It is not clear that this approach can produce enough value-added product from a mixture of dozens of compounds to make an economic difference. Some of the measurements could be more clearly presented. For example, the concentration of organics in the wastewater are shown as g/kg, but it is not clear whether this is per kilogram of lignin in the starting biomass or per kilogram of wastewater produced. If the latter, the handling of large volumes of water will impose a significant cost on the process. Second, the presentation would benefit from a simple mass balance flow chart: 100 g lignin to *X* g of aqueous-soluble convertible compounds to *X* g of a single intermediate to *X* g of a final product. This information is important for understanding the impact of this work and whether it makes sense to add such a unit operation to a thermochemical biorefinery. The TEA results could be more clearly presented. The impact on the $/GGE goals is based on the sales cost of adipic acid. However, no information is given regarding the production cost of adipic from the multistep fermentation or the assumptions behind the TEA evaluation. What percentage of the carbon needs to become adipic to achieve economic goals, yields, titers, etc.? What is the plan for the rest of the carbon? Additional issues arise from the work to make muconates as new performance-advantaged bioproducts. Model mixtures can be converted to mixed muconates at 90% yield, targeted as industrial plasticizers. Assuming that these compounds first get over the barrier of industrial acceptance, a customer would not pay the prices necessary to affect the $/GGE for a mixture when they could buy a pure compound that does the same thing or better. The same questions apply to the cyclic ketones that have been isolated from the waste streams and the value/utility of the diacid mixtures resulting from their conversion.

- The goal of this project is to valorize carbon waste generated by thermochemical biomass conversion by using an engineered strain of *Pseudomonas putida* to transform these low-value chemicals into high-value ones such as cyclopentanone and methyl muconate. The project involves two components: first analyzing the compounds within the carbon waste streams and then using *P. putida* to upgrade them. Overall, this is an exciting project with strength in analytic chemistry, metabolic engineering, and techno-economic analysis. Progress has been outstanding, particularly with regards to the strain engineering efforts. In the process, the team has been able to capture almost 90% of the carbon in the waste streams. In addition, the team is also using lipidomics to better understand the toxicity and tolerance mechanisms. This is clearly a necessary activity, though still in an early development phase. While the science is great, the impact is a little unclear. As the team notes, this is still early-stage research, so clear milestones are difficult to define as the state of technology is unknown. That said, having some high-risk projects within the BETO/NREL portfolio is considered a positive and should be continued if possible. Moreover, the team is making fantastic progress, so this is not a major concern.

- The project appears well thought out, organized, well managed, and well executed. The project's history of patents, publications, invention disclosures, and public presentations resulting from this work is quite good. The displayed progress and outcomes are substantial—analytics, carbon closure, and conversion, albeit synthetic/defined media utilization. The analytical developments within the project are impressive. Catalytic fast pyrolysis carbon closures of near 100% is really outstanding; however, the instrumentation litany required to do that is also considerable. Instrumentation applied this way as a guide and tool for fundamental research is well merited but not really practical outside of that space. Can the analytical learnings be boiled down to an industrial application or broadly available methodology similar to NREL laboratory analytical procedures? I understand the value of targeting wastewater treatment, but can this apply broadly or correlate directly to other process streams? The presentation indicated that substrate and upstream process changes significantly impact the bioconversion, which I interpret as a relevance challenge. In other words, it has to be robust or the napkin economics falter rapidly. Overall good job, well done!

PI RESPONSE TO REVIEWER COMMENTS

- We thank the Review Panel for their positive feedback and useful, constructive criticisms. One clarifying note: this process is not intended for lignin pyrolysis, but for pyrolysis of whole biomass, which is being pursued in the BETO portfolio. In terms of the industrial viability from an economics perspective, we presented TEA using the NREL pyrolysis models as our baseline to ascertain if this approach could be viable and add value to the thermochemical biorefinery. The definitive answer is that this approach, if successful, could add value to the thermochemical biorefinery, even if a relatively small amount of the original biomass is diverted to coproducts.

  In terms of the presented concentrations, we mention the "g/kg" concentration on a slide that shows the concentration of biomass-derived components in the aqueous waste streams. This number was presented to demonstrate that the range of organic species in the aqueous waste streams, as the reviewer notes, is quite low from a chemical processing perspective, especially for recovery or essentially anything but wastewater treatment in the current iteration of this process concept, but we emphasize that the reported ranges are ideal for biological conversions. Hence, these concentration ranges in the aqueous waste streams from pyrolysis form the entire basis for this project. In terms of the organic content from the starting biomass, this number was also presented, but likely fairly quickly, so we apologize if this was not clear. The starting biomass fraction that ends up in the aqueous waste stream tends to be ≤10%.

  In terms of a simple mass balance, the results of this will depend on the upstream process being modeled. As noted, ~10% of the biomass typically ends up in the aqueous waste stream from catalytic fast pyrolysis. We are targeting conversion of >90% of that, as shown in the presentation. These data were used in the TEA presented on slide 10 of the presentation.

  Regarding the clarity of the TEA, we are intending to report this in an upcoming peer-reviewed publication, wherein we will be able to go into much more detail relative to the short time spent on this in the Peer Review presentation. We stress that this is a low-TRL project as well, a completely new process concept for aqueous waste valorization in a thermochemical biorefinery, and that the TEA shown in the presentation serves as an indication of the viability of this approach. The information that the reviewer is requesting is available and will be reported in future peer-reviewed publications. The use of methyl muconates as plasticizers is being pursued in the "Synthesis and Analysis of Performance-Advantaged Bioproducts" project (2.3.4.501). Certainly there are many hurdles for the industrial acceptance of any new compound, and we are aware of that. However, as shown in the 2.3.4.501 presentation, methyl muconates offer performance advantages over currently used toxic plasticizers that are being phased out of use. More importantly, and as noted during the presentation, the challenging part of the Bioconversion of Thermochemical Intermediates project is not what products we make, but rather overcoming the toxicity barrier and funneling the majority of the carbon into single products—the end products we choose in the effort are merely exemplary. Once carbon is in central carbon metabolism, the tools of metabolic engineering and synthetic biology can be applied to make many different products.

  In response to the lack of material balance and conversion data not being provided, slides 11–13 show that we are able to biologically convert ~90% of the carbon in catalytic fast pyrolysis aqueous streams in engineered strains of *P. putida*. Moreover, the presentation showed the amount of organic material going into the catalytic fast pyrolysis waste streams.

  In terms of a thorough TEA and LCA to demonstrate process viability, we have conducted this, as was shown on slide 10 of the presentation. The TEA done to date demonstrates that this process concept is potentially viable, but certainly more bench-scale research is needed to achieve the metrics set by TEA. This is a wholly new process concept, and accordingly, it will be some time before it is ready for scalability.

In terms of the impacts, the overall purpose of this project is to mitigate or avoid costly wastewater treatment processes for the thermochemical biorefinery. Today, pyrolysis and catalytic pyrolysis processes generate highly toxic wastewater that is not readily amenable to anaerobic digestion (often needing a 1,000-fold dilution), and this waste stream, besides being a cost to the biorefinery, also represents a loss of valuable carbon from biomass. We intend to report more thorough TEA and LCA cases for this process concept going forward in the peer-reviewed literature, to bolster the scientific and engineering advances here in analytical chemistry and metabolic engineering. The comment from the reviewer about the accessibility of the analytical chemistry methods is valid—indeed, these are by no means trivial measurements to do. This is why we have worked with industrial partners in the past, as very briefly mentioned during the presentation, to help them to understand their aqueous waste streams. We have published these methodologies in the peer-reviewed literature as well, such that companies and organizations with adequate instrumentation can reproduce our results. Certainly the continued transfer of these methods to the broader community is a key effort in our upcoming efforts. Accordingly, we anticipate that this project can have even broader impacts for the industrial community.

LIGNIN UTILIZATION

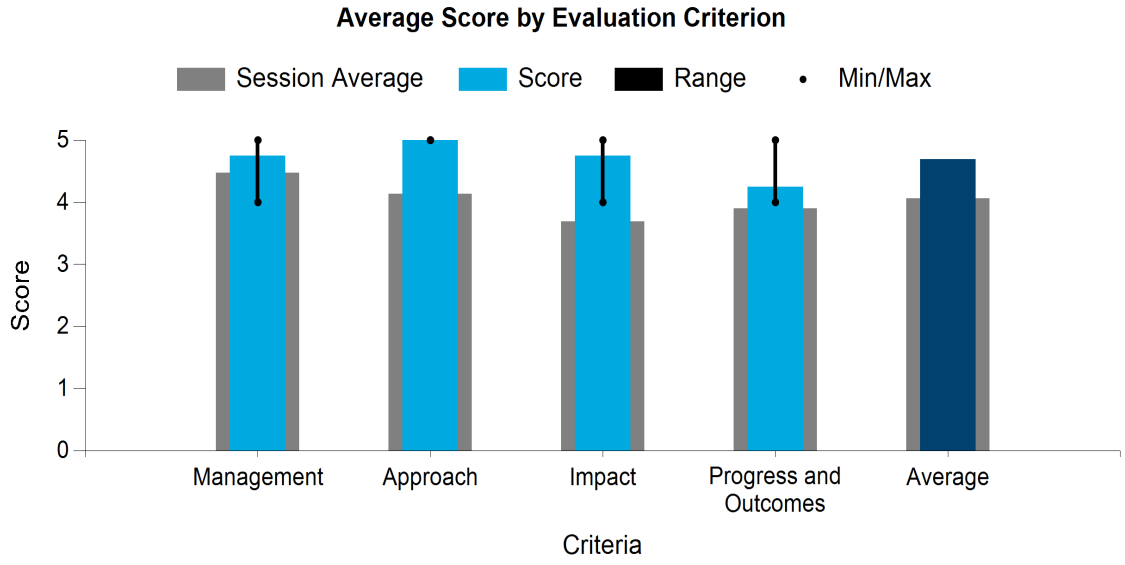
National Renewable Energy Laboratory

PROJECT DESCRIPTION

WBS:	2.3.4.100
Presenter(s):	Adam Bratis; Gregg Beckham; Michelle Nolker; Zia Abdullah; Courtney Payne
Project Start Date:	10/01/2019
Planned Project End Date:	09/30/2022
Total DOE Funding:	$4,350,000

Valorization of lignin has massive potential economic and sustainability benefits for the lignocellulosic biorefinery. However, challenges remain to realize lignin conversion to coproducts, especially related to selective, high-yield depolymerization to monomers and quantitative analytics on lignin, the latter of which is critical for accurate process modeling. Towards these goals, the Lignin Utilization project focuses on catalytic lignin deconstruction chemistries for C-O and C-C bond cleavage, development of analytical chemistry techniques to quantitatively characterize lignin, and syntheses of requisite model compounds for understanding lignin transformations. This work is done with and supports multiple BETO projects, including the Biological Lignin Valorization project, the Separations Consortium, and others.

Outcomes of Lignin Utilization for analytics and synthesis include (1) development of new mass spectrometry (MS) methods to characterize lignin dimers/oligomers in process streams, (2) deployment of a computational-experimental tool (with Biochemical Process Modeling and Simulation) to identify lignin-derived compounds with high fidelity from MS, and (3) delivery of >40 unique compounds. From a catalysis perspective, we have developed new oxidative approaches to cleave C-O and C-C bonds and produce >50% bioavailable aromatic monomers for biological funneling for the BLV project and have developed recoverable bases for base-catalyzed deconstruction of lignin.



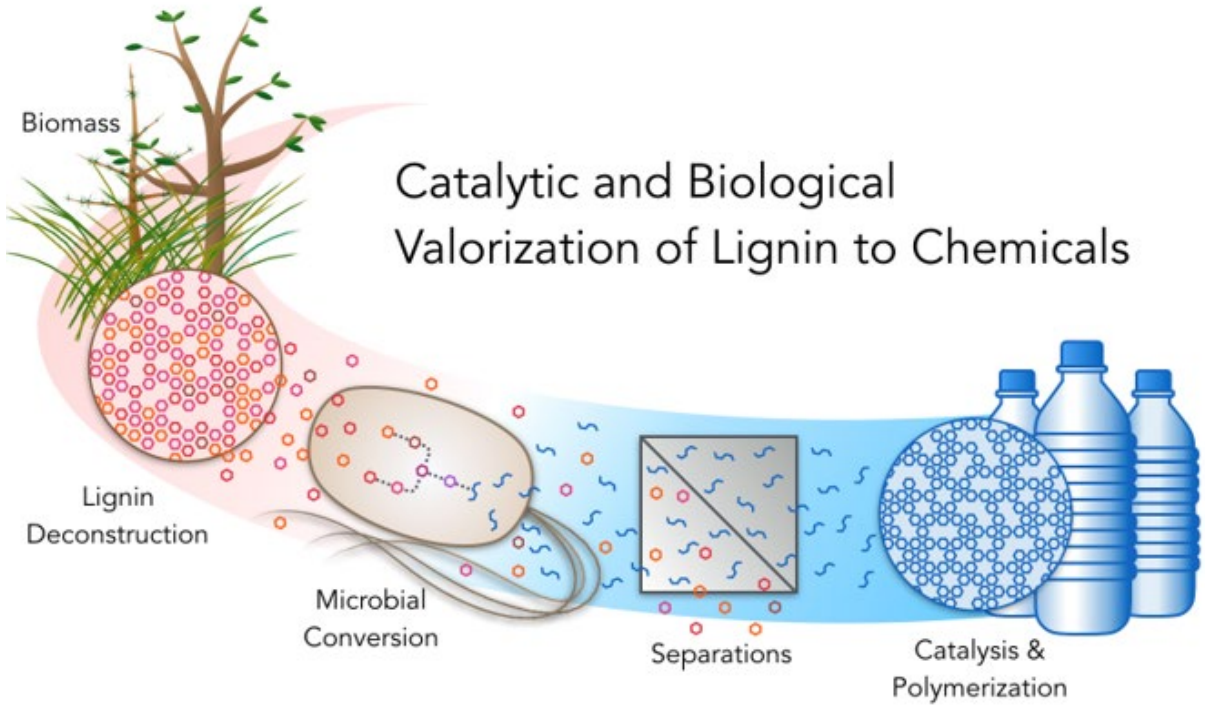


Photo courtesy of NREL

COMMENTS

- Management and university partnership provide a balance that is critical to developing lignin biorefinery. Tasks and subtasks are provided. Risks and risk mitigation are addressed. The approaches taken are novel and provide BETO with a good test model for valorization of lignin. The impact on catalytic development requires much needed and timely good analysis, and the establishment of a library of lignin compounds and derivatives is critical to success of this approach. This database will also enable other lignin valorization projects that aim to derive products from lignin. Progress and outcomes look promising as results reported so far, with two oxidative pathways developed, as well as the use of new analytical tool and new base with promising recovery identified. The available and cost of $Sr(OH)_2$ may be an issue. The researchers do not indicate whether $Sr(OH)_2$ is currently used at any wood pulp plants, and if not, why that is not the cases. The project needs more information on material balance and conversion yields for a good TEA, as well as more information on TEA and LCA that compares the kraft process with the use of $Sr(OH)_2$ side by side. On the overall, this is a good project with proof of concept demonstrated. It's hard to assess commercial viability without material balance.

- Strengths: The high-level impact of this work is clear. The project provides centralization of several efforts addressing the BETO goal of converting lignin to high-value products, and as such, plays an important role in BETO's biorefinery operating scenario. The inclusion of a robust set of analytics is a notable strength of this effort. For this project, the analytical work provides background information used in developing approaches for the catalytic, oxidative cleavage of recalcitrant C-C bonds in lignin, leading to more tractable low-molecular-weight fragments. More broadly, inclusion of this research activity is a good complement to reductive lignin deconstruction ongoing in NREL's Lignin First project, as well as other lignin deconstruction projects in the portfolio. The effort in synthesizing model compounds as analytical standards and coupling that work with MS highlights the power of MS for rapid lignin analysis, as has been demonstrated in the literature (e.g., Kenttamaa, Purdue). Importantly, these techniques are also made available to potential industrial stakeholders. The presentation indicates that the work is moving beyond the DMR lignin to include kraft lignin as a possible feedstock. This is an

improvement over earlier versions of this project, which seemed to be limited to the DMR approach. Demonstrating applicability of the lignin conversion technology to a wide range of known sources is an important step for increasing the impact of the work. The efforts to develop new lignin oxidation processes via polyoxometalate catalysis and autooxidation have shown some success in the ability to cleave recalcitrant C-C bonds in lignin. This holds the potential for improving the amount of lignin that can be converted into monomeric, low-molecular-weight compounds. Coupling this work with the exciting results from the Lignin First project offers the potential of increasing the yield of useful materials from the biorefinery's lignin fraction. The team reports interesting results on base recovery in the DMR process, and have found that $Sr(OH)_2$ is a good substitute for NaOH with several operational advantages. Significantly, the new approach gives a higher yield of monomers and lower cost because of its simplified recovery.

Areas for improvement: More clarity around the interest in kraft lignin would be helpful, given its importance as an energy source for the pulp and paper industry. The PIs indicate that they will take 1%–2% of the chemical recovery stream from a kraft mill as feedstock, but is not clear whether that is isolated kraft lignin or kraft black liquor. If the latter, it will be important to define the amount of black liquor being used, as mill operators are unlikely to give up a significant fraction of their black liquor fuel. More information on the operating scenario with the paper industry would also be helpful—for example, by translating this amount of kraft lignin into potential pounds of a final, single product, the number of mills needed to actually make an impact on $/GGE, and the envisioned means for collecting and centralizing disperse sources of black liquor/kraft lignin. Evaluating the impact of the project's new developments is more difficult, as the presentation did not include any TEA results, especially in the conversion of kraft lignin. This complicates evaluation of the potential impact of the work, and more economic modeling will improve the program. Results on polyoxometalate oxidation efforts show, consistent with similar literature efforts, that C-C bond cleavage proceeds to give low yields and mixtures of products. Use of real kraft lignin reveals molecular weight reduction, but further low yields of useful monomers or low-molecular-weight derivatives. Autooxidation gives higher yields with simple models, and employing this approach to RCF oil also shows molecular weight reduction, but no analytical data as to the composition of the resulting oxidized oil are given. Part of the issue is that both polyoxometalate and autooxidation are methods for bleaching/delignifying cellulose pulp, where the objective is simply to break lignin into small, easily removed pieces without consideration of the yield or further utility. More details on the composition of the oxidized lignin mixtures will be helpful, but the literature suggests that complex mixtures of products will result.

- The goal of this project is to develop processes for deconstructing lignin along with analytics and model compounds. In these regards, the project plays a central role in BETO's lignin valorization portfolio. The team is highly capable, understands the challenges, and is making excellent progress. In addition, the analytic portion of the project has the potential to greatly impact the field. Overall, this is an outstanding and well-managed project. No concerns or issues are raised.

- The presentation was excellent, and I really liked the thoughtful breakdown of the project, milestones, impacts, objectives, responsibilities, risks, and reporting. With all the various entities and activities within projects, the presentations can be disheveled. This project is well managed and clearly communicated, which to me carries a great deal of value. The project is well integrated and the industrial segment collaborations and partnerships speak volumes to this project's competence. The analytical developments have been substantial. Laboratory analytical procedure dissemination for analytics and synthesis methods will be a great offering for the global community. Internal development of the beam-MS and MS lignin library with experimentally generated fragmentation data are great achievements. The Lignin Wrangler not only possesses a great name but provides much needed functionality for lignin analytics. This team is filling the lignin analytical toolbox for the entire industry's usage. Having only one analytic chemist when it is so critical to development rate seems to be a bit of a bottleneck. Of

course, staff of that caliber can be tough to find, and I'm confident that resourcing allocation has been contemplative. Great project, well done!

PI RESPONSE TO REVIEWER COMMENTS

- We thank the Review Panel for their constructive feedback and positive comments on the Lignin Utilization project. We are very excited about the potential for this project to contribute in multiple ways to the ultimate valorization of this important biopolymer. We apologize that this was not clearer in the presentation, but we note that the Lignin Utilization project has always intended to provide catalytic deconstruction approaches that could be broadly applicable to technical lignins as well as biorefinery lignins. The DMR process, as the reviewers noted, is an important component of the BETO biochemical conversion process development, but the Lignin Utilization project certainly aims to be much more holistic in its impact and application. Our efforts on kraft lignin with polyoxometalate-mediated catalysis are fairly nascent at present. That said, we fully agree and recognize that kraft lignin is an important substrate, and after we are able to close mass balances on kraft lignin depolymerization processes (from isolated kraft lignin, to clarify), we will conduct TEA and LCA on this approach to understand if this is a potentially viable route. The reviewer's suggestion in terms of how to approach the TEA is very much aligned with our standard analysis approach. As noted above, we intend to provide deconstruction approaches that can be applied broadly, not just to kraft lignin, though kraft lignin is a useful substrate in terms of its availability for research and as a condensed lignin structure to study cleavage of recalcitrant C-C bonds. The autoxidation efforts are underway as a major component of the Lignin Utilization efforts (and polyoxometalate oxidation as a parallel effort), and detailed mass balances will be soon reported in forthcoming publications. We agree that yield measurements and mass balances are critical. However, as the reviewers know, these measurements take a substantial amount of time to do rigorously for lignin. In terms of products, we fully agree that lignin oxidation products will be heterogeneous—this is why the Lignin Utilization project is working very closely with the Biological Lignin Valorization project to use microbes downstream of oxidative depolymerization to be able to produce high yield of single lignin-derived products. Regarding the use of reversible bases, we are conducting interviews with industrial representatives to understand the potential viability and potential pitfalls for the reversible base concept in the biorefinery context. As described during the presentation on slide 18, the reversible base concept appears from a TEA and LCA perspective to be viable, at least for use in a DMR-type process. In terms of assessing the commercial viability of the project, we note that we have conducted rigorous TEA and LCA already, as shown on slide 2 of the presentation, which indicate substantial potential in lignin valorization for biochemical conversion processes with lignin utilization.

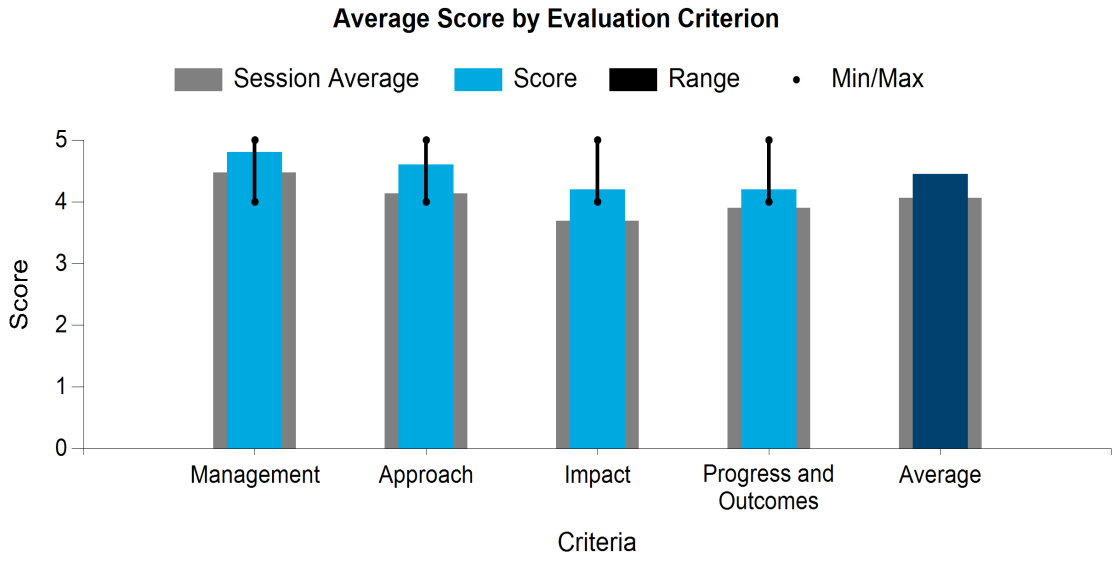
BENCH-SCALE INTEGRATION

National Renewable Energy Laboratory

PROJECT DESCRIPTION

WBS:	2.4.1.100
Presenter(s):	Adam Bratis; Nancy Dowe; Zia Abdullah; Courtney Payne; Jessica Krupa
Project Start Date:	10/01/2020
Planned Project End Date:	09/30/2023
Total DOE Funding:	$750,000

Bench-Scale Integration develops and optimizes fermentation processes to produce bio-based fuels and chemicals from biomass sugars. The project uses fermentation science to achieve high titers and production rates—for example, by manipulating how the microorganisms are fed biomass sugars and nutrients, modifying fermentation conditions (pH, temperature, aeration) or developing online control strategies that increase production rates. For this period of performance, we conducted small-scale fermentation research using strategies mentioned to produce 2,3-butanediol from biomass utilizing NREL's proprietary *Zymomonas mobilis* microorganism. BDO is a versatile chemical that can be catalytically upgraded to a variety of hydrocarbon fuels and chemicals. The 3-year project goal was to increase BDO titers from 50 g/L (2017 level) to 125 g/L on biomass sugars by 2020. We successfully achieved this target, which is a 150% increase in titer, thus showing the commercial potential of *Z. mobilis* while reducing the cost to downstream processing. The project also showed yearly progress improvements to the process design case using a whole biomass slurry. Future work will focus on scaling to produce large quantities of BDO broth for separations and upgrading R&D and improve the titer and rates from biomass whole slurry. Our goal is to produce 100-g/L BDO titer from whole slurry at >1,000 L by 2023, which will meet the technical targets set by our techno-economic analysis.



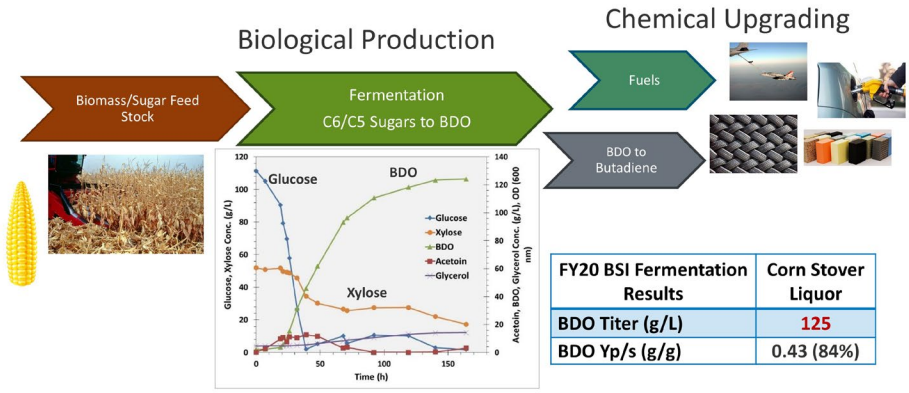


Photo courtesy of NREL

COMMENTS

- This is a balanced team approach with good coordination and leveraging with other groups (e.g., strain development, engineering, process development, end-product uses, pretreatment). Risks and risk mitigation is addressed in great detail. The approaches used in fermentation development and rapid analysis to meet targets looks very good. The project is within the scope of BETO's goal of enabling cost-effective production of biofuels and chemicals. Using near-infrared (NIR) to monitor fermentation is useful, timely, and innovative. Project impact to BETO is relevant to its stated mission. Industry interest with ExxonMobil is worth pursuing, as production scale-up plans to larger scale will require the involvement of an end user to optimize catalytic upgrading for that scale. Bio BDO from biomass will still be economically challenging as compared with petroleum and sugar/starch fermentation. A side-by-side comparison of these would be helpful in providing technological and economical barriers that need to be overcome. Progress and outcomes are promising. The reviewer is puzzled by the statement that the whole-slurry fermentation has poor reproducibility. Is this due to variability in DMR provided or instability of the strain used? It would be helpful to provide a good explanation, as it has implications to strain development and/or pretreatment process. The overall impression is that the team has had a good success record in achieving high titers of 2,3-BDO and yield that approaches targets and TRY to meet TEA/LCA. The incomplete use of pentoses reflect that the strains need further engineering. The efforts to address redox imbalance has been abandoned, so the fermentation process has to be microaerophilic with air blanket and/or by increased back pressure to ensure oxygen is available to cells. The ability to use whole broth in catalytic upgrading is promising. It suggests that targeting the reduction or eliminating glycerol production by the strain for improved yield and conversion of 2,3-BDO are things that need to be accomplished to ensure optimal catalytic upgrading.

- Strengths: This project is an important contribution to the program's carbohydrate conversion effort in that it is examining engineering solutions to improve the production of chemical intermediates. This project area has played a central role in the BETO program for several years, and is now closely linked to Zhang's work with engineered *Zymomonas*. The project's inclusion of a risk register is a strength and a more detailed evaluation of potential barriers than seen for other projects. Process engineering offers an important complement to the program's microbiological work in that it investigates methodology and

infrastructure necessary to transition a biological conversion to pilot and eventual commercial application. As such, it occupies a crucial position between the lab and industrial stakeholder, showing stakeholders that laboratory results can also have industrial application. It also demonstrates that engineering solutions can have a significant impact on bioprocess productivity and TRY. Finally, the incorporation of rapid NIR analysis is a strength of the program in that it provides real-time monitoring of process modifications. The project has made good progress in the production of BDO. The scale-up of the process to 100 L is notable, and has afforded the production of kilogram amounts of isolated product at 87 g/L. The project has met the 125-g/L target from the DMR corn stover feedstock under optimal conditions, and can get reasonable yields even using whole DMR slurry.

Areas for improvement: It would strengthen the presentation if there was more discussion about the dependence of BETO's fuel production scenario on the success or failure of this effort. Zhang's presentation indicated that several of the approaches tried for improvement at the lab scale were not successful. If the engineering solutions don't meet goals, is the program's high-level scenario in trouble? More information on the impact of these lower titers on the potential for success would be helpful—i.e., is 87 or 92 g/L viable? Despite the target, is there a value that is close enough? What is the effect of the mitigation plans on the ultimate cost of fuel? How dependent is the program on making BDO work—i.e., is there a plan B? The presentation would be improved with more economic information about the production cost of BDO in comparison to conventional BDO production from 2-butene via epoxidation. Slide 12 indicates the potential of BDO as a chemical coproduct. Thus, the same general questions regarding conversion of a known marketplace product to a fuel are pertinent. The rationale behind making cheap BDO as a single product and then turning it into a lower-value fuel could be explained more clearly.

- The goal of this project is to develop and optimize fermentation processes for different bioconversion processes with a focus on scale-up and de-risking technologies. Overall, this is a valuable service and integrates well with the other activities within the BETO/NREL portfolio. Current activities are focused on developing microaerobic fermentation processes for 2,3-butanediol production from lignocellulosic hydrolysate at 100-L scale. Overall, the team is making good progress and meeting their milestones. In addition to these pilot-scale studies, the team is also developing NIR and Raman spectroscopy for real-time process monitoring and control. This is viewed as a positive. While no major concerns are raised, the project would be strengthened by developing more technologies. The work with NIR and Raman is clearly a step in this direction. It would be great to see more activities along these lines, such as off-gas analysis and, assuming it is feasible, computational modeling.

- This project has the important role in de-risking the scale-up of bioreactors for biofuel production. The current focus on 2,3-BDO production with *Zymomonas mobilis* creates a nice integration with other projects in NREL and in the BETO portfolio. In particular, finding strategies to optimize how aeration is used to maximize 2,3-DBO titers and production rates seems to be essential for achieving the overall 2030 goals for the MFSP. The reported progress is impressive. Reaching titers of 125 g BDO/L in fed-batch reactors fed DMR-processed liquor (without solids) and 92 g BDO/L using DMR-processed whole slurry, both at a 100-L scale, shows that the optimization approach is working. The comparison of feeding the bioreactor with whole slurry after enzymatic hydrolysis or whole slurry before enzymatic hydrolysis (with enzymes added) is interesting but may require different optimization strategies, specifically in relation to enzyme loading and pH that were not reported. The work with near-infrared sensors for rapid monitoring of the fermentation process is promising.

- This project is loaded with relevance aimed at the end game; the industrial perspective is spot on. There is holistic research front to back, bench to industry, in order to de-risk technology. The goals are lofty—$2.47 MFSP design target case—but the progress and outcome's advancements enhance reviewer confidence. The achievements of 125-g/L titers in fed-batch mode, nearing design case technical targets, NIR development, and kilogram-quantity production of BDO are very good indeed. Those achievements,

combined with knowledge being gained on scaling aeration, vessel sizing/geometry, agitation setup, and the feedback from the separations team, has the project in good stride and poised for additional success. Even the project's mission statement "We want robust and industrially relevant fermentations for bio-derived products with reduced carbon intensity to lower greenhouse gas emissions and improve sustainability" is really great and well received. The analytical development related to NIR is great and could eventually be applied broadly. NIR is a very useful tool, and even though it is not inexpensive for an online system, it is still cheaper than off-gas MS. I am curious, though, why dissolved oxygen is not enough for process control. Also, does the NIR have the utility of off-gas MS? I am excited at the prospect of drop-in conversion processes at corn ethanol plants and the evaluation of solid-state fermentation (multiple fermentation modes) on multiple substrates. It is clear that advanced control over sugar feed rates and aeration levels to balance redox are needed, although Gen1 doesn't currently have this level of intricacy. Please continue to push strain robustness in all aspects, especially kinetics. It is true that tank space is relatively cheap; however, 120–165-hour fermentations present challenges. It is useful that BDO is less toxic, although ethanol's toxicity and rapid kinetics provide value than shouldn't be overlooked. It is my opinion that the fermentation is scalable, and so are sterilizing media and precise oxygen control, those features can be done, sure, but they are not currently being done cheaply at scale. The microaerophilic process scaling mitigation plan is inadequate. Just because Genomatica was able to scale their fermentation to a pharmaceutical-grade, industrial-scale aerobic fermentor on dextrose, that is way different than whole-slurry biomass processed in a $2/gal installed CapEx Gen1 fermentor. All I am saying is that—and you are likely aware—it is significantly different, and to say it can be done here because of Genomatica isn't enough. If the BDO TEA is using a pharma-grade fermentor and dextrose then so be it, but I don't think that it is. Overall this is a great project, well done!

PI RESPONSE TO REVIEWER COMMENTS

- We would like to thank the Peer Reviewers for their time and thoughtful reviews. We appreciate the constructive feedback on the project's vision, mission, and objectives: to develop industrially relevant fermentation processes for bio-derived products that de-risk scaling, lower carbon intensity, and improve sustainability. We also appreciate the reviewer's acknowledgment of the 100-L scale-up that produced kilogram amounts of product and meeting the 125-g/L titer target. Our results show that our optimization efforts are working and that fermentation engineering solutions can positively affect titer, rate, and yield.

  The project will continue to use a risk register to address technology development barriers and continue developing analytical methods like NIR and Raman spectroscopy to aid in fermentation control and enable scaling. Both the risk register and the NIR work were positively received by the reviewers. The reviewers provided excellent feedback for the project's future work and ways to improve future project presentations. As the reviewers pointed out, a deeper dive into the economics to present a comparison between starch-based fermentation, biomass-derived fuels and coproducts from BDO, and the equivalent fossil-derived fuels and coproducts is warranted. We plan to use NREL's pilot-scale bioreactors to address scaling microaerophilic slurry fermentations to aid in scaling beyond 9,000 L; developing online and rapid at-line analyses will be important to this effort. Our early work to feed and mix biomass slurries to reach higher solids concentrations for higher BDO titers has been a challenge to produce consistent results at 0.5-L scale, and we will continue to address this issue. Much like wind and solar have benefited from value engineering over the past 20 years and are now producing some of the cheapest electricity, lower BDO production costs and improved titers, rates, and yields can still be achieved through fermentation engineering in the absence of strain development. Thus, this project can still provide a viable pathway for BETO to achieve their fuel production goals.

CONTINUOUS ENZYMATIC HYDROLYSIS DEVELOPMENT

National Renewable Energy Laboratory

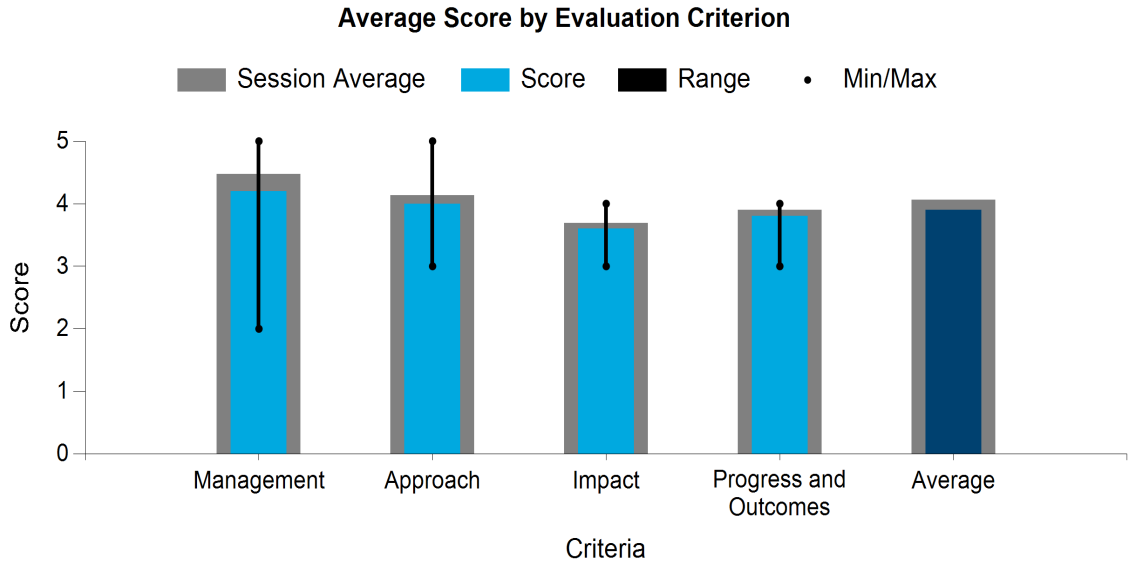
PROJECT DESCRIPTION

The Continuous Enzymatic Hydrolysis Development project aims to reduce the cost and commercialization scale-up risks of biorefinery sugar-lignin production through development of a deployable continuous enzymatic hydrolysis (CEH) process. Through the use of external cross-flow membrane filtration 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 achieved through a series of reactor-membrane unit stages.

WBS:	2.4.1.101
Presenter(s):	Adam Bratis; Jim McMillan; Zia Abdullah; Courtney Payne; Jessica Krupa
Project Start Date:	10/01/2020
Planned Project End Date:	09/30/2023
Total DOE Funding:	$1,650,000

This 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 than traditional batch enzymatic hydrolysis (BEH). The project's primary objective is to reduce the cost of CEH to be compellingly lower than conventional BEH—10% lower in year 1 and 20% lower in year 3 (end of project). A related objective is to expand CEH's operating envelope to increase process efficiency. Through more thorough de-risking and demonstration of CEH, in conjunction with building a suite of modeling and optimization tools that allow for more facile rigorous in silico evaluation of novel CEH designs and modalities, this project intends to elevate industry interest in adopting CEH as an improvement over conventional batch processing.

This project was merit-reviewed in FY 2020. It's now in its first year of a new 3-year plan spanning FY 2021–2023.



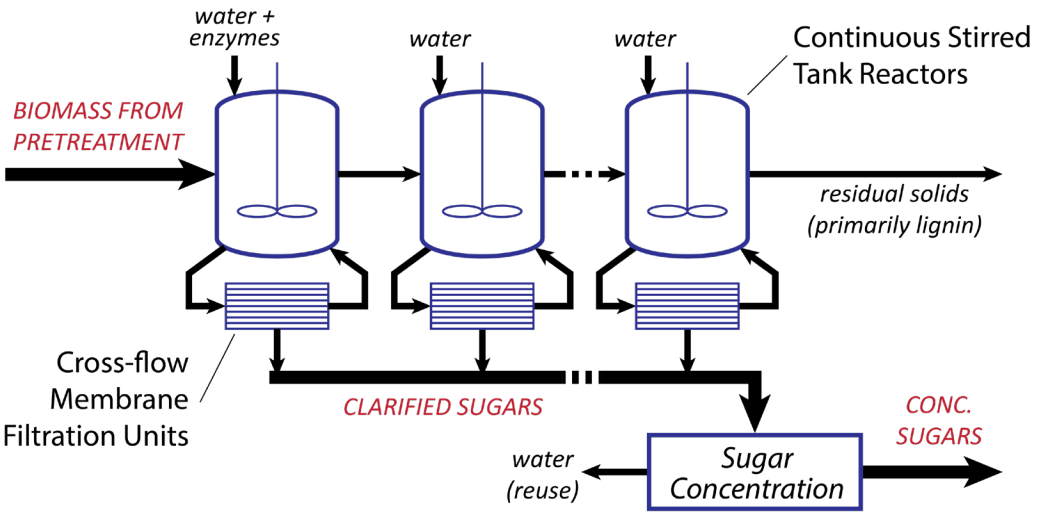


Photo courtesy of NREL

COMMENTS

- Management team is well leveraged with other working groups. Risk mitigation strategy is in place. This project provides a good approach to validate the use of continuous enzyme hydrolysis. If successful, this approach can offer advantages over batch hydrolysis in reducing operating expenses/CapEx from reduced enzyme dosing. CEH, if sustainable over many cycles, is a better fit for large-scale deployment as compared with batch reactor operation. Enzyme supplier costs continue to be significantly high even in the best-case scenario. Enzyme loading and turnover is not discussed, so this needs to be considered and addressed. Solids loading is still lower than desirable, as higher solids have been demonstrated at pilot scale with acid- and organosolv-treated corn stover. Feedstock handling and limitation to pumping is an issue with DMR. Can the paddle reactor operation be validated with carboxymethyl cellulose/cellulose gum, which is inexpensive and readily available for a proof of concept? This can allow for demonstration enzyme stability over many cycles and enable measurement of loss of activity of some of the components in the commercial blend being tested. Better economics will require high solids loading, improved enzyme performance, staged enzyme loading, reduced energy use, and improved membrane and pump operation. It will also provide a baseline assessment that can compare NaOH DMR and DMR produced by two-stage chemical treatments. This can help improve impact and industrial relevance. Progress seems reasonable. PI should consider use of ceramic membranes that can be cleaned and reused, as cost of membranes (fouling, longevity) is an issue. Increasing solids loading should help reduce evaporation costs for the enzymatic hydrolysis slurry. On the overall, this project is worthwhile and done at a reasonable scale to validate the concept.

- Overall, this project received a lower score due to perceived pragmatic issues; however, this work is vital and I absolutely recommend continued development. The presentation data suggesting 80%–85% conversion yields in 24 hours is a remarkable feat. The valuation shift to MSSP from MFSP is valid; creation of clean, cheap sugars will open many doors. Considering the process is continuous, contamination or sterility enhancements could be an item of contention regarding risk identification and mitigation strategies. At relatively low percent total solids loadings, dilute sugar levels, limited concentration pressures, and contamination control would seem to be essential in this fertile environment. Collaboration with Mike Himmel for high-temperature, alkaline, acidic enzyme systems and the lignin group with respect to solids utilization post-hydrolysis might add value here or be currently underway. Similarly, cycling up of unwanted large (>50-kDa) soluble and insoluble compounds could occur with microfiltration retentate recycling, or smaller unwanted soluble compounds if ultrafiltration recycling is employed. Longer run campaigns might elucidate this phenomenon. It

appears that the CEH process will still require solid/liquid separation post-hydrolysis, albeit smaller, but still necessary for residual lignin recovery. Perhaps the lignin is recovered from the microfiltration retentate, but the process flow diagram does not indicate this. The PDF shows unconverted solids removal from the hydrolysis tank somehow. A better illustration of the lignin retentate stream (washed or concentrated) and solids purge in the process could be helpful; it is not clear how residual lignin solids will be selectively separated from fresh corn stover. This leads to the crux of my concern regarding enzyme recycle strategy. The issue mentioned briefly in the quad chart, selective separations, is not clearly communicated. If the ultrafiltration system is not required for recycling because the enzymes are bound to the solids, as suggested in the presentation, the solids have to leave the system eventually. If compositional analysis suggests there to be no unconverted glucan/xylan in the residual substrate, then perhaps enzyme recycle is possible with this demonstrated configuration. Finally, with regards to impact and comparative economics, it would be helpful to know if the higher enzymatic hydrolysis yields shown are due to benefits from CEH or from lower solids loadings compared to the BEH results.

- Strengths: This is a straightforward project that has good potential to streamline the production of fermentable sugars and reduce their cost through transition from fed-batch to continuous operation. This translates into a potentially significant reduction in the $/GGE. This project is also a logical next step to investigate in parallel with ongoing organism engineering and process improvements for sugar production. Preliminary economics support this contention, with higher sugar yield offsetting the initial higher cost of installation. A single-stage reactor has been assembled, based on in silico virtual engineering and TEA to help guide construction, and is in operation, having been tested on dilute acid feedstock at >8.5% solids. Initial tests with DMR feedstock reveal that current solids level is limited to <10%. As a result of this observation, the PIs plan to carry out more simulation experiments to optimize the process. This is a strength and another illustration of the strong integration of computation and experiment within the program.

  Areas for improvement: The PIs are targeting slurries with 10% solids. To provide a benchmark, it would be useful if the PIs could present industrial analogies that carry out selective, continuous transformations at high solids levels to get a sense of the challenge. The PIs indicated cement pumping as a possible analogy, while also acknowledging that there will need to be trade-offs (in future evaluations) between equipment costs and sugar yields. Information on whether the restrictions of DMR material negatively influence the potential for success of the overall biomass-to-biofuel process would be helpful. This is important, given the emphasis that NREL has put on the DMR process. A couple of minor items: on slide 5, project partners are listed as informal. In addition, on supplemental slide 22, the team implies that they are having ongoing problems actually getting an industrial partner on board. What does this say about the industrial response to this effort, if anything?

- The goal of this project is to develop a continuous process for the enzymatic hydrolysis of pretreated (dilute acid and DMR) lignocellulose. Overall, the project has a strong management plan, has clear goals, and is making good progress towards meeting their objectives. The project is still relatively new, so it currently difficult to evaluate whether the team will ultimately achieve their objectives. With regards to the execution of the project, no concerns are raised. The approach also seems reasonable. However, the impact is somewhat unclear. The project would greatly benefit from additional justification for a continuous process. Such processes introduce additional operational complexities (e.g., startup, stability, and process interruptions), especially when considering the challenges associated with material transport. In addition, unlike a traditional petrochemical process, the feedstocks are heterogeneous. The justification was unclear during the review process beyond providing TEA numbers without discussing the underlying assumptions and limitations.

- This is a challenging project that seeks to develop CEH technology using reactors in series and membrane filtration in each reactor to separate the free sugars from the slurry containing the biomass and the enzymes. Great progress has been made with the single-unit experimental setup, which has allowed

identification of where to focus to overcome the challenges of working with the DMR slurries and operating filtration units at higher solids content in the slurry. The virtual engineering part of the project, which uses the output from the single-stage reactor-filtration unit to simulate flow-through operation in a multistage system, is more of a black box. From first principles of reactor engineering, the benefits of a multistage system are due to higher reaction rates that can be achieved in the initial stages of the system (because of higher substrate concentrations), which in turn could result in a smaller overall reactor volume to achieve the same conversion efficiency compared to a continuous single-stage system. Virtual engineering would allow simulations to be performed assuming different numbers of stages in series, which could result in different outcomes in terms of total volume needed to achieve the same conversion efficiency. Do the investigators see a benefit of such analysis, or has the project been preset to simulating three units in series? It is also not clear how reactor volumes compare between batch hydrolysis and the simulated CEH system, and what the impact of tankage size is on the predicted installed costs. Other projects in the BETO portfolio are aiming at using enzyme loadings of less than 10 mg protein per gram of cellulose. Is this project maintaining that same target for enzyme loading? Would success in demonstrating CEH feasibility contribute to the goal of reducing the enzyme loading, or would the process require higher enzyme loadings? Finally, integration with the Biochemical Platform Analysis project could be improved to facilitate how projects describe the results of TEA. This project uses a normalized metric of the MSSP instead of the MFSP that is used in several other projects within the BETO portfolio. The change in metrics makes it difficult to compare with other projects; the use of a normalized metric may be useful within the project itself, but again, makes it difficult to compare to other projects. If the MSSP is the preferred metric, reporting could benefit from information about the 2030 goal for MSSP that the Biochemical Platform Analysis project uses, and how far from that goal this project currently is. Likewise, the Biochemical Platform Analysis project could better highlight the 2030 goals for MSSP that this and other projects in the portfolio may be using.

PI RESPONSE TO REVIEWER COMMENTS

- We thank the reviewers for their insights and helpful, constructive comments and feedback. We recognize that the ability to efficiently pump slurries at high insoluble solids levels is required to realize significant economic benefits of using CEH versus traditional BEH. We remain focused on real-world pretreated lignocellulosic feedstocks rather than model delignified feedstocks like carboxymethyl cellulose, as these materials have quantitatively different rheological and enzyme binding characteristics (e.g., different upper limits at which slurries can be pumped, as well as different enzyme-substrate adsorption/desorption behavior). Nonetheless, the use of model feedstocks will be pursued for longer-term enzyme stability tests during CEH operation if supply of DMR pretreated feedstock becomes problematic. We also agree that further proof-of-concept CEH operation over longer times and at higher insoluble solids levels is necessary, and we have planned and are already performing experiments on this using the existing industrially relevant (mini-pilot) unit-stage CEH equipment setup.

  Membrane selection remains another key area of focus. We plan to further explore the effects and sensitivities of CEH performance to different membrane chemistries and nominal pore sizes, and this work will also consider ceramic membranes if they are available in suitable form factors and sizes. Our strategy has been informed by examining existing industrial slurry handling processes, particularly membrane-based wastewater treatment processing, and we used prior wastewater treatment knowledge and experience to guide our research and system design. Evaluation of trade-offs involved in CEH processing will extend beyond CEH operation to also examine how differently pretreated feedstocks (dilute acid vs. DMR pretreatment) perform.

  As far as formal partnering goes, ongoing relatively low petroleum and transport fuel prices are contributing to industry being slow to adopt biomass-to-sugars technology. However, we do have industry-associated partners facilitating the project—i.e., both Porex, Inc. (current membrane manufacturer) and an independent membrane separations consultant from industry.

This project absolutely sees value in analysis and accordingly is proceeding on an "analysis first" basis. We have been using a variety of different analysis and modeling techniques, including complex and integrated mass and energy balances and TEA, to analyze a multitude of factors impacting the economics of CEH: reactor volumes, number of unit stages, enzyme and solids loadings, membrane permeation rates and lifetimes, etc. Prior process simulations and associated TEA have guided CEH development and identified areas of cost sensitivity or knowledge uncertainty for R&D focus (e.g., membrane performance). With the help of a more detailed virtual-engineering-based representation of each component of the CEH process, we will be able to simulate all aspects of the process with a greater degree of accuracy and confidence in results that meaningfully translate to larger scales. Initial and ongoing TEAs are being done in the context of/to be consistent with BETO's biomass program; for example, using the program's nominal baseline enzyme loading target. This said, we believe there is potential to ultimately reduce enzyme loadings in CEH compared to what is required for BEH. Most significantly, at similar enzyme dosages, through reducing sugar product feedback inhibition on the enzyme system, CEH has the ability to achieve faster conversion and higher sugar yields than BEH, and this may prove the more powerful economic lever than reduced enzyme loading. It is also to be noted that the Biochemical Platform Analysis project also maintains the so-called sugar model (that estimates MSSP rather than MFSP), and that the sugar and integrated biorefinery models are consistent up through sugar production. To date, TEA has indicated CEH is economically favorable to BEH assuming it can be reliably operated at specified performance levels. With the help of more detailed virtual engineering, we hope to further elucidate the effects and sensitivity of the CEH process to additional factors such as contamination and other scale-up issues.

A strength of the approach with respect to contamination is that sugars do not accumulate to high levels in CEH, and like BEH it is operated at temperatures of 50°C or higher, which is also beneficial to keeping down potential contaminants. To clarify, the presentation data labeling were misleading in implying 80%–85% conversion yields were obtained in 24 hours. Rather, this was reporting monomeric sugar yields (rather than total solubilized sugar yields) obtained in BEH over 7 days after removing sugars that had been produced after 24 hours. We recognize that there are many trade-offs involved in CEH, and our approach is leveraging the strength of innovative process design combined with the power of virtual-engineering-based TEA to analyze and select the overall best process configurations for further testing and development. Major trade-offs such as recycling enzymes with separate membrane systems (e.g., using multiple membrane types) or optimizing a single membrane slurry filtration-based design are being evaluated and will be used as necessary to enable the best overall process design to be realized.

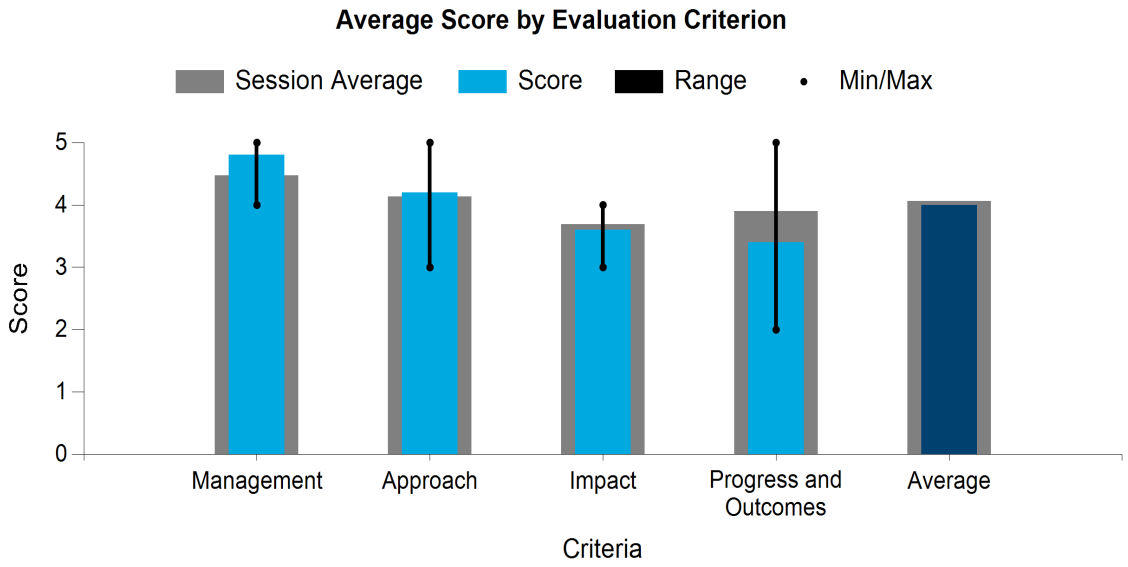
TARGETED MICROBIAL DEVELOPMENT

National Renewable Energy Laboratory

PROJECT DESCRIPTION

WBS:	2.4.3.102
Presenter(s):	Adam Bratis; Min Zhang; Zia Abdullah; Courtney Payne; Jessica Krupa
Project Start Date:	10/01/2018
Planned Project End Date:	09/30/2021
Total DOE Funding:	$2,900,000

The goal of the Targeted Microbial Development project is to develop microbial pathways capable of producing high-carbon-efficiency intermediates amenable to economic separation and catalytic upgrading to hydrocarbon fuels and chemicals. *Zymomonas mobilis* is known for its high specific glucose uptake rate, rapid catabolism, and high ethanol yield. It has been engineered to efficiently convert the second and third most abundant plant-derived sugars, xylose and arabinose, to ethanol at high yield. With its ability to utilize most biomass sugars, even in hydrolysate environments, it is now important to enable this microorganism one of the leading platforms for biomass conversion. By applying metabolic engineering and synthetic biology tools, we engineered *Z. mobilis* to produce 2,3-BDO together with ethanol. 2,3-BDO can be chemically upgraded to fuels and other chemicals as coproducts. We further demonstrated success in redirecting carbon flow by deleting the ethanol synthesis pathway, which enabled the organism to produce 2,3-BDO exclusively. We are working to further improve pentose utilization, as it is critical in achieving high yield and high rate for a cost-competitive biomass sugar conversion process.



COMMENTS

- This is a good, balanced management team with expertise in metabolic engineering, fermentation process development, microbial genetics, and biochemistry. Strategy with tasks and subtasks is provided. Challenges and risks are highlighted. Approaches to engineer a 2,3-BDO strains that grows anaerobically with improved pentose utilization are sound. Addressing redox imbalance is critical for a successful project. The impact of engineering a 2,3-BDO-producing strain is limited, as currently there are no commercial entities that can produce and utilize biomass hydrolysates. It is also not clear that the

production of 2,3-BDO from hydrolysate by fermentation is cost-competitive with sucrose/glucose/starch. A TEA can provide this information for a side-by-side comparison for batch and fed-batch best scenarios for corn stover hydrolysate vs. sucrose/glucose/starch derived with *Zymomonas*. Progress and outcomes are reasonable; however, there are still some remaining challenges to engineering a robust anaerobic strain that can utilize pentoses efficiently as arabinose and xylose are partially used. In 2018, an NREL study (https://www.nrel.gov/docs/fy19osti/71949.pdf) did provide a side-by-side comparison for the cost of both 2,3-BDO and butyric. It would be useful to update this report with latest findings based on strain engineering and current TRY for both processes.

- Strengths: The program is complementary to other organism development activities in the BETO portfolio and is examining alternate biochemical approaches to BDO as a fuel intermediate. Focusing on *Zymomonas* as the key organism is a strength, as the team draws on extensive NREL experience in its modification for EtOH production. Additionally, the focus on *Zymomonas* offers good opportunity for stakeholder discussions, as it is well recognized for industrial applications. The PIs have been successful in modifying *Zymomonas* to eliminate the normal EtOH production from C6 sugars and turn it toward BDO production. At the same time, work has continued to improve utilization of C5 sugars in the same process. The team identified key issues in redox balance as barriers to optimal sugar consumption and developed reasonable approaches to overcome these barriers. The team has met its goal for arabinose consumption, albeit at low rates. Overall, this work demonstrates NREL's strengths in strain development and optimization to include the incorporation of CRISPR (clustered regularly interspaced short palindromic repeats) and mechanistic investigation via cell-free systems as experimental tools.

  Areas for improvement: The project faced setbacks in redox balance and arabinose use, as the planned approaches did not pan out. This project faces similar questions to others making chemicals from carbohydrates and then converting them to fuels. The rationale behind making BDO as a single product and then turning it into a fuel that will be of lower value than the BDO itself is not clear. Further, the project would be strengthened if the TEA could provide a simple comparison of the projected production cost of BDO from biological routes vs. from petrochemicals (e.g., from epoxidation of 2-butene). Identifying a reasonable split between chemicals and fuels for optimal economic performance of the biorefinery would strengthen the presentation—for example, modeling a biorefinery making fuel, BDO, and butyric acid in appropriate ratios to assure profitability. The amount of progress on this project was less than expected. The PIs identified challenges and experimental approaches to overcome them, but the reported results generally were not successful. Current work is still running into problems with xylose and arabinose metabolism. Accordingly, it is important to understand the overall dependence of the larger program to this work—i.e., whether these setbacks are major hindrances to the fuel effort.

- The goal of this project is to produce the platform chemical 2,3-butanediol using *Zymomonas mobilis*. *Z. mobilis* is an attractive microorganism for producing chemicals given its high sugar uptake rate and low biomass yield. However, it is native producer of ethanol. The NREL team was able to eliminate ethanol production in *Z. mobilis* and redirect carbon towards 2,3-butanediol. They were also able to generate impressive titers and yields. This is a major technical achievement because *Z. mobilis* was only considered for ethanol production prior to their work. It can now be engineered to produce other, more valuable chemicals such as 2,3-butandiol. The project is being managed well. The tasks are appropriate and the team is making good progress. The work is also well integrated with other NREL projects. Overall, no major concerns were raised. The impact score was somewhat reduced because 2,3-butanediol is a relatively easy molecule to produce in many microorganism. That said, producing this compound in *Z. mobilis* is a major accomplishment. Current progress is focused on the coproduction of H_2 and succinate and co-utilization of pentose sugars. The impact of the former is still unclear. The latter is a common problem when dealing with plant-based sugars. The NREL teams has worked on similar problems in the past, so they are ideal to tackle this problem. Lastly, the efforts to develop CRISPR-Cas9 for *Z. mobilis* are promising and would great improve the utility of this microorganism.

- The project focuses on enhancing 2,3-BDO production with *Zymomonas mobilis* by engineering strains for better redox balance and better utilization of xylose and arabinose. The team developed elegant approaches to engineer *Z. mobilis* for production of H_2 or production of succinate as strategies to gain redox balance without the need to add oxygen to the culture. In parallel, a separate project was aiming at developing process optimization solutions by finding operational conditions that minimized the concerns with oxygen addition. The genetically engineered approaches were not sufficient to improve growth of *Zymomonas* in the absence of oxygen, and as a result, a no-go decision was made to stop further attempts to genetically engineered redox balance into *Z. mobilis*. This is a good use of the go/no-go decision point. Progress is being made on investigating arabinose and xylose utilization by *Z. mobilis* and on enabling a CRISPR system for pathway engineering in *Z. mobilis*, which will be used to test hypothesis related to arabinose and xylose utilization. Looking forward to seeing whether those strategies work.

- The project seems well managed and on the right track, but still a substantial way from reaching the end-of-milestone targets. It is great to see the in vitro cell-free extract assay utilization in an effort to understand sugar transport and metabolism limitations—similarly, CRISPR for enhanced pathway engineering. I am hopeful these employed systems will lead to significant developments. Great results for glucose and xylose utilization were presented, which make near-term impacts tangible. I am very happy to see Gen1 corn ethanol diversification as a mentioned near-term opportunity. The shortcomings with arabinose and fermentation times (150 hours) are significant. Arabinose conversion prioritization is paramount not only with respect to the challenges the sugar presents to downstream catalytic upgrading, but also to contamination pressure. Wild-type *Lactobacillus* love arabinose and can quickly ruin a good day if arabinose is not utilized. More clarification on why the succinate pathway work was stopped would be helpful, as the pathway appeared successful. State of the art describing cell mass diversion to improved yields and strain engineering to anaerobic functionality were not clearly defined.

PI RESPONSE TO REVIEWER COMMENTS

- We thank the reviewers for the overall positive feedback, encouragement, and insightful comments. We appreciate their acknowledgment of the importance and technical challenges for engineering a robust anaerobic strain that can utilize xylose and arabinose efficiently. Although the two approaches we explored to balance the redox did not meet our final objective of anaerobic fermentation, we acquired considerable understanding to help us design alternative strategies. We stopped the succinate pathway work because we could not overcome the technical limitations of expressing active fumarate reductase, which is the last step of the pathway.

  With enabling a CRISPR system for pathway engineering in *Z. mobilis*, as well as the cell-free system to identify the target limiting steps, we are encouraged to speed up our pathway engineering efforts. As related to TEA, we (the Platform Analysis team) have focused to date on our base case approach for the integrated stover-to-fuel pathways in support of program SOT development. Moving forward, we will plan to evaluate alternative sugar sources to highlight opportunities to integrate with existing industrial technologies, specifically optimization/trade-offs for batch versus fed-batch processing. We agree that identifying a reasonable split between chemicals and fuels for optimal economic performance of the biorefinery would strengthen the presentation. We presented summarized analysis results earlier in the FY 2019 review that show significant savings when methyl ethyl ketone is produced as coproduct.

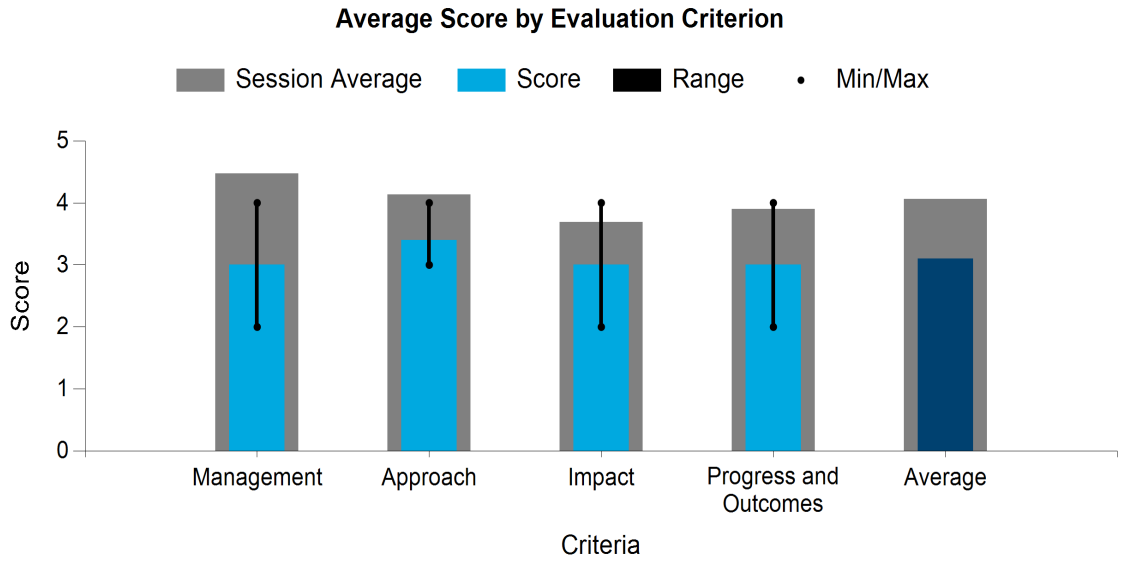
PROCESS INTENSIFICATION FOR THE REDUCED COMMERCIAL CAPEX OF BIOFUELS PRODUCTION (PRICE CAP) USING DYNAMIC METABOLIC CONTROL

Duke University

PROJECT DESCRIPTION

WBS:	2.4.3.200
Presenter(s):	Michael Lynch
Project Start Date:	09/01/2016
Planned Project End Date:	12/31/2019
Total DOE Funding:	$1,691,595

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



COMMENTS

- I am very happy to see the focus of this project, the realization that CapEx is a major hurdle to commercial advancement, is well received. The proposed CapEx per annual gallon of capacity at $0.50 is an incredulous metric, and any progress made to approach that metric is impactful. The presentation was quite basic but did provide and illustrate some necessary components for thorough review such as

TEA discussion and process flow diagram. High-optical-density fermentations were achieved in respectable times, although media formulations were not clearly detailed in that phase of testing. Sugar co-utilization was achieved, although starting sugar concentrations are not near commercial relevance and byproduct accumulation is concerning. Target terpene production rates and cell recycle have also been advanced in a reasonable and timely manner; however, the lack of semicontinuous, integrating processing and end-of-milestone production rates (25 g/L-h) were not available or clearly illustrated.

- Strengths: The concept behind the work is straightforward in that is recognizes how CapEx can make or break new bioprocessing technology, particularly for first-start operations. This project addresses that issue by investigating new methods of process intensification for growth and production using farnesene as the target product. To address these stages, the PIs define several reasonable criteria for investigation and optimization (slide 6)—e.g., CapEx can be driven down with rapid biomass growth in stage 1 and low mass/heat transfer requirements in stage 2, among other criteria. The project appears to have made progress toward their stated operational goals. Biomass production has been increased by modification of the growth medium (slide 14) and biomass production is able to consume all cellulosic sugars (slides 17 and 18), leading to mevalonate, xylitol, and pyruvate production (slide 19).

  Areas for improvement: It is difficult to evaluate the impact, as the presentation does not contain sufficient detail. Economically, the presentation suggests that successfully reaching their operational goals will have a strong influence on CapEx (slide 10). But missing from the glide path is a description of yields, rates, and other assumptions used to generate this projected CapEx reduction. The novelty of this work is not clear, other than the CapEx context in which the project is placed. For example, the goals on slide 11 are typical for improvement in any bioprocess—high production, use of all sugars, high production rates, continuous processing, etc. In other words, if these goals were met in any project, the CapEx would come down. At the end of the presentation, the reader feels as if he/she is seeing just another bioprocess optimization project introduced by a rather novel context on CapEx. There is no mention of the organism being used or any sense of whether the products being generated are formed simultaneously, or whether they can be made selectively. Moreover, there is no sense of a baseline against which it can be determined whether these observations are significant improvements or not. During farnesene production, it appears (slide 23) that the process actually ends up with a mixture of terpenes, which may require a separate separation (possibly increasing CapEx) depending on the planned use of the products. The project started operation of a continuous fermentation system, which could offer some significant benefits to CapEx, but the recycling encountered a number of difficulties and is still being optimized to include CapEx reduction by building inexpensive reactor systems via 3D printing. How this would translate into a commercial scale is very unclear. Overall, this presentation appears incomplete—few details are given, the results are minimal, and it's difficult to understand what's really being investigated and what's been achieved. Based on this presentation, the impact of this project appears to be low.

- The goal of this project is to demonstrate semicontinuous two-stage production of terpenoids from cellulosic sugars. With a semicontinuous two-stage process, the team seeks to provide a demonstration for how to reduce the CapEx of biofuels production. To achieve this CapEx reduction, the team will use the approach of dynamic metabolic control, in which the growth phase of the microbial culture is physically separated from the production phase. For the growth phase, they plan to use a smaller reactor and achieve high density of microbial cells. For the production phase, the plan is to use a flow-through system with cell recycle. The project will benefit from a clear explanation of the rationale for how the proposed approach reduces CapEx. Is it the dynamic metabolic control? Is it the separation of the process into two stages? Is it the continuous operation in the production reactor? For example, CapEx comparisons against a process that only uses one stage but continuous operation or a process that uses two stages but batch operation in both reactors could be useful to help explain the approach. It is already common to separate growth phase from production phase, but this can be done in a single reactor. It is not clear what the benefit of separating these phases into two reactors is. Why would the mass transfer

requirements be higher during the growth phase? Mass transfer of what material? I could understand this if the mass transfer limitation is related to aeration of the cultures. Is this process using aerobic conditions? What microorganism is used? There are other projects within the BETO portfolio looking at hydrolysis and fermentation in flow-through systems, and there may be benefits of creating collaborations or communications with those teams.

- The goal of this project is to reduce CapEx costs associated with fermentation through process intensification. The basic strategy will involve high-cell-density fermentations coupled with co-sugar utilization and two-phase metabolic control, where growth is decoupled from production. The latter is the key scientific advance, though the details regarding the method are vague. A key strength of the proposal is that it is motivated by TEA. In addition, the team has developed a web-based TEA simulator. The project seems on track with regards to the stated milestones. However, these were not clearly motivated. The main weakness of the project is that the impact is unclear. In addition, it is not clear how generalizable the results are. Part of the problem was the work was poorly presented, making it difficult to review. As consequence, it was difficult to be excited by this project.

- The overall project plan is outlined. It is not clear if this is work done by the PI with other collaborators on board. DMC is a company where PI is a partner. The contribution and role of the company in the work is not highlighted. No risk mitigation plan is outlined. The overall approach is sound and has been used in the past at industrial scale. Semicontinuous and continuous processes are hard to maintain indefinitely, as typically productivity and yield drop. Cells need to be provided with maintenance to extend the stationary productive stage. Contamination can also be an issue, as contaminants adapt to use end products for carbon and energy use. Dynamic metabolic control is not a novel idea. The impact from a significant reduction in CapEx, if achieved, can be important to reduced target cost for low-value biofuels and chemical commodities. It is not clear if the cost reductions in slide 6 have been achieved or are based on modeling. The PI needs to clarify this point. The listed accomplishments indicate that biomass levels ~50 g cell dry weight/L has been achieved; the co-consumption of all cellulosic sugars has been shown in growth phase, but there is still a need to demonstrate that for stationary phase. Demonstrating a productivity rate for terpene at >0.75 g/g cell dry weight-h still needs to be demonstrated; semicontinuous processing and process integration is also pending, so has not been done. These targets were not met by 9/30/20 as planned. The overall impression is that this project is trying to demonstrate in parallel the production of mevalonate and terpenes from mixed sugars. It is not clear if the project is using a mock hydrolysate or a hydrolysate that is prepared in lab by mixing the different sugars and inhibitors. Publication record indicates that there are many contributors that are not listed as collaborators on the update. The PI should list focus areas and contributions made by his numerous collaborators.

BIOCHEMICAL PROCESS MODELING AND SIMULATION

National Renewable Energy Laboratory

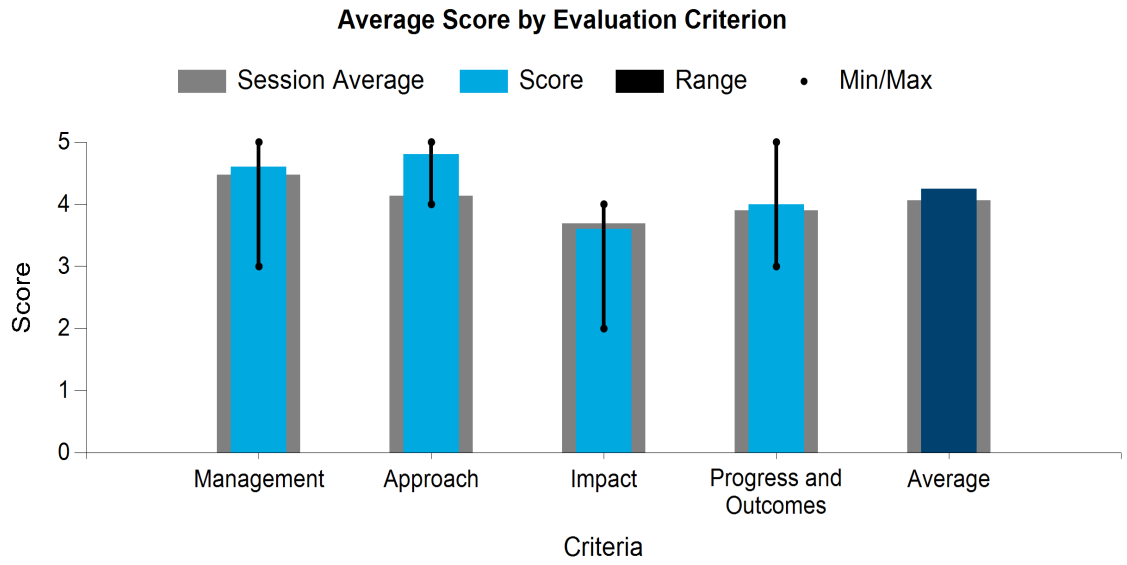
PROJECT DESCRIPTION

WBS:	2.5.1.100
Presenter(s):	Adam Bratis; Yannick Bomble; Zia Abdullah; Courtney Payne; Jessica Krupa
Project Start Date:	10/01/2018
Planned Project End Date:	09/30/2021
Total DOE Funding:	$2,950,000

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 simulation that could otherwise not be solved or would take too long with experiment alone to reach BETO's targets. We have produced solutions that have resulted in determining the most likely fatty acid derivative for passive transport out of bacteria that upgrades biomass, and we have also designed enzyme mutations for enhanced lignin upgrading. Metabolic models have been developed to tune the activity of butanediol 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 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 microaerobic production at all scales and have been instrumental in improving the accuracy of techno-economic analysis models. This project is essential in the process of selecting the final processes for 2022 and 2030 biofuel production targets.

More specifically, we have recently (1) predicted bond dissociation energies of >25 commodity plastics via density functional theory and determined the mechanism of polyethylene terephthalate (PET)-degrading PETase enzymes; (2) developed a computational tool for lignin modeling for bioconversion; (3) developed thermodynamic and kinetic models of 2,3-BDO production in *Z. mobilis* that explains glucose and xylose utilization and provides guidance to improve TRYs; (4) developed machine-learning methods to predict protein promiscuity and mutations to further improve microbial and enzymatic driven process; (5) developed tools to convert omics data from high-throughput experiments into actionable strategies to improve processes in industrially relevant hosts; (6) developed new methods to enable high-fidelity simulation of aerobic fermentation at industrial scale and resolve mismatch of time scales 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).



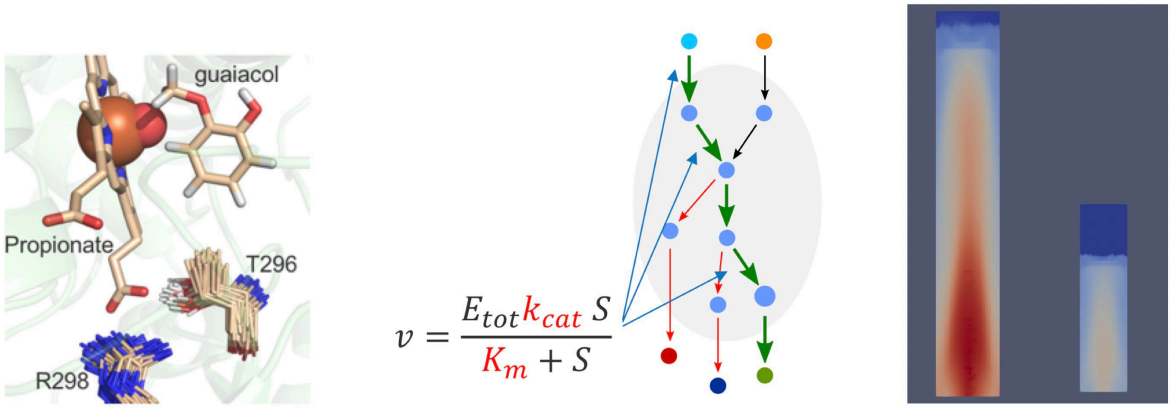


Photo courtesy of NREL

COMMENTS

- All catechism aspects are critical and appear to be getting logical and reasonable consideration. The progress and outcomes have shown direct benefits to a multitude of projects. Collaboration with 13 projects is a substantial undertaking, and this magnitude of workload will need to be resourced appropriately. It feels like perhaps the project group is spread thin. Self-selection with successful projects appears to be taking place, which is not a compliment or a criticism, but rather purely an observation. I would envision most PIs will see the conspicuous value within the Biochemical Process Modeling and Simulation team and look to engage in these strategies and tools to enhance their own outcomes. It is great to see the creation and deployment of tools, not only for research purposes or for BETO directly, but for broader, multiple industry use such as the publicly available reactor models and new computational approaches. The project's history of invention disclosures and publications are strong benchmarks. To some degree, the project is purely computational, even though it is informatics to a broad suite of users. The project staff might benefit from some cross-training or cross-functional activities. To get a better perspective and a deeper understanding, we find it to be valuable for our accountants to regularly tour the production facilities. Great project, well done!

- Management plan is adequate and requires good communication with other groups that can benefit from the tools being developed. Mitigation and risks are not addressed adequately in the review update. Several approaches have been deployed in several areas to assist with model prediction. It is not clear that this effort is adequately funded to deliver timely information. It is also not clear whether the team gets involved directly in some of these projects to fine-tune the models while gaining insights from hands-on experimental work. Modeling outputs and projections can be improved if inputs are validated by engaging modelers directly in the research. The relevance to BETO's mission is excellent, as there is a need for timely delivery in a cost-effective manner. Gaining insights and improving technology transfer for scale-up industrial opportunities will require active involvement and will be dependent on personnel and resource availability. Having personnel in the mix with industrial experience will ensure more success upon transfer. Progress and outcomes are provided for several projects that rely on improved catalysts. Not clear though if we are close to whole-cell system engineering, but the tool is helpful in identifying metabolic bottlenecks and targets to improve redox balance. On the overall, this project is a serious effort to assist other projects where engineering of better enzymes/catalysts is necessary in a focused way. Simulating engineering of cells growing on mixtures of sugars in the presence of inhibitors and several end products in batch, continuous/semicontinuous, and fed batch under different aeration levels is still challenging. This project, while high on innovation, will require many years prior to scale-up and commercial deployment. This will require continued investment in high-risk cell-free technology development.

- Strengths: This project is a valuable core service center for the overall program. One of NREL's strengths in recent years has been the embrace of computation as a tool for experimentalists. These in silico efforts have proven their value in catalyst development and organism engineering, and as a basis for better focus of research efforts. Multiple modeling approaches are available across a wide range of granularity, affording insight to transformations at the molecular scale as well as large-scale reactor modeling. It's a worthwhile activity that crosscuts many of the projects in BETO's portfolio, and one that should be continued as part of the overall program. The project is actively involved with all of the projects within this program area, holding annual meetings with the project PIs to help define areas where modeling can be useful, and how it might be incorporated into the annual operating plan. While most of the work is in a service mode, there are opportunities for the project to carry out leadership in new areas to expand the utility of computational modeling. This latter area is important in order to maintain the viability of this research area. In response to comments from previous peer reviews, the PIs offered some concrete examples of how modeling has been translated into experimental work—e.g., work on lignin-converting enzymes and vanillin, and modeling the slow rate of xylose consumption by *Zymomonas*.

  Areas for improvement: It is a little hard to judge the specifics of this program, as the presentation spent most of its time describing the potential of modeling, including some examples that have become publications in high-impact journals. But despite the results from lignin enzyme engineering and *Zymomonas* improvement, justification of this research effort will be strengthened by continuing to develop solid examples of how modeling has produced demonstrated improvements in TRY, chemical yield, catalyst performance, etc. This is a challenge that all service centers face. The tools are available, but there is still a responsibility to convince the researchers to use them. Demonstrating a close link between computational results and improvements in experiment will lead to routine inclusion of computational modeling in most projects.

- This project provides modeling support for the various activities within the BETO/NREL portfolio. The potential impact is clear, as modeling can provide new insights and greatly accelerate technology development. It also provides a valuable service for others. Overall, the project is doing well. The management structure is clear, with tasks clearly defined among groups. The team is working on a number of different projects ranging in scale from proteins to fermenters. Given that there are so many subprojects, it was difficult to evaluate progress. Not clear whether there is an easy fix to this problem,

as all of the different activities provide valuable services and impact different areas within the BETO portfolio. One area where the team should consider focusing more on is reactor modeling and computational fluid dynamics. Such simulations can greatly aid in fermentation scale-up. Also, such models are being ignored by most academic researchers (especially in the United States), so this is one area where the team can really drive the field and advance the state of technology. Lastly, it was unclear whether the team was developing open-source tools that will benefit the broader community. Disseminating their tools will improve the impact of this project.

- This project uses modeling at multiple scales to help direct experimental research by other projects in the BETO portfolio. Success stories were reported on modeling that help the rational design of enzymes for lignin valorization and the creation of tools for lignin analytics, predictions that will improve rational design of enzymes for plastic biodegradation, and computational analyses of the effect of oxygen input rates on the production of 2,3-BDO. The project appears to have a positive impact on the productivity of other BETO projects.

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. 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. The risk associated with this project are twofold; the first is to consider too many projects and not focusing on the ones where modeling can have impact, which could lead to unproductive use of our resources. This is why we take the time to think about our approach in light of what our experimental collaborators in BETO need. The second is to not yet have the appropriate software, methods, and/or hardware to tackle a problem, which usually prompts us to seek fruitful external collaborations, which we have done successfully to BETO's benefit in the past. Given that this is a computational project working with many other experimental efforts in BETO to enable the cost-efficient production of biofuels, biochemicals, and biopolymers, we do not anticipate commercial deployment directly from this effort but in partnerships with other efforts in BETO. Computational fluid dynamics 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. Scientific dissemination is an important aspect of our research, and we do make all the codes, force fields, and other computational tools available to the public. We do agree that cross-training can be very important. For the last few years, we have encouraged researchers work on this project to attend and actively participate in relevant experimental group meetings to get their perspective and also to better identify needs to remain relevant.

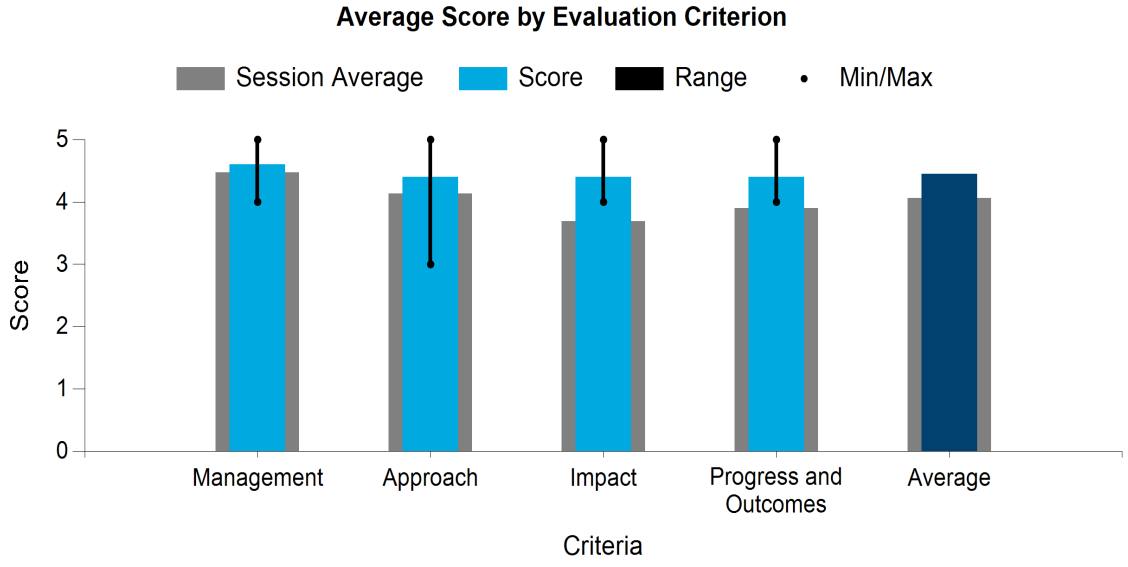
ANALYTICAL DEVELOPMENT AND SUPPORT

National Renewable Energy Laboratory

PROJECT DESCRIPTION

WBS:	2.5.1.101
Presenter(s):	Ed Wolfrum; Zia Abdullah; Courtney Payne; Jessica Krupa; Justin Sluiter
Project Start Date:	10/01/2018
Planned Project End Date:	09/30/2021
Total DOE Funding:	$2,155,000

The objective of the Analytical Development and Support project is to produce and maintain the critical analytical methods and tools that enable evaluation of emerging biofuels R&D at NREL and in the broader biofuels research community. Our project is divided into two tasks: one is to develop novel analytical techniques and improve existing methods, and the other is to maintain existing analytical capabilities at NREL and provide outreach to the wider community. The Analytical Development and Support project is world-recognized for our laboratory analytical procedures, which provide detailed procedures for 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 dialog with stakeholders allows us to provide robust, precise, accurate, and publicly available analytical procedures for better valuation of scientific tools, such as our recent accomplishment in developing a cellulose assay to support the U.S. Environmental Protection Agency and industry in calculating converted cellulose during starch ethanol production. We continue to develop analytical capabilities to support BETO's directives to research cost-advantaged fuels such as animal wastes and novel bioproducts like 2,3-butanediol.



Analysis	FY19	FY20
ASE Extraction	600	450
CHN	3300	2100
Compositional Analysis	500	400
FIS	1200	1500
Intermediate Solids	300	800
Near-Infrared (NIR)	400	800
Liquors Analysis		2200
UV-VIS	1800	1200
%Solids/Ash	4500	1500
Starch	300	200
Unique Samples	**5,772**	**4,608**
Total Analyses	**21,862**	**16,156**
Projects Served	**61**	**41**

Photo courtesy of NREL

COMMENTS

- Overall management slides provide sufficient details. As Analytical Development is a large group, it would be helpful to have more details on the management structure and go-to people and areas of expertise. While risks are not highlighted and identified in the presentation, the focus on good training of personnel with timely delivery is critical to consistency of results and relevance to stakeholders that benefit from it. The presentation provides a balanced approach between supporting existing projects and stakeholder requests with new method development. The overall impact of NREL on developing methods is well recognized by internal and external end users. One of these is the recent development of a method to measure cellulose/starch in corn fiber. The method developed has been adopted by the U.S. Environmental Protection Agency, for whom timely development and deployment has been of great value. Progress and outcomes cite the development and validation of near-infrared for 2,3-BDO fermentation as another example of analytical method development. Analytical Development has also taken the lead on method development on purely characterized cost-advantaged feedstocks used in anaerobic digestion that have a range of biomaterials. Analytical development is clearly necessary for the timely analyses of waste streams that present analytical challenges in current efforts to valorization. On the overall, the project continues to serve BETO well. It would be good to highlight industry engagements with more examples of how Analytical Development is meeting external client needs by providing timely analyses. This information can be readily obtained from procedure downloads by external users. Continued focus on rapid cost-effective methods that can be readily implemented at industrial scale as a goal for the effort needs to be highlighted. While wet chemistry validated methods are good in establishing feedstock composition, developing spectroscopic methods for rapid use in the field is an area of need, and the near-infrared BDO analyses in fermentation is a good example. Analytical development continues to be challenging and lacking at large-scale biorefineries.

- Strengths: This project provides a critical service function for the entire BETO program by offering and maintaining the analytical capability, methods development, and training for assays on the many feedstock and biomass conversion samples generated by the research teams. The team also has a clear process in place for method development and validation (slides 8 and 9), which has led to several of their laboratory analytical procedures being adopted as ASTM standards. Despite the COVID slowdown, the

team has been able to process more than 16,000 total analyses in FY 2020, including 2,200 liquors analysis, which looks like an addition to the team's analytical capability from FY 2019. The impact of this project is high. As the presentation points out, the analytical team is ensuring high-quality research data, the importance of which can hardly be overstated. High-quality data and reproducible, standard analytical techniques are a key part of NREL's reputation. The general acceptance of their publicly available laboratory analytical procedures within the research community is a good example of the significance of the groups work. The procedures provide a common framework for analysis of extremely complex material, allowing credible comparisons to be made between different research projects. The team's combination of analytical skills (wet chemical and instrumental), development of standard methodology, and clear emphasis on quality control continue to make this center a key component of BETO's program.

Areas for improvement: On slide 6, the presentation describes training carried out for staff. It would be useful to have a clearer picture of the split between training staff and carrying out analyses as a service function, and the amount of time required for instrument maintenance. The presentations quad chart shows a cut of almost $300,000 (nearly 30%!) in funding between FY 2020 and FY 2021. This had to have placed a significant burden on the team. More information on the rationale for the funding cut and how it was addressed would be useful.

- The analytical development and support project is a critical project that performs a service to other BETO projects by processing thousands of samples per year with about a 2-week turnaround time. The impact of rigorous method development by this team is clear and not only serves BETO projects, but also reaches industry, other labs, and academic units at a global scale. A risk is that potential adoption of the developed methods will depend on the use of instrumentation that is accessible to other potential users, so a focus on inexpensive and user-friendly methods is critical.

- The project is well managed and executed. It is true, NREL is a leader in biofuels analysis, allowing a worldwide community language; this is undeniable, and the team's recent compositional analysis publication should be recognized as proof. The analytical chemistry is foundational, and approved methods have the ability to complement and impact ongoing pathway approval efforts related to Gen1.5 renewable identification numbers via the Renewable Fuel Standard. With respect to the publication, the method development and validation efforts utilizing nuclear magnetic resonance and liquid chromatography–mass spectrometry add credibility, and the risk mitigation plan to simplify the core methods is merited. Industry will need to get it done (composition) with NIR, high-performance liquid chromatography, LECO, and gas chromatography. The recognition that MS and NMR widespread adoption being unlikely is well received. Continued NIR model development with model functionality on a range of instrument vendors such as Thermo-Fisher, Bruker, Metrohm, Perkin Elmer, and Zeiss would be of great benefit. Round-robin industrial Gen1.5 feedstock sampling and NREL-performed wet chemistry to build publicly available models could be a great industrial utility. The migration in development to cost-advantaged feedstocks will open a new portfolio and create significant value to industry lacking these tools. Lignin quantification in high-protein-content substrates is a legitimate pursuit; similarly, tools for yeast quantification within high-lignin or high-fiber substrates would have immediate industrial impact. Methodology to determine yeast vs. non-yeast protein mass, protein chemistry, protein arrangements, simplifying amino acid quantification methods, 1,3- and 1,6-beta-glucan quantification, and mannose oligo-saccharides from protein vs. structural carbohydrates, to name a few, would be impactful to the renewable fuels industry. Although the project was successful in achieving what was asked for, a one-pot method quantifying cellulose, starch, and xylan/arabinan is still very much needed. Excellent project and great job!

- The project provides analytical support for both internal and external partners, a critical need for any project. The services range from routine measurements and instrument training to methods development. In addition, the team is developing standardized procedures for biofuels/chemical research. This seems

to be a unique contribution that exploits the expertise and experience at NREL. Overall, this is a critical program for BETO/NREL. The team is making excellent progress. They are meeting their objectives and providing a critical service. The milestones are not clear, but this is difficult for this sort of program. Going forward, it would be great to see this program work with more external partners and further develop standardized protocols.

PI RESPONSE TO REVIEWER COMMENTS

- First, I would like to thank the reviewers for their time and comments. Engagement with stakeholders is very important to this project, and feedback such as this truly helps to direct our future research goals.

  This project is not directly responsible for covering the time of the analysts when performing the analysis that support the program; that funding comes from the individual projects that request the work. This project coordinates the work to ensure that all samples are analyzed efficiently and by well-trained and qualified analysts on instrumentation that works.

  This project is not as large of a group as it appears. It serves to coordinate the work of many researchers but is largely made up of only three people. Justin Sluiter manages the project, coordinates with external stakeholders, leads method development work, and trains staff on new methods. He is full-time on this project. Darren Peterson is tasked with monitoring the sample queue to ensure that we are meeting deadlines, ensuring proper training on protocols, and performing instrument maintenance. He estimates that he spends about half of his time on this project, split evenly between managing the sample queue, instrument maintenance, and training. Katie Michel joined the team with a diverse commercial analytical background in FY 2020. She assists with method development and optimization efforts. She has taken over the data management for the project.

  Because it was not yet public information at the time of the presentation, we were not able to discuss our funding situation and ongoing stakeholder collaboration efforts, as they are closely related areas. We are pleased to announce on May 3, 2021, a directed funding opportunity for industry and universities to leverage the Analytical Development and Support biomass compositional analysis capabilities. This opportunity is funded at $1.5 million over the next 2–3 years. We hope to engage directly with stakeholders to address analytical issues that they are facing. The stated areas on interest for this opportunity are characterization of mixed carbohydrate streams, development of rapid characterization for mixed carbohydrate and lignocellulosic streams, and development of analytical methods for characterization of wet organic wastes. The total funding available is $1.5 million. The ADS project had a reduced scope and funding the during 2021 to prepare for this directed funding opportunity effort.

  We are aware that the national laboratories are blessed with cutting-edge equipment and that not all stakeholders have equal access to instrumentation. Katie Michel, who has recently joined the group, has extensive experience in commercial analytical. Her knowledge in this area will help us to develop accessible methods.

  Analytical Development and Support sets milestones that reflect the diversity of work that we undertake. Often these are steps towards method development that support the program or numeration of samples and projects supported. The recent development of a cellulose-specific analytical method to support the U.S. Environmental Protection Agency and starch ethanol industry caused us to shift our milestones entirely to supporting that effort.

  The new analytical method published in *Cellulose* has not been adopted by the U.S. Environmental Protection Agency. It is under consideration, just like all other methods.

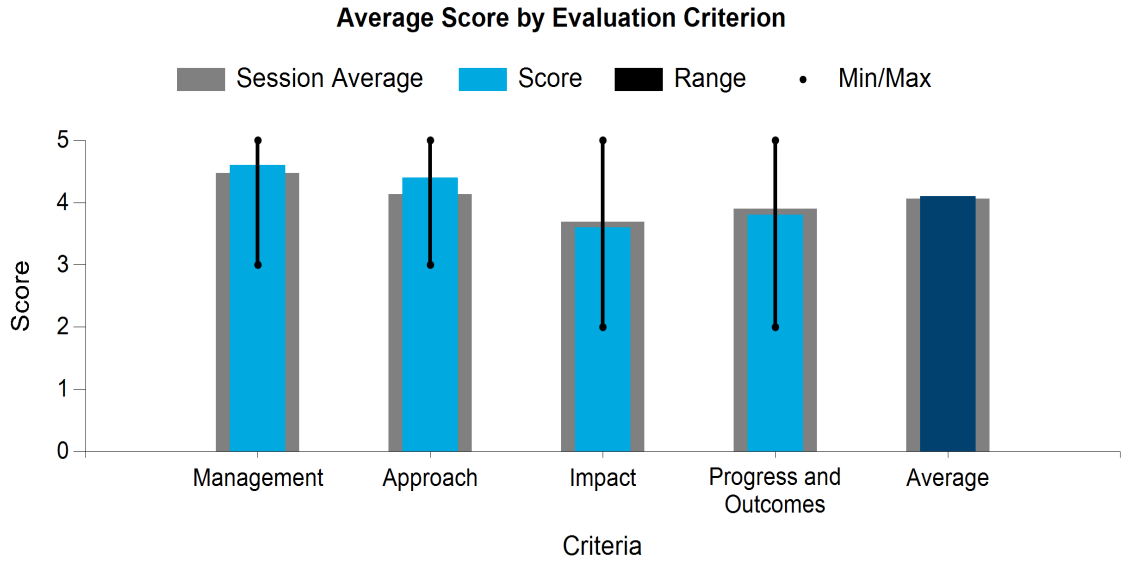
ENGINEERED REVERSAL OF THE ß-OXIDATION CYCLE IN *CLOSTRIDIA* FOR THE SYNTHESIS OF FUELS AND CHEMICALS

Northwestern University

PROJECT DESCRIPTION

WBS:	2.5.3.206
Presenter(s):	Michael Jewett
Project Start Date:	10/01/2018
Planned Project End Date:	01/31/2022
Total DOE Funding:	$1,600,000

Rapid population growth, a rise in global living standards, and economic competitiveness have intensified the need for sustainable, low-cost biofuels and bioproducts. Industrial biotechnology using microbial cell factories is one of the most attractive approaches to address this need, particularly when large-scale chemical synthesis is untenable. Unfortunately, designing, building, and optimizing biosynthetic pathways in cells remains a complex and formidable challenge for many reasons. First, design-build-test cycles for optimizing a given biosynthetic pathway can take on the order of weeks to months, requiring hundreds of person-years of research and development time to bring a new bioproduct to market. Second, most high-throughput platforms for testing engineered organisms focus on *Escherichia coli* or yeast, which limits the platform organisms, feedstocks, target molecules, and stable operating environments for development. Third, a focus on linear heterologous pathways limits co-development of multiple products. Here, this project will address these limitations via unique pathway tools and engineering strategies that enable rapid synthesis of next-generation biofuels and bioproducts from lignocellulosic biomass in *Clostridia*. The core of our unique approach is to reconceptualize complex biological systems engineering by combining in vitro and in vivo work to advance state-of-the-art pathway design, prospecting, and validation in an integrated framework. This framework will diversify the breadth of both products and platform organisms available to meet DOE and U.S. Department of Agriculture bio-based industry goals. We specifically aim to develop a new platform for engineering reversal of the ß-oxidation cycle (r-BOX) in *Clostridia* for synthesis of advanced fuels and bioproducts from biomass syngas produced by established gasification technologies. The environmental, community, and rural economic development impacts will be assessed for implementation in the Southeast region of the United States, utilizing regionally abundant forestry residues as feedstocks.



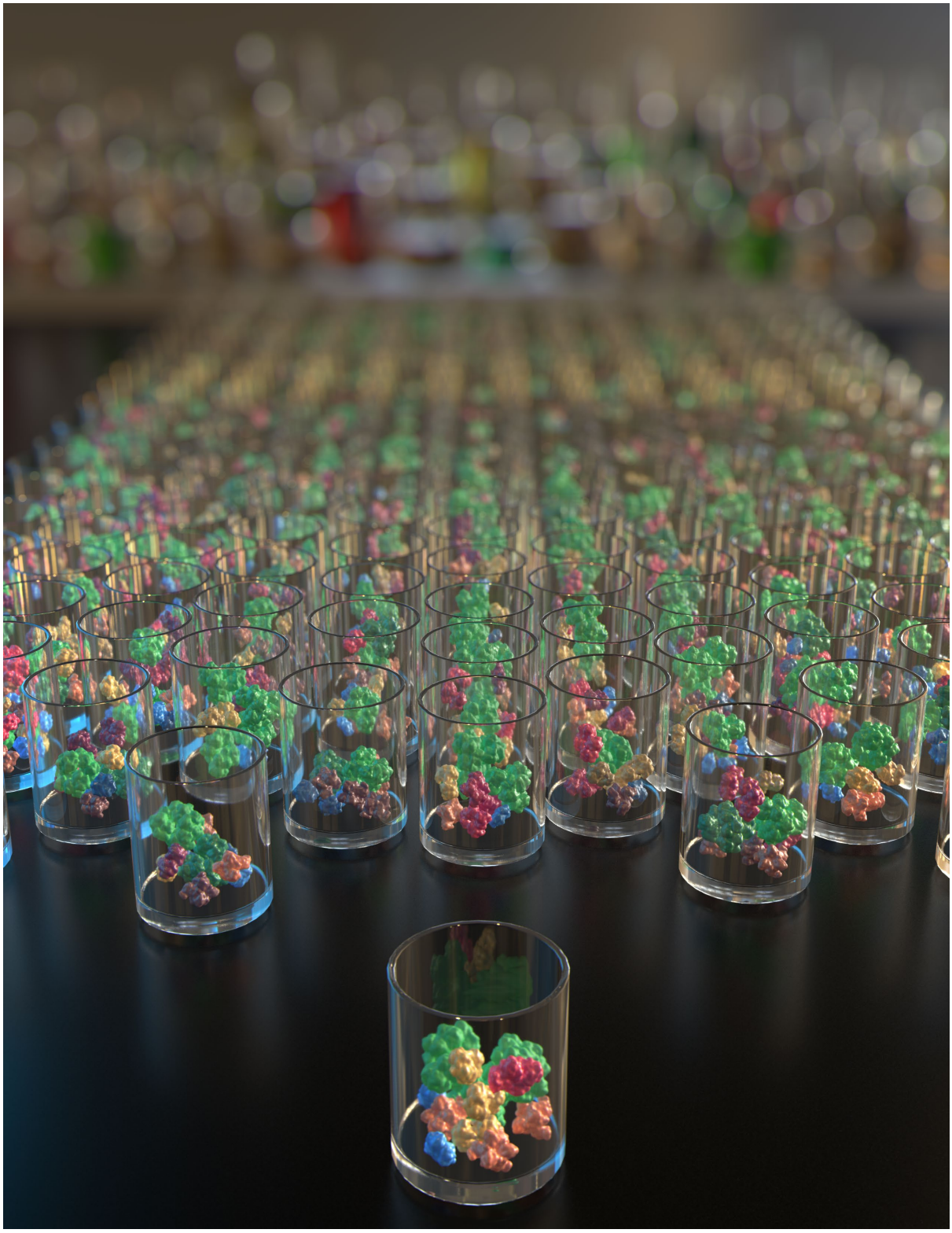


Photo courtesy of Northwestern University

COMMENTS

- Management consists of three universities and a private company with separate tasks and deliverables. Risks and mitigation and communication strategy are outlined. Approaches use knowledge base reverse engineering of the ß-oxidation cycle in industrial strains of *Clostridia* using pathway design, cell-free extract, and validation. The approaches are innovative (design, build, test) and within BETO's focus areas. The impact of these approaches can be assessed at the end of this project, but so far good progress has been made with two selected end products (hexanol, octanol); however, TRY are not within commercial viability at this stage of development. Progress and outcomes indicate that the project's initial tasks have been completed. As a proof-of-concept project, this is a promising project. Reverse engineering of ß-oxidation cycle into *Clostridia* will pave the way for a wide range of products, but this project represents a long-term investment in R&D. The commercial viability is not within reach, as the productivity target is low. As such, this is a high-risk project but hard to assess return on the investment in R&D. The environmental and socioeconomic impact component is interesting but cannot be assessed fully unless commercial viability is established. Impact of this project can be assessed better once a TEA/LCA can be carried out, but with present targets will need be possible without longer-term investment with realistic TRY.

- Strengths: The core technology in this work is syngas fermentation, using syngas from conventional lignocellulosic gasification. The high-level concept makes some sense, in that biomass gasification generates a well-defined starting material—syngas—which avoids the problems of multiple carbohydrate- and lignin-derived structures generated in other approaches. The technology has been available for decades, and gives access to both fuels and chemicals depending on how the syngas is converted. The inclusion of LanzaTech as a partner is a strength and indicates that industry senses an opportunity. Further, Lanza possesses a commercial gasification facility and a huge number of different sequenced *Clostridia* strains for screening; this is an excellent combination of facilities directly pertinent to the project. The general scientific approach is interesting. The PIs will reverse the beta-oxidation cycle (r-BOX) in *Clostridia*, giving access to a wide range of chemical structures (slide 12). Achieving this goal will employ a cell-free step for prototyping organisms and subsequent whole-cell processes to produce chemicals.

  Areas for improvement: Biomass gasification remains more expensive than gasification of coal. Thus, this work may be interesting scientifically, but does not address the question of why one would gasify lignocellulosics if they could get syngas from conventional sources. The impact of syngas CO/H_2 ratios was also not mentioned. This ratio can vary widely depending on the gasification technology used, and needs to be clarified. Post-presentation discussion revealed that different CO/H_2 ratios have been examined, but the outcome was not clear. Critically, there is no mention of TEA for this project, making assessment of the possible impact much more difficult. This issue becomes particularly acute when seen alongside the project's goal to generate 100 mg/L/day of an r-BOX product, which is a surprisingly small amount. Greater justification of how titers of that level translate into an economically viable process would strengthen the presentation. The process also gives mixtures of products (slide 42; butanol and hexanol) in a roughly 1:1 ratio. While the number of products is markedly less than many of the mixtures in alternate biomass conversion efforts being reviewed, it still ends up being a coproduct scheme. Surprisingly, the presentation makes no mention of TEA, production costs, etc. This omission gives the reader no sense of the commercial opportunity, which is critical in a BETO-supported program. A further important issue was the inconsistency between messages in the presentation and those in the subsequent discussion. The presentation clearly indicates a focus on gasification of cellulosic biomass (slide 5) to supply the feedstock for production of fuels, additives, and chemicals. These are large-scale operations, and thus a cost comparison to conventional coal-based gasification must be included. The post-presentation discussion switched focus and indicated that the work was looking at gasification of unidentified lignocellulose-containing waste streams. If it is waste streams, the operations would be smaller scale, disperse, working with low concentrations of material, and suggesting that the output

would never generate enough product to make a real impact. No comparisons or commercial operating scenarios were given, and moreover, the answers seem contradictory, which are considerable weaknesses of the project and omissions from the presentation.

- The goal of this project is to produce medium-chain fatty alcohols from syngas. The basic strategy is to reverse the beta-oxidation cycle. While this approach has been previously demonstrated in *E. coli*, it has not been shown in a gas-fermenting *Clostridial* species. Given the difficulty of genetically engineering *Clostridia*, the team has used cell-free systems for rapid pathway design and optimization. It is actually surprising how well this worked given that the cell-free system was based on *E. coli*. Overall, this is a beautiful demonstration of this technology for guiding strain engineering. The science is also fantastic. Perhaps the greatest strength of the project is the strong partnership between academia and industry, where bench-scale technologies are translated into real processes. Another strength is the outreach activities, consisting of workshops developed for rural economic agencies. Overall, this is an outstanding project and the team is making great progress.

- The project is interesting and relevant; direct industrial benefit appears to have been achieved. Utilization of synthesis gas, by *Clostridia* fermentation, from established gasification technologies for drop-in fuels, chemical building blocks, and fuel additives with a reported $14 billion U.S. market value is a worthy strategy. Product compound diversity is a great asset to this undertaking. The project seems to be on track to meet or exceed the end-of-milestone targets; however, the production metrics of >0.1 g/L/h at pilot scale in milestones 3.4–3.5 have not yet been achieved. LanzaTech is a great commercial partner and adds credibility and utility to this project. Similarly, the academic collaborations are noteworthy. In broad terms, with respect to impact, it would be insightful to know if any of these portfolio strains have made it to commercial use and what rigor or metrics were required to advance the established organism to industrial selection.

- This project is a great demonstration of the power of cell-free technology for combinatorial analysis of enzymes to create optimized pathways for integration into a microbial chassis. Reverse beta-oxidation is a known pathway in many *Clostridia* that ferment sugars to a variety of organic acids. The project has demonstrated the integration of reverse beta-oxidation into *Clostridia* strains that lacked this pathway. Furthermore, by selecting the terminal enzymes in the cyclic pathway, the team can direct the pathway from production of acids towards production of alcohols. It is also impressive that with optimal selection of terminal enzymes, the team can direct the cyclic pathway towards maximizing production of alcohols with a specific length. Overall, a great project.

PI RESPONSE TO REVIEWER COMMENTS

- We appreciate the reviewer recognizing the promising nature of our project and its innovative approaches. The r-BOX products can be coproduced with ethanol, already proven as a commercially viable product with this technology. Some of the r-BOX products have potential for relatively direct separations, improving the potential for early-stage commercially viable coproduction with ethanol. As we increase selectivity to products of interest, environmental benefits will be realized once the products enter the marketplace. The initial LCA indicates that using renewable power, along with low process requirements, will result in products with substantial GHG emission savings over their fossil counterparts. The majority of the TEA work package is back-loaded in the project, and based on the progress to date and commercial performance targets, the economics continue to be attractive. This will be de-risked with increasing technical progress, both during the project and during the subsequent scale-up and development.

  We appreciate the reviewer celebrating our project as outstanding, recognizing the strengths being brought to bear through integration, and highlighting that the science is “fantastic.”

We thank the reviewer for these comments and assessment that we are on track. We agree that the final productivity targets have not been met, but plan to complete the project milestones 3.4 and 3.5 by project end as planned. A key goal of this project was to establish functionality of the r-BOX pathway in *Clostridium*. Having achieved this goal, we have de-risked an important step and can quickly accelerate to high levels of the target products of interest.

We thank the reviewer for their positive assessment.

We appreciate the recognition of multiple strengths. Thank you for the comments regarding gasification and the economic implications of using coal versus biomass as a feedstock for gasification. Syngas produced from coal gasification has a lower cost of production than syngas produced from biomass due to the lower commodity price for coal ($/Btu). But this "cost" should not be looked at in isolation. The true cost of syngas produced from coal will be burdened by the additional costs associated with inherent environmental and health costs attributable to coal extraction, ash production, heavy metal mitigation, and of course release of fossil carbon to the atmosphere. Biomass, on the other hand, is not burdened by these externalities, and in addition, fuels produced from biomass gasification and gas fermentation will benefit from financial incentives such as California's Low Carbon Fuel Standard. While superficially less expensive, syngas from coal gasification is not advantaged, and unlike biomass, is not renewable. In the absence of carbon capture and sequestration, products derived from coal gasification will have higher emissions than products derived from biomass. LanzaTech will discuss options with the Bioenergy Technologies Office for how to best address and compare costs between coal and biomass.

Regarding H_2:CO ratio, the reviewer is correct that depending on feedstock type and technology, the ratio of H_2 to CO in syngas can be variable. Importantly, and unlike traditional Fischer-Tropsch catalysts, LanzaTech's proprietary gas fermentation process and microorganism is capable of operating on a wide range of H_2:CO ratios. LanzaTech has successfully demonstrated this capability on different feedstocks at various scales with multiple partners around the globe. Regarding scale of commercial deployment, to clarify, LanzaTech's technology is flexible and can be deployed to match the feedstock opportunity. Both small-scale, lower-volume waste streams and larger-scale, higher-volume waste streams can be valorized depending on the specific project characteristics. Likewise, the r-BOX pathway offers flexibility and product options (hexanol at the lower end of the cost; there are a lot of high-value products that might not need such high volumes). Coproduction of r-BOX chemicals with ethanol is the likely route to commercial viability. In order to meet the U.S. Renewable Fuel Standard 2, or potential future low-carbon fuel standards, the ethanol must have low life cycle greenhouse gas emissions, which will not be possible with coal as the feedstock. On the pathway toward large-scale commercial viability, utilization of carbonaceous waste streams can provide opportunities for low-cost feedstocks that are consistent with the smaller-scale production appropriate for TRL 8 and TRL 9 systems.

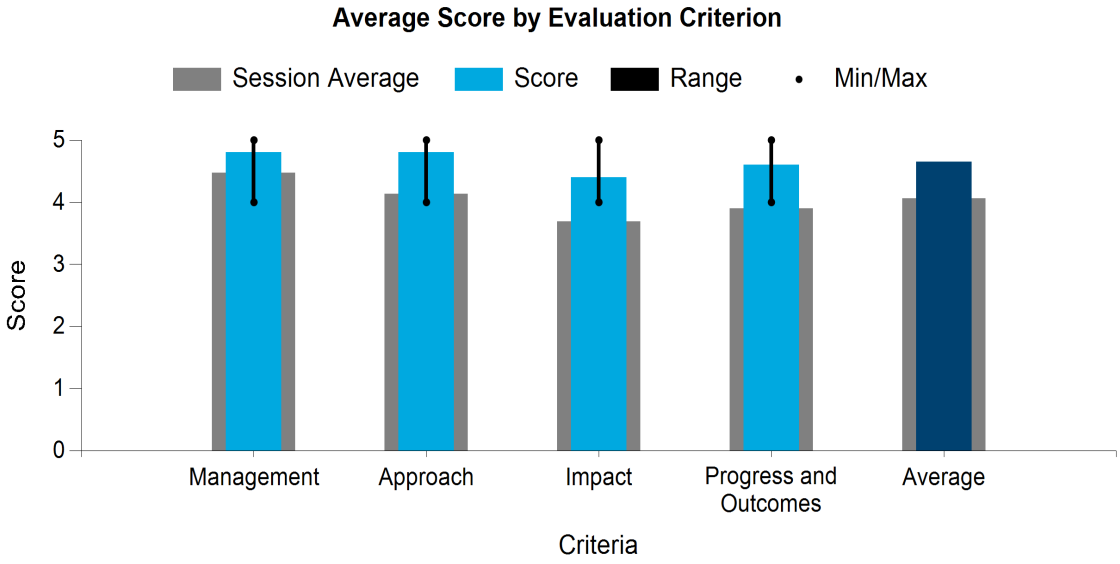
ENZYME ENGINEERING AND OPTIMIZATION

National Renewable Energy Laboratory

PROJECT DESCRIPTION

WBS:	2.5.4.100
Presenter(s):	Adam Bratis; Mike Himmel; Zia Abdullah; Courtney Payne; Jessica Krupa
Project Start Date:	10/01/2018
Planned Project End Date:	09/30/2021
Total DOE Funding:	$2,700,000

The FY 2020 SOT economic model for the DMR BDO process indicates that the production and use of biomass-degrading enzymes represents ~10% of the MFSP of BDO. We are thus working to enhance the performance of the dominant cellulase enzyme, Cel7A. In FY 2018–2019, we selected 100 promising genes from the "Cel7A wheel of life" and cloned them into *T. reesei* using a constitutive promotor. This natural diversity screening resulted in the discovery of several enzymes exhibiting improved characteristics relative to the industry standard Cel7A from *T. reesei* (Tr). Growth of the transformed host on glucose ensured that the Cre1-induced cellulase expression cascade was suppressed, thus enabling purification of the target gene product. The first top-performing enzyme found was from *P. funiculosum* (Pf). Other top-performing enzymes were identified from *T. aculeatus*, *T. terrestris*, and *A. oryzae*. Computational modeling predicted the structural subsites responsible for the improvements, which were cloned into the sequence of PfCel7A. The recombinant enzymes were expressed in *T. reesei*, purified, and tested. Testing was not possible with SOT relevant substrates, so the best-case substrate was used: solids from the DMR process subjected to a novel dilute alkaline wash to remove the "lignin shield." Two chimeric Cel7A enzymes show improved performance (1.2 to 1.35 times) relative to PfCel7A, which in turn showed considerable improvement (1.6 times) relative to *T. reesei* Cel7A. These new enzymes have been provided to our industrial partner for evaluation.



Cel7A Chimera Showing Beneficial Subsites and Cellodextrin Substrate

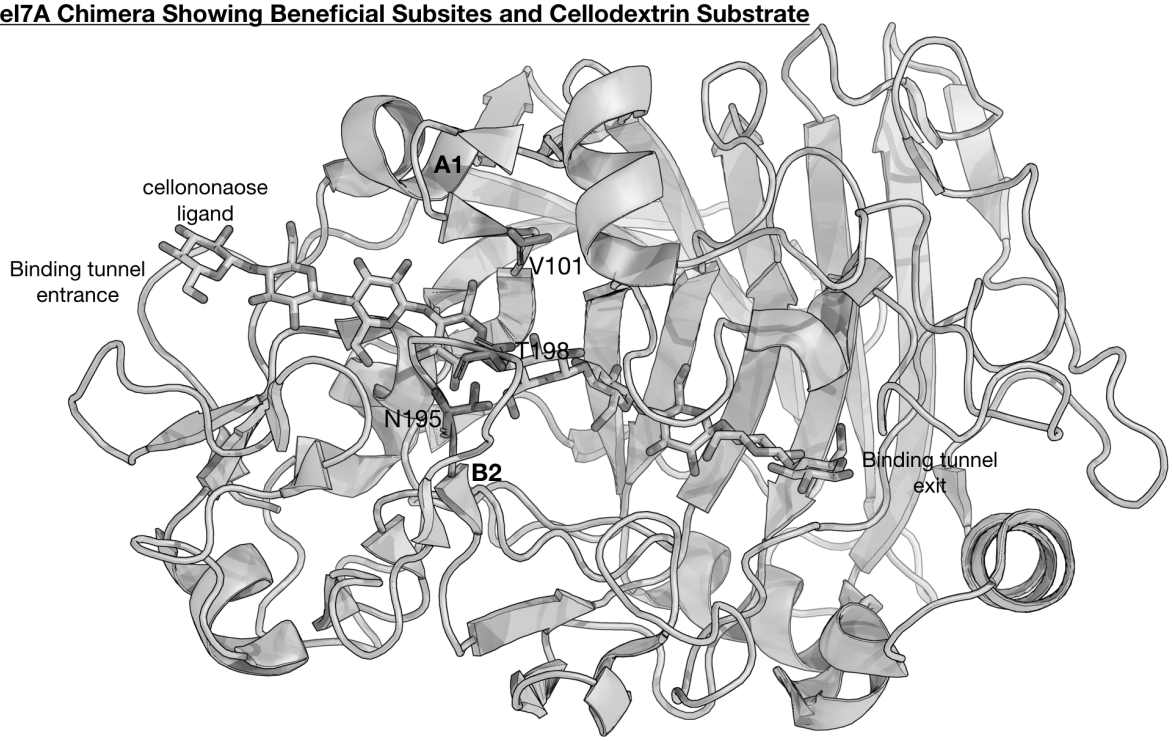


Photo courtesy of NREL

COMMENTS

- Good management structure with defined roles, tasks, and subtasks. Risks and a mitigation strategy are in place. The project deploys multiple parallel approaches that are innovation-driven with go/no-go decision points. The project will have impact by developing an improved cellulase on selected DMR-acidified and base-washed corn stover. The team still needs to resolve the uncertainty around the usefulness and impact to other biomass feedstocks that use other types of pretreatments. The impact of this project is confirmed by externally provided support letters. Progress and outcomes are illustrated in slide 28, as the new chimeras have significant measurable improved performance over wild-type enzymes. These results are promising and will enable reaching the stated goals for 2021 for enzyme performance. On the overall, this is a good, impressive effort on engineering a better class of Cel7A. The team has shown that by deploying multiple approaches and tools, a better-engineered Cel7A protein can be obtained. The performance of the improved enzyme was demonstrated using modified acid-hydrolyzed and washed DMR corn stover that has reduced lignin content. The applicability of this enzyme preparation to other feedstocks, if confirmed, can assist in further cost improvement needed to cellulase enzymes. Beyond the improvement of Cel7A, the current cost of cellulase enzymes will continue to be high, so additional improvements in cost reduction for the production of sugars from biomass is necessary. Finding ways to reduce crystallinity during pretreatment can help reduce protein loading. The use of additives to reduce enzyme loss to nonspecific binding to DMR and to increase surface area, as well as recycling of enzyme/splitting enzyme dosing, are worth considering. Using a hybrid secondary acid treatment with cellulases are other things to consider also.

- I am very glad to see the ambitious project goal expectations were surpassed. The 11% xylose yield improvement, which was achieved in 1 year, is almost hard to believe—well done. It is not clear that all yield improvements are attributed to enzyme platform progress, DMR/pretreatment enhancements, or combinations thereof. The project's industrial partners are well regarded, which illustrates the value this team is bringing to market. Validation from Sarah Teter regarding NREL molecules in current 1.5G product discovery speaks for itself. The track record for patents, publications, and public presentations

resulting from this work is excellent. The efforts to evaluate the 2019 Peer Review comments are well received. It is great to see dilute acid and multiple DMR strategies contrasted, but I would like to see more process insight, as washing appears expensive and water is not a renewable resource at some facilities. Rate kinetics were mentioned to be slow, and productivities are also low; it was beneficial to hear this, as industry will want to understand this. Encouraging data were exhibited with conversion improvements. Similarly, mention of stress relationships and practical industrial aspects of operations or common process upset scenarios such as percent total solids and substrate loading, end-product inhibition, and temperature and pH ranges will be important to highlight at some point. Near-term impact with Gen1.5 feedstocks was mentioned, although no specifics were given related to the enzyme engineering or optimization. It is known that fiber contains a reasonable fraction of arabinan, which can be easily converted with industrial yeast strains to ethanol. For producers utilizing corn fiber feedstocks, featuring this area of work would be impactful. The retraction could be due to the rescheduled investigations on glucuronoarabinoxylan linkages. I am very excited for the work to come. Good job!

- Strengths: This project does a terrific job of highlighting BETO's links between fundamental science and practical applications in the biorefining industry by drawing on NREL's extensive expertise in organism and enzyme engineering. The potential impact of this research on biorefinery operation is significant, as it targets new cellulase systems, a major cost contributor to the final biofuel MFSP. At the same time, the work offers scientific impact through generation of new fundamental knowledge, demonstrated by publication in high-impact, peer-reviewed journals. The team's subsite approach, enabled by the Megatron, gives a sophisticated approach for importing subdomains into *T. reesei*, inducing properties necessary for improved performance over current cellulases. In particular, the team has been able to introduce three mutations into chimeric systems and engineer an organism with a 1.55 times improvement in the production of fermentable sugars. Significant progress has been made toward project milestones, and importantly, suggests new directions for the ongoing work. Of particular importance, and linked to other projects in the program, is the proposed transition from whole-cell to cell-free technology. Commercial interest in the project was demonstrated by industrial letters of support.

  Areas for improvement: The presentation would be strengthened if it could address the issue of potential diminishing returns. For example, slide 8 shows a reduction in projected cost of about 16% from FY 2019 to FY 2022, but then only a 3% drop in the following eight fiscal years. There also appears to be a typo on the slide, in that MFSP reduction = $7.49 in each column except the first, where the FY 2019 SOT is stated to be $7.79.

- This is a fantastic project and a clear highlight of the BETO portfolio. The team is making outstanding progress and doing really interesting and cutting-edge science, from both an applied and fundamental perspective. It is also great to see the team working with companies such as Novozyme. Lastly, the results with the king chimeras are very impressive. No concerns are raised by this reviewer.

- This project is making exciting progress on engineering better cellulases by combining discovery of new enzymes from natural enzyme reservoirs, exploring mutations that improve activity, and making chimeras to combine subdomains from different enzymes. Improvements on hydrolysis are contributing to the predictions of reduction in the MFSP of the biofuel. Nice work!

PI RESPONSE TO REVIEWER COMMENTS

- We appreciate all the reviewer comments. We would like to thank them for a very thorough review and for their support. In response to concerns about reducing cost and crystallinity, reducing the crystallinity of the feedstock is indeed one way that pretreatment can further continue to reduce enzyme loadings and thus cost. But cellulose has been designed by nature to resist such processing steps, so usually high-energy mechanical treatment or more expensive chemistry is required. Higher disruptive energy means more horsepower, and thus electricity use and related GHG challenges. More caustic chemistry certainly will work to swell and modify cellulose, but usually these molecules are expensive, explosive, and toxic

to microbes and factory staff. In developing DMR, we have attempted to "thread the needle," balancing pretreatment efficacy, cost, and process yield. Note that the improved Cel7A mutants should enhance any processing step that deconstructs lignocellulose to mixed sugars, including corn fiber and even short fibers from pulp and paper processing. Acid treatments have the problem of condensing lignins remaining in the slurry, which leads to lower-value lignin. It is true that the Gen1.5 applications appear to be very promising, especially the new Cel7A enzymes, and our work to identify recalcitrant glucuronoarabinoxylan linkages and enzymes able to hydrolyze them that do not exist in current commercial formulations. This linkage work has been moved to later this year, and hopefully reported in a milestone in FY 2022.

Regarding deep-dive biochemistry of the Cel7A mutants, this work is now underway. I think I mentioned during the talk that some of the critical data were generated late in the year—this occurred when we had suitable quantities of all purified enzymes, enough of the DMR pretreated feedstocks (from several modified procedures), and enough staff in the lab. Yes, COVID did slow down pretty much all lab work in 2020. We will examine performance as a function of percent solids, temperature, product inhibition, and the overall formulation. This work should result in a nice high-impact paper. The final alkali washing step is very interesting. As one might expect, an alkaline process is sensitive to *any* pH excursions that might occur along the way, as this will result in partial "replating" of lignins (probably as lignin carbohydrate complexes) on the cellulose microfibrils. We spent some time demonstrating this was the root cause of the reduced conversion of the DMR solids sent to Andritz for disk refining (solids acidified to stabilize the biomass during shipping). This final dilute alkali wash can be omitted if more lignin is removed from the solids early on, which is why LTAD has recently focused on additional steps such as ozonolysis and dual alkaline extractions. Finally, our modest yields from *T. reesei* were considered sufficient for the objective of this work—i.e., to produce enough protein upon which to conduct research. We would normally consider scale-up of desirable enzymes to be the specialty of the enzyme companies, but in this case, we will consider proposing to DOE that we pivot some effort next year to building more effective production strains.

The topic of diminishing returns is very real. However, projects like improving cellulase performance will "float all boats" in most biological commercial biomass conversion processes. But another thing that may be worth noting is that even though the percentage reductions may become smaller, on an absolute basis the "best case" FY 2030 MFSP that we show still represents a $0.23/GGE reduction compared to FY 2022 and a $0.50/GGE reduction compared to the latest FY 2020 SOT basis, which would definitely contribute a meaningful amount of assistance in further reducing the dependence on what we need from lignin upgrading (i.e., every little bit counts given the heavy lift we have on the lignin side). Moreover, the recently appreciated application of better cellulase formulations to Gen1.5 (corn fiber) is an example of this. So, work that may be reaching value asymptotically in the Gen2 process (here, with DMR pretreatment) may have a much larger impact on other biomass processing scenarios. The underlying question is, how much more do we need to know about fungal cellulases and how much better does their performance have to be? For better or worse, this question must always be linked to overall process specifications, such as which feedstock, pretreatment, and enzyme formulation is under consideration? When I wear my basic science hat, I think it is very interesting that we were able to find a half-dozen or so superior Cel7A enzymes in nature, compared to the *T. reesei* enzyme. At the outset of this project, I would not have predicted this outcome. Also, the fact that we were able to build active and accurate mixed chimeras reflects the skill of the team, but is nonetheless sort of surprising as well. I only recently realized that what we did reflects natural mechanisms and consequences of horizontal gene transfer in the biosphere! I hope our work here will inspire a new general approach to enhancing critical commercial enzymes using these strategies.

CELL-FREE AND IMMOBILIZATION TECHNOLOGIES

National Renewable Energy Laboratory

PROJECT DESCRIPTION

WBS:	2.5.4.101
Presenter(s):	Adam Bratis; Yannick Bomble; Zia Abdullah; Courtney Payne; Jessica Krupa
Project Start Date:	10/01/2018
Planned Project End Date:	09/30/2021
Total DOE Funding:	$2,700,000

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 biological conversion of feedstocks to biochemicals.

More specifically, we will develop new technologies and routes that could be used to produce high-value biochemicals such as 1,3-propanediol, 3-hydroxypropionic acid, 2,3-BDO, or polyhydroxybutyrate from biomass-derived C5/C6 sugars or lignin, but also from waste byproducts such as glycerol. This project will lead to significant innovation and new concepts and rational design of pathways and enzymes. Within this project, we will develop new metabolic enzyme cascades that will represent natural or artificial combinations of enzymes to produce the desired biochemicals from a variety of feedstocks. We will also develop basic design 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 will include a TEA of cell-free approaches to provide the sensitivities of the process to enzyme loading, activity, pH, reactor volumes, and cofactor recycling rates. Finally, we will focus on further increasing stability, operating lifetime, and efficiency of the pathway enzymes by immobilization on support surfaces. We will focus on immobilizing pathway enzymes or combinations of enzymes on several different conducting polymers and evaluate the effect on stability and operating lifetime. As more combinations become available, we will conduct a more systematic study of the means of immobilizing these enzymes. This preliminary work will enable the in-depth study of cofactor recycling at these interfaces using mediators for electron transfer. Taken together, these approaches will enable process intensification, continuous operation, lower capital and separations costs, and end-product flexibility, and thus have the potential to contribute significantly to BETO's goals of cost-competitive biofuels and bioproducts.

To date, we have demonstrated (1) the conversion of pyruvate to 2,3-BDO (four enzymes with cofactor recycling, no additional cofactors needed) >90 g/L (2 g/L/h) of 2,3-BDO from pyruvate without optimization in <48 hours; (2) the assimilation of xylose in our cell-free system in top glycolysis towards the production of 2,3-BDO; (3) the promise of converting glycerol to 1,3-propanediol and 3-hydroxypropionic acid based on enzyme tethering to protein scaffolds; (4) cofactor recycling at electrodes and assessed long-term cofactor stability; and (5) the conversion of glucose and hydrolysate to 2,3-BDO (with cofactor recycling) at titer of ~30 g/L (2.5 g/L/h) of 2,3-BDO from glucose and ~16 g/L (1.4 g/L/h) of 2,3-BDO from hydrolysate.

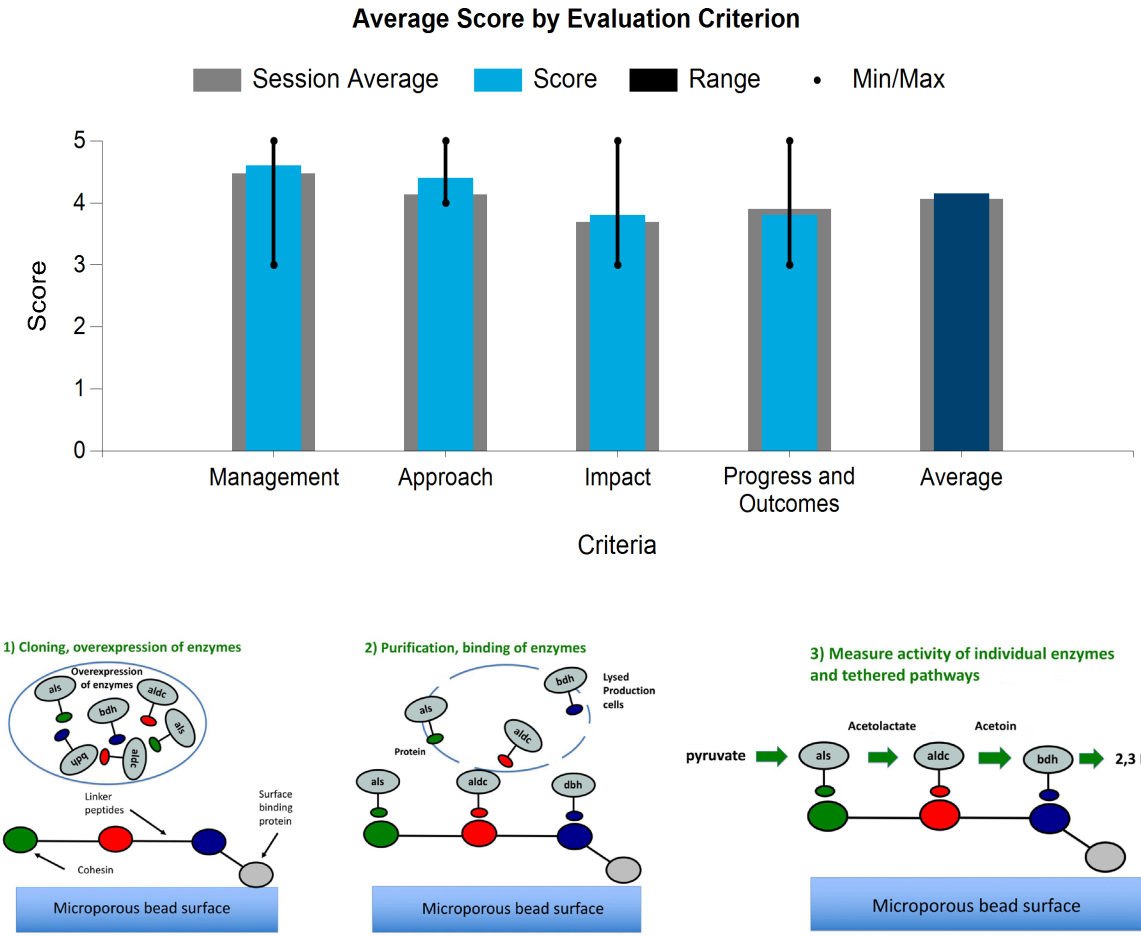


Photo courtesy of NREL

COMMENTS

- Good management organization with tasks and subtasks. Highlighted risks are enzyme stability, cost, and cofactor cost, as well as processing costs. The project deploys two parallel promising approaches for cell-free enzymes and immobilized enzyme catalytic pathways. These innovative approaches are made possible by BETO's focus on supporting new technologies that can enable future cost-effective production of biofuels and chemicals. Technological development, while challenging, is consistent with BETO's drive for innovative disruptive technologies. Industrial impacts are hard to assess with hydrolysate. It is not clear how much enzyme protein/cofactors would be necessary as compared to the production using whole-cell catalysis. The impact of toxic inhibitors and degradation products on cell-free enzymes is not well understood. There is no indication that contamination of cell-free preparations does not also present a challenge, and how this can be addressed and controlled in in vitro synthesis. Progress and outcomes look promising for protein stability, engineering, and improvement without added cofactors with cofactor reutilization. Products made and titers indicate that cell-free manufacturing for 2,3-BDO with no factor addition has been demonstrated from glucose, mock hydrolysate, and hydrolysate with reasonable yields. Titers so far are not comparable to that reported for fermentation using whole-cell engineered *Zymomonas mobilis* strains. This is a challenging, high-risk approach, even though some of the early results are encouraging. Among key accomplishments, the project team highlights several achievements such as the demonstration of the conversion of pyruvate to 2,3-BDO at a titer that is >90 g/L with a productivity of 2 g/L/h without optimization in <48 hours using four enzymes without additional cofactors, and the assimilation of xylose using the cell-free system to direct flow

towards 2,3-BDO and the conversion of glucose and hydrolysate to 2,3-BDO (with cofactor recycling) at a titer of ~30 g/L with a productivity of 2.5 g/L/h from glucose, and ~16 g/L with a productivity of 1.4 g/L/h from hydrolysate. These accomplishments support that cell-free and immobilization technologies are promising approaches. Commercial adoption and scale-up will still be challenging.

- Strengths: This project and others examining cell-free conversion technology is a promising, longer-term approach for streamlining bioproduction of chemicals and fuels. Its inclusion in the portfolio highlights BETO's unique position in examining new bioprocessing technology considered too risky for industry. The project reports interesting results and demonstrates valuable fundamental research. Pyruvate can be converted to BDO using a cell-free, four-enzyme system at 90 g/L, meeting and exceeding production milestones. The result on enzyme immobilization and that wild-type activity can be recovered by modifying the length of the tether between the enzyme and its support is scientifically interesting. Pathways to 1,3-propanediol, 3-hydroxypropionic acid, and diacids have been developed, suggesting wider applicability if operational challenges (cofactors, enzyme production) can be overcome. The effort incorporating biomimetic cofactors for electron transfer is particularly interesting from both a scientific and cost standpoint, as is the project's demonstration that the cofactors can exhibit high stability. Early-stage results also show that the cofactors can be recycled at electrodes, as needed for a more economical approach. These features are central to developing long-lived and economically viable cell-free systems.

  Areas for improvement: The key barrier to this approach is recycling of the cofactors (expensive) needed for redox balance. Large-scale production of enzymes is also a real challenge (for example, in processes requiring 23 enzymes!), but surprisingly, was not mentioned in any detail. More clarity around the high-level operating scenario would be useful, as would additional detail regarding the assumptions behind the TEA (slide 13). Without more information and justification, it is hard to believe that these cost targets could be reached. Electrochemical processes, here suggested for the recycling of cofactors, will be considered exotic within industry. It would be useful if the PIs could point to examples of large-scale electrochemical conversions of the type planned in this work, other than long-established approaches (chlor/alkali, hydrodimerization of acrylonitrile). As is the case in several of the NREL projects, it would be helpful to hear more about why BDO is being used as a fuel instead of a stand-alone chemical. The most informative comparison would be a side-by-side projection of production costs from cell-free, conventional biochemical, and petrochemical approaches.

- The concept and proposition have the potential to be unprecedented and far-reaching. The interactions with other BETO projects, national labs, academia, and the excitement from industrial partners is a testament to the concept potential. The project's challenges and solution plans are strategic and logical. The current state being bench scale with defined media and sterile processing signals that the technology is early in the development cycle, but everything appears to be on track.

- The goal of this project is to produce 2,3-butanediol from hydrolysate using a cell-free system. Cell-free systems are a promising technology for producing biofuels and chemicals without the need of living cells. They are especially promising when dealing with toxic substrates or products. However, there are significant concerns whether cell-free systems can scale due to the costs of the enzymes and cofactors. Some believe that these components can be made sufficiently cheap, especially when the enzymes can be recycled and cofactors regenerated. Others do not. As a consequence, the impact is unclear. Despite the concerns raised above, this is an exciting project. The project is well managed with clear and compelling milestones. The team is also making good progress and reaching their milestones. They are also addressing the enzyme recycling and cofactor regeneration challenge, thus addressing one of the primary concerns regarding cell-free systems. The only potential weakness is the choice of product: 2,3-butanediol is relatively inert, and most microorganisms can tolerate high concentrations. Perhaps the team should consider producing a toxic molecule, thus taking advantage of one of the primary strengths of cell-free systems.

- This project is evaluating the feasibility of using in vitro cell-free systems for production of biofuels from lignocellulosic biomass hydrolysates. As a proof of concept, the project is focusing on producing 2,3-BDO at 40 g/L and productivity greater than 1 g/L/h. Good progress has been demonstrated with a cell-free system that produced 16 g/L of 2,3-BDO from hydrolysates (diluted hydrolysates?) with a productivity of 1.4 g/L/h. This system used 23 different enzymes to assemble the pathways from glucose and xylose to 2,3-BDO. TEA has been used to make predictions of the effect of enzyme stability (number of cycles that can be done with a batch of enzymes) and the cost of cofactors (to assess the effect of cofactor recycling). The metric used is "bioconversion and fuel productivity cost," although it is not clear what this metric is measuring (is it $/GGE of biofuel, or something else?). This project will be able to provide valuable insights into whether cell-free systems with a large number of enzymes could be competitive with microbial conversion processes or whether the opportunities for cell-free systems would be better focused on specialized short pathways to be used in combination with other microbial and/or chemical processes. A suggestion for this project is to also evaluate its feasibility using LCA. For instance, while it may be the case that some cell-free systems operate with no CO_2 evolution, the production of enzymes and cofactors may have a non-negligible CO_2 footprint that needs to be considered. This project, with its connections to other BETO projects, seems to be the proper place to perform such analysis.

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. Regarding enzyme production and costs, we are confident that scale-up production can be achieved using dedicated production strains. Some enzymes will need to be expressed intracellularly, but 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/encapsulation. We already know that some enzymes remain active for days in solvents without immobilization/encapsulation, which bodes well for the prospects of enzymes operating for 30+ days once stabilization approaches are put in place to enable continuous operation. The same strategies used to increase enzyme operating lifetime can be used to increase the enzyme tolerance to toxic compounds encountered during production. Some enzymes will always be more susceptible to degradation, and new process strategies such as modular reactors have to be developed in the future to be able to selectively swap these enzymes for smooth and continuous operation.

  Regarding the selection of 2,3-BDO, as our project was new to BETO, we decided to select a product that would be aligned with other projects that relied on fermentations for 2,3-BDO production to directly compare our titers, yields, and productivities and demonstrate that cell-free systems can be cost-competitive even when considering a product that can produced by microbial fermentations at high titers and that has low toxicity. We are now moving to more toxic and valuable products to fully demonstrate the benefits of cell-free biocatalysis over fermentative processes for these types of products.

  Contamination could indeed be a concern, as these cell-free systems are intended to be used for weeks at the time. There are several approaches that can be used to avoid such contamination. First, cell-free processes are intended to be used for the production of cytotoxic intermediates or products, or to convert toxic feedstock streams, which will help limit the potential for microbial contamination. Second, cell-free systems are being developed to function at higher temperatures than traditional fermentative processes (using immobilized enzymes and synthetic cofactors), which could also further limit the potential for microbial contamination.

  The cost targets in our TEA are indeed in $/GGE biofuels. Here we focused on the "bioconversion and fuels" component of the total fuel cost, as this is where most of the benefits of cell-free production will

be seen. With enzymes operating for 100 production cycles, we can see a 10% benefit with cell-free systems over traditional fermentations, even for a nontoxic product such as 2,3-BDO. We expect this comparison to be more advantageous for cell-free production with toxic products. The consideration of LCA is indeed a really good suggestion. We do anticipate that cell-free processes can be designed to increase carbon efficiency but also limit or reuse any CO_2 emission evolved during the production process. Additionally, cell-free processes will have much higher volumetric rates, which will help lower water treatment needs. It is also correct to point out that enzyme production has a CO_2 footprint that has to be carefully considered. In this regard, increasing the stability and operating lifetime of these enzymes is crucial to reduce overall CO_2 emission originating from their production.

We do agree that there are significant obstacles that need to be overcome for cell-free biocatalysis to become a reality at the industrial scale. However, enabling this technology at that scale could be a game-changer for the production of biochemicals and bioproducts leading to reduced production cost and less carbon-intensive processes. One of these obstacles is cofactor management, but we and others are demonstrating that cofactors can be recycled and are stable for multiple production cycles, thus leading to reduced cost. This cost can also be further reduced by enabling the use of cheaper and more stable synthetic cofactors. Regarding the use of electrochemical processes for cofactor recycling, we agree that these approaches could be a priori considered challenging in industry. However, electrochemical conversions are already widely used in a host of industrial-scale processes, including the production of many value-added organic and inorganic products. In the bioelectrochemical area, microbial fuel cells, in which electrons are transferred from bacteria to an anode, have found commercial applications in wastewater treatment. In the past year, researchers have efficiently scaled up an electrochemical cofactor recycling system from 4 mL to 500 mL and also showed that 5-L reactors can be readily achieved with larger electrodes. This work shows that scale-up could later be achieved, especially given the smaller reactor volumes associated with cell-free processes. Therefore, we believe the time is now to develop electrochemical processes for high-value, enzyme-catalyzed products.

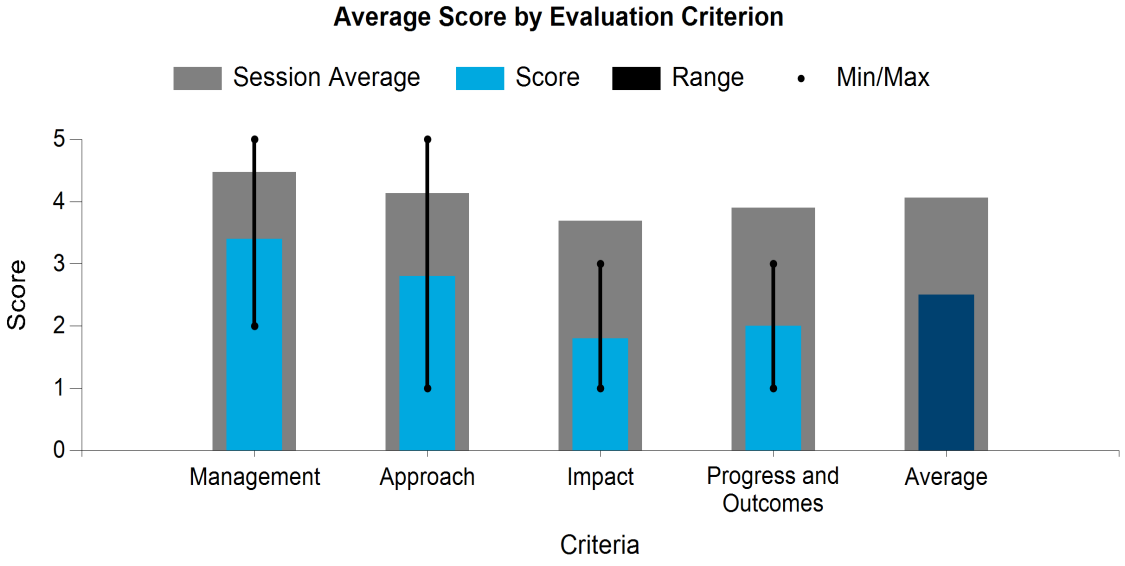
A TWO-CHAMBER GROWTH AND PRODUCTION SYSTEM FOR ROBUST CONTINUOUS BIOPROCESSING

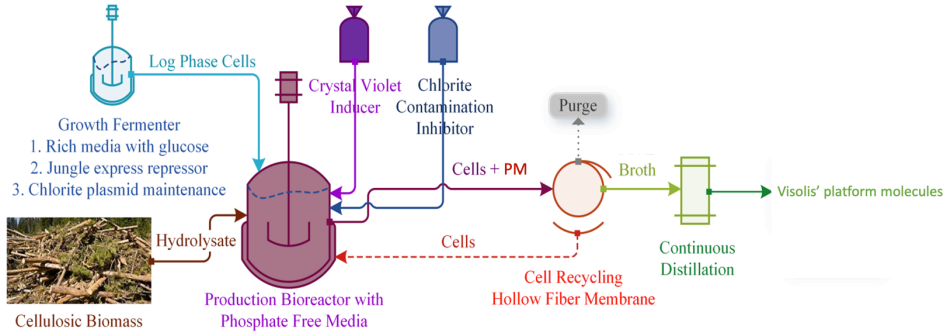
Pow Genetic Solutions, Inc.

PROJECT DESCRIPTION

WBS:	2.5.6.201
Presenter(s):	Ouwei Wang
Project Start Date:	10/01/2019
Planned Project End Date:	12/31/2022
Total DOE Funding:	$2,468,821

In the last century, the petrochemical industry has enabled the manufacturing of ubiquitous products that form the backbone of modern society, ranging from pharmaceuticals to everyday household goods. Petroleum-based processes, however, are unsustainable and contribute to global climate change. Biomanufacturing of renewable alternatives has largely not been able to compete on price, as the underlying production process, batch fermentation, has not changed in decades. The project team envisions a process that overcomes two major challenges of continuous fermentation—genetic drift and contamination—with an innovative bioprocessing method. This novel process will effectively solve these two problems by combining three emerging technologies: (1) the introduction of Jungle Express, a highly controllable and economical genetic switch technology that minimizes genetic drift and enables efficient decoupling of growth from production phases; (2) an economical biocide-based resistance mechanism that prevents biological contamination during prolonged continuous fermentation; and (3) a two-chamber fermentation process. We will harmonize these technologies in a proof-of-concept process and take it to pilot scale for the biological production of Visolis' bio-based platform molecule, a bio-intermediate for a range of renewable products including monomers and polymers, solvents, and fuels.





Overall Process Diagram of the proposed two-stage continuous bioprocess. *The crystal violet inducible, chlorite-resistant* platform molecules *(PM) production strain is grown in the growth fermenter with optimal media to maintain cells at mid-log phase. Cells are continuously pumped to the production bioreactor, in which the presence of crystal violet triggers Jungle Express, the genetic switch inducing PM production and simultaneously inhibits growth and competing pathways. The broad-range biocide chlorite prevents contamination in both tanks, and cellulosic hydrolysate provides the carbon source for PM production. Cell recycling prevents the biocatalyst from washing out. A reverse osmosis membrane dewaters the production tank to maintain the continuous mode for sustained productivity. After levels of 150 -200 g/L build up in the supernatant, PM is sent to a continuous distiller to obtain pure PM for subsequent catalytic upgrading to monomers and fuels.*

Photo courtesy of Pow Genetic Solutions, Inc.

COMMENTS

- I appreciate and am encouraged with the project ideas and concepts. The novelty of strain engineering for biocide resistance allowing continuous fermentation will be a significant achievement. I agree that bioproducts through aerobic fermentation don't compete well economically and that the project's solution could eventually be a viable pathway. Chlorite treatment was mentioned as a microbial control, as was Fermasure from DuPont. To be clear, Fermasure is chlorine dioxide, which is different than chlorite. Furthermore, chlorine dioxide resistance in organisms is well understood industrially. It is not clear how 83% fermentor CapEx reduction can be shown while the project plans on utilizing or requiring sterile fermentations. Continuous sterilization can be done at scale, although it is not being done cheaply at scale today.

- Strengths: This project is investigating new methodology for continuous bioprocessing. At a high level, these efforts offer an opportunity for process streamlining and a reduction in CapEx, all of which will be reflected in the $/GGE value of biofuel. The focus of their project is the elimination of genetic drift and bioreactor contamination and construction of a novel two-stage continuous bioreactor to improve process efficiency.

  Areas for improvement: The presentation, unfortunately, is largely uninformative. A preliminary TEA is included, but the processes being compared and the assumptions used in the evaluation are not defined. Thus, it is not clear that this approach will have a significant impact on operating expenses. The PIs apparently do not have a large number of results to present, making it difficult to assess progress. Slide 25 indicates that they do have a biocide-resistant strain but do not mention their target product. Mevalonolactone is mentioned in the abstract but not further discussed in the presentation. There is also no description of what markets the product will target, or their size. In general, the PIs emphasize the potential of their work, but really present very little in the way of hard data or results, making credible evaluation of the impact difficult. The actual amount of information pales in comparison to many of the other projects under review, giving a poor sense of what works and what doesn't.

- The goal of this project is to demonstrate a flow-through fermentation system for the continuous production of a proprietary molecule at a rate of 2 g/L/h. The milestone for this system is to sustain this level of production for at least 72 hours. It is not clear from the project description what the rationale for the two-chamber system is. It is also not clear how the two-chamber system will be operated, so it is difficult to comment on the approach or impact of this project. The project has made progress on initial tasks but has been significantly delayed due to the COVID-19 pandemic. There are other projects within the BETO portfolio looking at flow-through systems, and there may be benefits of creating collaborations or communications with those teams.

- The overall management plan and risk mitigation is in place with go/no-go milestones. Approach taken is interesting but needs further validation. Semicontinuous processing and continuous production of low-value commodities is already done at commercial scale. Most of these suffer from reduced productivity over time with the selection of strains that are adapted to poor growth conditions. It is not clear how the approaches used will address that. Funding by BETO is useful in establishing feasibility, but it is still too early to gauge success and impact, as well as commercial potential. Use of biocide resistance presents a potential downside and needs to be addressed, as it will contribute to increased resistance to chlorine-containing compounds in the environment from wastewater discharges. Special handling of chlorite (as salt or generated from chlorine dioxide gas) is required. Progress and outcomes are limited, as the project has slowed down due to the current pandemic. The turbidostat is operational, but there is still the need to optimize conditions where cell mass is in balance, so cell washout/cell death does not occur using a hydrolysate. It is not clear what the target platform molecule is. The commercial viability of the overall approach needs validation, and the key assumptions need to be reviewed. This is a high-risk project with questionable returns without additional validation. Results of testing using hydrolysate in turbidostat need to be demonstrated soon. No information is provided on the organism used.

- This project was difficult to evaluate because no details were provided. The goal is to develop a two-stage continuous fermentation process. However, the approach was unclear, entirely lacking in any detail, and the discussion was superficial. Few results were presented beyond previously published work not associated with this project. None appear to substantively advance the state of technology. Also, based on the presentation and discussion, the team appears to lack expertise in fermentation engineering and was unfamiliar with industrial practices.

PI RESPONSE TO REVIEWER COMMENTS

- We thank the reviewers for their thoughtful feedback and comments. The biggest issue raised is the lack of detailed information in the presentation, which makes this project difficult to evaluate. We apologize for this problem; as for-profit entities, we have to be careful to not release proprietary information to the public domain that could hinder future patent applications. We also followed the instruction to not provide proprietary information, which unfortunately restricted our discussion of the rationale of the two-chamber system, the product's identity/market size, the potential impact of the work, and detailed data. We aim to revise our approach in future peer review and disclose as much data as possible without negatively impacting the intellectual property application. We further addressed each specific comment below.

  We agree with the reviewer and aim to biologically detoxify the chlorite (ClO_2^-) to molecular oxygen and chloride before wastewater discharges. This was achieved by using a chlorite dismutase expressing *E.coli* at Pow. We do not expect to generate chlorine dioxide in our process.

  We agree with the reviewer concerning the turbidostat, and we are in an active investigation to optimize the conditions for the turbidostat. Fortunately, the biggest problem we are having now is that our strain is growing too fast/robust, and thus consumes more medium than we expected. We agree with the reviewer and aim to test using hydrolysate in the near future and determine the stability of our setup.

We intentionally kept the name of our platform molecule vague for intellectual property concerns. We apologize for the confusion.

We respectfully disagree with the reviewer on the comment that the team appears to lack expertise in fermentation engineering and was unfamiliar with industrial practices. One sub-awardee, the Advanced Biofuels and Bioproducts Process Development Unit, part of Lawrence Berkeley National Laboratory, was established by DOE to help the industry to ramp up the bioeconomy. They had worked and scaled hundreds of industrial fermentation processes and provided intellectual support on this project, with a strong emphasis during the scale-up stage. In addition, Visolis had successfully scaled the mevalonolactone production used in this project to a 9,000-L demo scale. Pow Bio also has worked with multiple companies to date to explore the feasibility of (fed) batch and continuous production and process development. We do apologize for not making this background information clear in the presentation.

We thank the reviewer for the comment on communications with other teams, and we would greatly appreciate the opportunity to connect with other teams and collaborate.

We thank the reviewer's encouragement and optimism on the project ideas and concepts.

We thank the reviewer for this comment on chlorite treatment. Fermasure was brought up during the Q&A of the presentation to answer the question if the addition of chlorite will cause bleaching or oxidizing effect to nutrients in the production medium. Fermasure was mentioned as a use case to suggest the chlorite bleaching effect is not expected to happen. We agree with the reviewer that the active compound of Fermasure is chlorine dioxide. However, chlorine dioxide itself is a toxic, reactive yellowish-green gaseous compound above 11°C, and thus Fermasure-stabilized chlorine dioxide, which is sodium chlorite in basic buffer solution, is used and added to the fermenter. The stabilized chlorine dioxide is activated in situ by lowering pH, or acid-producing bacteria, the contaminants. More detail can be seen in the Dupont Fermasure intellectual property below. The stabilized chlorine dioxide may be provided as a solid material, such as a composition comprising an alkali or alkaline earth metal chlorite powder, inert ingredients, and optionally dry activator such as a dry acid. Preferably, the metal chlorite is an alkali metal chlorite; more preferably sodium chlorite.

"Prevention of bacterial growth in fermentation processes," section 6, US patent US9926576B2, DuPont US Holding LLC. The linked presentation below from Dr. Derrick Okull from DuPont also provides the technical detail of Fermasure, which states on slide 3: "Fermasure is a solution of 15–25 % Sodium Chlorite; Chlorine dioxide is formed under acidic pH conditions." https://distillersgrains.org/wp-content/uploads/2019/05/5-Okull-Stabilized-Chlorine-Dioxide-Fuel-Ethanol-Fermentation.pdf. We also would like to emphasize that we do not expect to generate chlorine dioxide in our system, as pH will be controlled and we rely on the activity of chlorite to inhibit the contaminants.

We agree with the reviewer that continuous sterilization or sterilization is energy-intensive and expensive. The 83% reduction is compared with the original fed-batch case of Visolis' current production process, which also requires sterilization; thus, sterilization was not a changed variable.

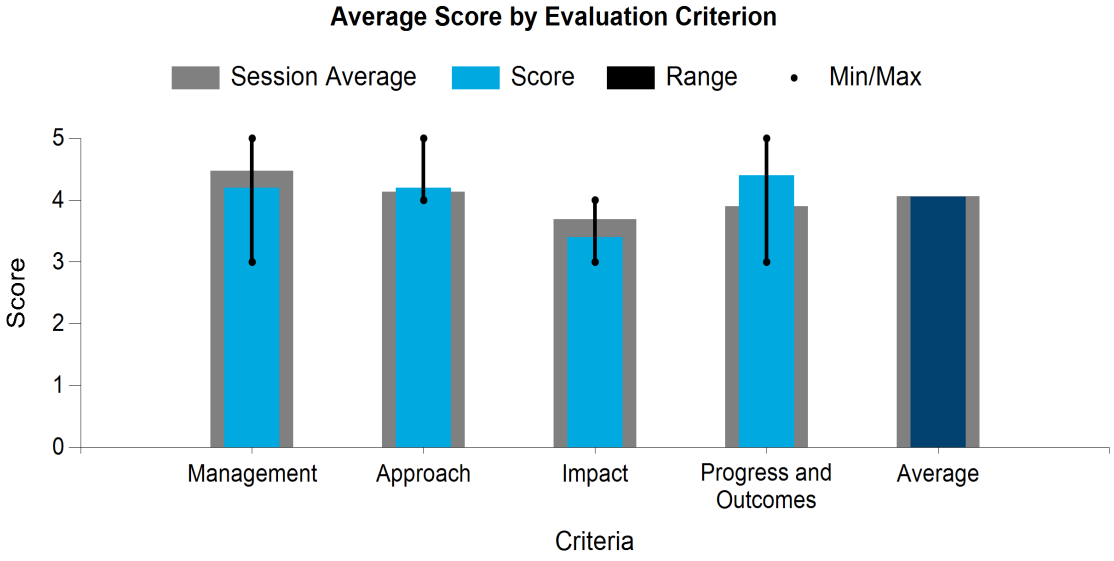
TOWARDS ECONOMICAL CELL-FREE ISOBUTANOL PRODUCTION

Invizyne Technologies, Inc

PROJECT DESCRIPTION

WBS:	2.5.6.203
Presenter(s):	Tyler Korman
Project Start Date:	10/01/2019
Planned Project End Date:	12/31/2022
Total DOE Funding:	$2,078,605

Many bio-derived compounds could replace petroleum products if efficient bioproduction systems can be developed. Approaches with engineered microbes often fail to achieve stringent production metrics required for cost-competitive petroleum replacement. In an effort to break through biological barriers, Invizyne is developing cell-free bioconversion methods. Invizyne's cell-free systems often exceed what is possible in cell-based systems. In one example, we achieved isobutanol productivity from pure glucose at >3.5 g L/h, titer of 275 g/L, and yield >90% over 100 hours. The goal of this project is to broaden substrate scope and lower costs by producing isobutanol from cellulosic glucose using inexpensive cofactors at productivity and titer nearly an order of magnitude higher than cell-based efforts. Incorporating cellulosic sugars and reengineering cofactor requirements of specific enzymes represent challenges that must be overcome. To this end, we have demonstrated significant isobutanol production from cellulosic sugar in addition to using cheaper cofactors. Overall, development of a highly efficient, long-lived cell-free process will have an impact on both industry and academia, causing many to rethink how biomanufacturing is performed and what types of bioconversions are possible when limitations of cells are removed from the equation. If successful, this project will greatly advance the metrics by which future cell-based and cell-free systems will be evaluated.



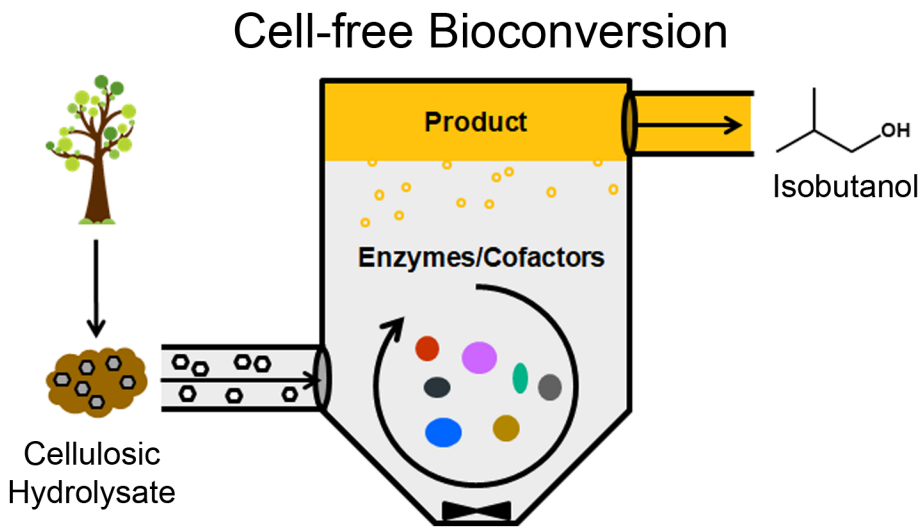


Photo courtesy of Invizyne Technologies, Inc

COMMENTS

- Management team consists of a collaborative effort between Invizyne and James Bowie lab at the University of California, Los Angeles. Dr. Bowie is also a founder of Invizyne and has been affiliated with the company for over 10 years. One of his areas of expertise is synthetic biology and whole-pathway protein engineering for fuels and chemicals using cell-free extract. Risks and mitigation are not addressed, but it maybe inherent in challenges listed. Cell-free synthesis of fuels and chemicals is a promising approach to the synthesis of fuels and chemicals. This is the primary approach used here, and it is being validated for the synthesis of isobutanol from glucose and cellulose fraction from a hydrolysate. Supporting this project is within scope of BETO's mission in enabling fuels and chemicals from cellulosic feedstocks. The technology, if shown to be robust enough and validated at a commercial scale with good economics, can be a game-changer. That impact is being recognized by leading scientific journals. The concept of cell-free synthesis using enzymes has been around for some time and has been used for a range of products (chemicals, pharmaceuticals, nutraceuticals, sweeteners, vegetable oil processing etc.). Whole-pathway synthesis presents a new challenge and opportunity but will take years to develop and validate. Progress and outcomes are promising and milestones have been provided and achieved. On the overall, the team could benefit from having an enzyme/cofactor supplier as a partner in this venture to achieve better biocatalyst cost structure, as it is identified as a major cost contributor. Having industrial partners can enable faster rapid deployment of technology and allows for greater leveraging of resources. It also brings in other areas of expertise that would be needed for scale-up. It is not clear if NREL is preparing and providing hydrolysate and what the actual preparation method being used is. Is it enzymatic hydrolysis DMR? More information on the composition of hydrolysate and validation with a range of cellulose cakes from acid pretreatment is needed as well. Cell-free synthesis offers several potential advantages over whole-cell systems. These advantages need to be demonstrated at scale. It is not clear why 500 g/L of isobutanol is the target. Does isobutanol impact cell-free-synthesis enzymes? Enzymes inside whole cells are also operating free or within a compartment and are shielded from dehydrating agents and free radicals. Hydrolysates will be challenging that way. The recovery of end products is also assumed to be easier in absence of cells, but in the case of isobutanol is not straightforward. That requires that enzymes/cofactors be immobilized or encapsulated for ease of separation with end-product removal to enable recycling of enzymes. There is an added cost, as it requires barriers for ease of operation. TEA needs to assume real-world situations based on real-world experience where pH and temperature excursions are common. Most industrial commodity-scale enzyme operations are challenging at that scale. Contamination is common. The existing processes are run at

high enzyme loading and highest temperature at optimal pH. Better understanding of current processes is warranted prior to further development. Again, the team can be benefit from partnering with an enzyme company that has been developing industrial applications. I highly recommend to BETO having cell-free enzyme synthesis also reviewed by industrial end users and existing enzyme companies to ensure that further development and validation in this area can be commercially viable. It is also recommended narrowing scope of products to prioritize short-term, mid-range, and long-term deliverables.

- Strengths: This appears to be a promising direction for improving chemical production by bioprocesses. The PIs point out that a properly designed cell-free approach can lead to higher TRY, and as an illustration, the project targets an impressive end-of-project milestone of 500 g/L isobutanol production, a 25-times increase over the best whole-cell approaches. The project reports good previous results—for example, titers with 10-times higher values, and the ability to use/recycle cofactors that are much cheaper. The concept of making bioconversions perform more like chemical reactions is a compelling justification of the work. The PIs' initial work has resulted in publications in high-impact journals, highlighting its potential. Most of the key tasks in the project have met or exceeded the desired targets. The presentation of actual results is a little sparse, but they indicate at a high level that all milestones are being accomplished or are on schedule. Importantly, they indicate that glucose in a cellulosic hydrolysate is almost as good as pure glucose, which is consistent with the concept of making this approach behave more like a chemical transformation.

  Areas for improvement: The potential impact of this work suffers because no comparative TEA is given for bio-isobutanol vs. conventional production. This would be an easy comparison to make given the availability of TRY information, and thus its omission is surprising. Further, the process costs for generating enzyme, its isolation, etc., is missing. This could be a significant cost contributor and needs to be a central part of the project's effort. It would be useful to have an idea of the PIs' plan for transforming a wider slate of biorefinery sugars—glucose, xylose, and arabinose mixtures—as described in other presentations.

- The goal of this project is to demonstrate the use of cell-free systems for production of isobutanol at titers and productivities higher than what can be accomplished using microbial cultures. The milestones appropriately target titers that are much higher than titers obtained with live cultures, which is one of the potential benefits of using cell-free systems for biofuel production. The project appears to be on track and making steady progress. The team has demonstrated isobutanol titers as high as 275 g/L from pure glucose and isobutanol production from real lignocellulosic hydrolysates (titers reported without scale, so it is not known what current titers are with hydrolysates). This project will benefit from some level of interaction with other BETO projects working on cell-free demonstrations, and from TEA and LCA of the developed cell-free technology.

- The goal of this project is to make cellulosic isobutanol using a cell-free system. These systems provide a powerful technology for producing biological molecules without the challenges associated with living systems, a key one being product toxicity. However, it is still unclear whether this technology can be economically scaled. The team acknowledged this issue and is clearly aware of the challenges. However, the answer will be unknown until someone tries. Aside from general concerns about cell-free systems, the team is making outstanding progress and achieving remarkable results. No concerns were noted. Overall, this is an exciting project and provides a well-justified bet within the BETO portfolio on a promising technology.

- The project theory of cell-free biotransformation is really exciting, and I agree that eventually these will be real conversion alternatives. I am encouraged with this project's concept and approach. The aggressive claim of "By the end of the Project we will have a cell-free system that outperforms any previous microbial system for the conversion of a biofuel from cellulosic feedstock" is a radical statement, and one I feel is actually achievable with this project in relative terms. The project appears to

be on schedule and well situated for meeting or surpassing the defined metrics. The goal of 500 g/L isobutanol at good productivity and a reasonable scale is lofty, but again, progress towards that is on track. The cofactor recycling system is a paramount platform feature—similarly will be cofactor generation. More detail or clarity regarding cofactor production and costs would be helpful.

PI RESPONSE TO REVIEWER COMMENTS

- We would like to thank the reviewers for their careful and considerate evaluation of this project and their recognition of the large potential impact of cell-free enzyme systems to produce chemicals in the future. We are particularly excited about results where isobutanol was produced using sugars derived from enzymatic hydrolysis DMR corn stover provided by NREL. Because we are using stable enzymes, the system is much more stable to the isobutanol produced as well as to inhibitory compounds within a hydrolysate. This is the power of cell-free biocatalysis. We appreciate the suggestions to establish partnerships and establish robust sources of enzymes and cofactors to reduce costs and ensure a sustainable pipeline of input materials, and we are actively evaluating potential options. Other advances that may also enhance system performance such as immobilization and product separation strategies are also being evaluated with the help of collaborators at NREL. While perhaps outside of the scope of this project, additional technologies that enhance the performance of cell-free biocatalysis is an active area of study. Finally, as we hone the cell-free process from a technical standpoint, we continue to evaluate and incorporate new information from vendors, collaborators, and experimental results into a TEA that can help identify additional areas for improvement. We also agree with the reviewers that as the technology matures, a detailed TEA is essential to identifying products that can be commercially viable in the short, medium, and long term given the state of cell-free technology and the advances being made.