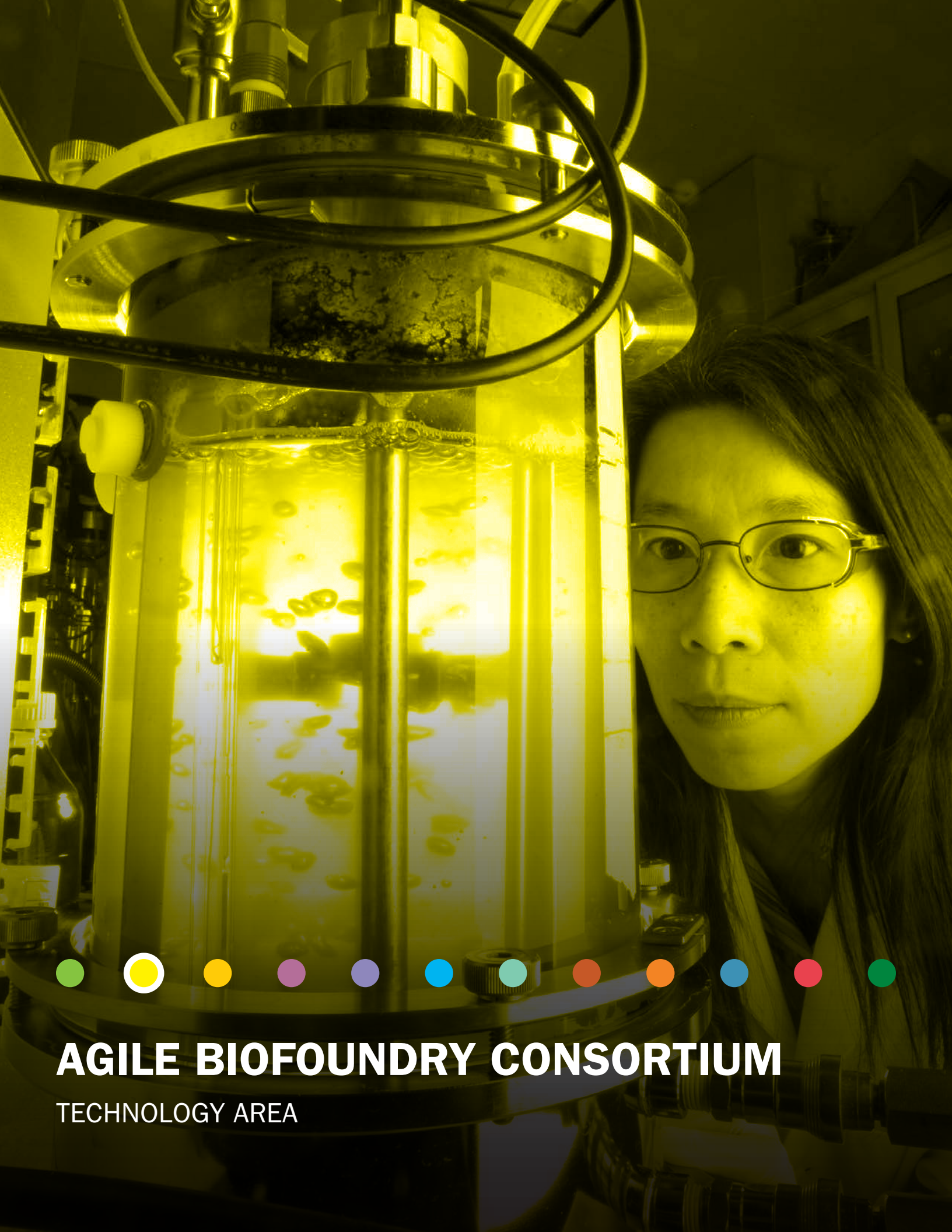

AGILE BIOFOUNDRY CONSORTIUM

TECHNOLOGY AREA

CONTENTS

INTRODUCTION

The Agile BioFoundry (ABF) Technology Area 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 17 presentations were reviewed in the ABF session by seven external experts from industry and academia. 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-agile-biofoundry.

This review addressed a total U.S. Department of Energy (DOE) investment value of approximately $65 MM, which represents approximately 10% 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 Jay Fitzgerald as the ABF Technology Area Review Lead, with contractor support from Boston Government Services. In this capacity, Marykate O'Brien was responsible for all aspects of review planning and implementation.

AGILE BIOFOUNDRY REVIEW PANEL

Name	Affiliation
Pamela Peralta-Yahaya*	Georgia Institute of Technology
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Fuzhong Zhang	Washington University in St. Louis
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ABF REVIEW PANEL SUMMARY REPORT

Prepared by the ABF Review Panel

INTRODUCTION

The Agile BioFoundry (ABF) is a distributed consortium of seven national laboratories: Lawrence Berkeley, Sandia, Pacific Northwest, Los Alamos, Argonne, Oak Ridge, and the National Renewable Energy Laboratory (NREL). The ABF's mission is to reduce the time needed to scale up the bioproduction of advanced biofuels and renewable high-volume chemicals through the development of integrated capabilities across the national laboratories. Specifically, the ABF's goal is to reduce bioprocess scale-up time by 50% compared to the average of 10 years, thereby supporting the advancement of the U.S. bioeconomy. To do this, the ABF focuses on developing enabling technologies to accelerate the design-build-test-learn (DBTL) cycle around engineering microbes for molecule production. The ABF funds internal projects, as well as partnership projects with industry and academia via directed funding opportunities (DFOs). ABF also participates as a team member in funding opportunity announcements (FOAs) directly funded by DOE. Within internal projects, ABF has four technological subareas: (1) DBTL, (2) integrated analysis, (3) host onboarding and development (HOD), and (4) process integration and scale-up. For this review, internal projects related to infrastructure, target and host engineering, HOD, and industry outreach were presented. A total of seven DFO projects and five FOA projects were also presented, which included a mix of small and large companies as well as academia. The three modes of advancing ABF goals were evaluated during the BETO Peer Review process.

STRATEGY

The ABF has a defined strategy, with a clear mission, goals, and technical targets. Over the last five years, the ABF has successfully established a program that develops industry-needed enabling technologies and hosts, gives access to scale-up infrastructure, and engages with industry toward the development of the bioeconomy. Still needed, from a strategic planning perspective, is a thorough analysis of key barriers to industrial fermentation-based manufacturing, how those barriers inform ABF technological areas, and to what extent DFO/FOA and internal ABF activities address these barriers. Further, the panel suggests establishing easily understandable metrics to track success. For example, important measures of work performed (e.g., number of hosts onboarded or equipment acquisitions completed) were provided. However, additional metrics for the ABF's role in the growth of the bioindustry need to be clearly defined. Such an internal analysis could help the ABF rebalance its portfolio and set new technical milestones for the next five years.

The panel agrees that the ABF needs extensive interactions with industrial partners to achieve its goals. Both DFOs and FOAs were judged as appropriate funding mechanisms. External partnerships strengthen existing ABF skill sets and give small companies access to cutting-edge technologies to accelerate their pathway to product commercialization. The selection of the DFO partnership projects tries to strike the right balance between an individual company's commercial interest with broader commercialization impact to the bioeconomy. The panel agrees that there are additional opportunities to leverage, through the DFO and FOA processes, at the project distribution level in terms of project risk, and potential for advancing ABF capabilities. Specifically, DFOs and FOAs could take more risk by incorporating more innovative technologies, ensuring that ABF meets its goals. Often, DFOs and FOAs were seen as benefitting the partner companies more than advancing ABF capabilities. In some cases, it was unclear the benefit to the entire bioeconomy rather than the selected company. Finally, the time scale for industry-led projects (18 months to 3 years) was seen by some as too long as companies need to show rapid progress, which in turn requires very rapid deliverables (e.g., 1 month) from ABF. For this reason, it was suggested that the ABF should increase industry awareness and use of Strategic Partnership Projects (SPPs).

ABF integrates input from industry and diverse stakeholders to develop its strategy, regularly vetting it with the industry advisory board (IAB) and using external reviewers to vet DFO proposals. The IAB involvement could be stronger, and it could be brought in to advise with greater frequency. Further, the IAB is not fully representative of the breadth of industries and product cycle stages that form the bioeconomy. More generally, the ABF has not prioritized diversity and inclusion in how it engages industry. Finally, dissemination of ABF capabilities is currently not sufficient. Improved dissemination would improve the type and number of industry and stakeholders giving input into program strategy, FOA topics, and lab calls.

No major technical gaps were identified by the panel. The panel agrees that all the technology areas the ABF is currently working on support the development of a robust bioindustry. Technological areas with most impact were judged to be HOD as well as host engineering, in particular the beachhead concept that emphasizes the development of high-flux pathways toward beachheads rather than a commercial product, with the vision that companies would further engineer these strains to produce the final product. Nevertheless, going forward, the ABF should verify that companies want to use the strains leading to the beachheads. Machine learning approaches to accelerate the DBTL cycle were judged as promising. The techno-economic analysis (TEA) and life cycle assessment (LCA) of chemical bioproducts that could be derived from beachheads will help industry determine the commercial viability of a product and set appropriate target objectives for bioproduction. Other highly impactful areas were the generation of chemical biosensors for high-throughput screening, rapid DNA design and construction, proteomics and metabolomics infrastructure, and the development of publicly available tools and databases to leverage ABF findings. Standardized metrics for the DBTL cycle composition and time should be used by all projects. This would allow the identification of key challenges, which is the premise to the entire ABF effort (i.e., improving the speed of the DBTL cycle to improve the speed of bioproduct scale-up). Via the presentations, it was not clear to the reviewers if DBTL cycles had improved since the last Peer Review.

In the coming years, the ABF has an opportunity to better define its role in the bioeconomy in order to fulfill its mission effectively and efficiently. A fee-for-service (i.e., SPP) model was seen by some as more appropriate for some industrial interactions, helping ABF accelerate its process. SPPs could help support foundational work at the ABF that may not be directly integrated into a FOA or DFO. Further, SPP funds could also be applied to reduce academic cost-share burden. Such an approach must be counterbalanced by the fact that ABF may lack the capacity to evolve more innovative projects. Finally, with the recent establishment of the Department of Defense-led Bio-Industrial Manufacturing and Design Ecosystem, BioMADE, the ABF may also wish to define its role more clearly in the bioindustrial ecosystem and avoid duplicating work and effort underway in BioMADE.

STRATEGY IMPLEMENTATION AND PROGRESS

The ABF is funding a range of projects that are closely aligned with its mission. The strategy was well conceived and sensibly prioritized. The range of project partners, from small startups to large mature companies as well as academic laboratories, allowed the ABF to tackle technical challenges at different stages. The portfolio of projects related to different goals of the ABF. HOD was a particularly relevant project, and a more extensive dissemination of these results would have a greater impact to the broader community and stakeholders. It was noted that, potentially, advancing a reduced number of hosts to the highest tier (high-throughput screening capabilities) might be more helpful than onboarding many organisms at the lowest tier. The work-around beachhead was also identified as particularly relevant because it should reduce the time to industrial development, albeit it has not yet been proven to do so. Additionally, multiple projects focused on addressing scale-up bioproduction issues.

Improvements in the ABF capabilities could be assessed with additional metrics pertaining to the speed of DBTL cycles, number of experiments per month, or FOA/DFO contributions to DBTL capabilities. Without such metrics, it was difficult to quantify the overall success of the ABF. The ABF is on the path to, but is not yet at the level of, rapidly offering bioindustrial services to industry. Working toward this goal is part of the ABF's mission.

Projects were judged as being on the leading edge of the work within the field, enabling science that pushes the capabilities of what one laboratory is able to do. Projects are being worked on diligently and are hitting the set targets and goals. The overall scope of work, ambition, and challenge level are on par. However, there is room to choose projects that encourage more innovation and for projects to have a clearer alignment with filling the goals identified by ABF. It was particularly impactful to be able to access all the ABF resources via a centralized system. Although all projects were in line with ABF goals, some had only a limited benefit in expanding ABF capabilities. Some members of the panel saw requiring lignocellulosic feedstock for all ABF projects as a limitation because it was judged to be of limited immediate utility to industry, and possibly against the goals of reducing time to market.

The ABF is likely to meet its goals and targets based on its current portfolio. However, the panel urged the ABF to prioritize the identification of metrics that define success for the ABF, and to identify its long-term goals. The project portfolio fits well within the overall mission of the ABF. However, it is important to ensure that each of the internal projects, DFO projects, and FOA projects address gaps in knowledge or process identified by the ABF. A systematic analysis of the different approaches the ABF is taking to ultimately accelerate the scale-up of bioproducts would ensure that the funded projects are filling critical areas of need. Such an analysis would not only identify successful approaches and generate lessons learned that could be generalized for the scale-up of different products but would also identify criteria for closing out unsuccessful projects so that scarce resources could be reallocated.

The ABF team is managing projects well, staying focused on the goals and mission of the program, and leading to beneficial outcomes to the performers and government. A more specific determination of success metrics and overall ABF long-term goals would result in improved benefits for performance and government.

RECOMMENDATIONS

Establish clear metrics around ABF goals and outputs.

The panel unanimously concurred that the ABF should systematically analyze its medium- and long-terms goals with an eye toward identifying gaps in knowledge and process to ensure a high impact on progressing the U.S. bioeconomy. Currently funded projects fall within the ABF's mission; however, the contribution of each project toward addressing a specific technical gap was not always clear. Further, performing such an evaluation in a continuous fashion will allow the ABF to identify new barriers, ensuring that it will always be working at the leading edge of the field.

The panel also agreed that ABF should develop clear metrics around overall ABF success and the success of specific ABF areas. If the ABF's goal is to reduce the time in bioprocess scale-up by 50%, then the metrics at the forefront should be those that are relevant toward this outcome, rather than peripheral measures (e.g., number of onboarded organisms, new equipment acquisitions, academic publications) that do not allow the evaluation of the effectiveness of the ABF to its stakeholders and the government. In particular, standardized metrics across different projects to assess the improvement of the DBTL cycles over time should be implemented. For example, how many strains can be assembled in one day, how many omics experiments can be run in a month, how often are ABF hosts and tools used outside ABF. These metrics, and others like them,

should be used to not only assess future projects but also current projects to determine their continuation. Overall, the ABF should see an improvement in the metrics—in particular speed and cost reduction—on improving the bioproduction of new molecules over time.

Partnership projects should be strategic, with a look at helping companies mature, advancing ABF capabilities, and the entire bioindustry.

The panel concurred that the DFO and FOA projects allowed the ABF to partner with companies and academia to fulfill different roles depending on the partnerships. Most of the presented partnerships were seen more as using ABF existing capabilities and significantly benefitting industrial collaborators rather than advancing ABF capabilities (e.g., accelerating the DBTL cycle) or providing benefits to the entire bioindustry. In the same spirit, the ABF is providing outstanding support to smaller companies by providing access to capabilities, such as metabolomics and machine learning, that are unavailable to them. The ABF should also start demonstrating that these contributions are indeed helping those companies mature. Overall, the ABF should measure if their own capabilities are improving over time and be able to correlate a reduction in the scale-up of molecules with each subsequent molecule produced.

The panel members agreed that the larger the government investment, the more ABF should be advancing the state of the art and its own capabilities, or choosing projects that would allow them to fill some of the gaps in knowledge or process identified as critical to accelerate the scale-up of bioproducts. Part of the problem with addressing this second challenge is that the ABF has not systematically analyzed the bioindustry to identify key barriers and developed a plan to specifically address those barriers via internal project funding or partnership. Moving forward, the panel recommends the ABF reevaluate the barriers in industry by strongly engaging stakeholders and industry, to reevaluate what is most needed to reduce the time to scale-up as a bioproduct. This will allow the ABF to not only reassess its priorities at the five-year mark, but also more deliberately choose the projects with which it engages.

The panel members agreed that the ABF should start testing whether some of their hypotheses for reducing the time to scale-up a bioproduct are indeed valid. Beachheads are a large emphasis of the ABF, and partnership projects should start evaluating the value of access to these beachheads in order to determine if the concept needs be refined or abandoned. A similar argument can be made around host onboarding development. The ABF is currently onboarding more than 15 hosts and moving only some of them to higher tier (i.e., tools that allow for high-throughput screening). Partnership projects should start evaluating if this approach is the most impactful or if the reverse is more appropriate: focus on a smaller number of hosts but advance them all to a higher tier.

Increase dissemination of ABF capabilities and outreach to stakeholders.

The ABF is building a public infrastructure that should be available to all stakeholders. The ABF includes participation of industry leaders on the IAB and as DFO partners. There is an opportunity to increase awareness among industry and academia on how to engage with the ABF or ABF capabilities. Disseminating the knowledge and access to those capabilities will accelerate the rate at which companies move through the start-up process and make it or fail in the market. The ABF should not only develop software or repositories, but also other reference materials such as manuals and virtual seminars to spread the knowledge gained at the ABF, in particular around HOD. The panel also recommends that the ABF use more extensive and more targeted channels to advertise the different funding mechanisms (e.g., fee for service, SFO, FOA).

Finally, the panel recommends that the ABF better engage with industry and utilize its IAB. The IAB should encompass all sectors of the bioeconomy, from small startups to large companies. It should also engage with

companies in different parts of the bioeconomy, from suppliers to distributors to end product manufacturers. A robust bioeconomy will hinge on the advancement of all these sectors.

ABF PROGRAMMATIC RESPONSE

INTRODUCTION

The program would like to thank the reviewers for their time and thoughtful comments throughout the review process. The program responses to reviewer recommendations are found in the following section.

Recommendation 1: Establish clear metrics around ABF goals and outputs.

The program appreciates that the reviewers identified this opportunity to improve tracking of progress made by the consortium. Measuring and tracking the ABF's progress is key to the ABF's overall mission and goals, and provides an opportunity to shift activities in response to changing priorities. The program particularly appreciates the suggestion to run an opportunity analysis to identify gaps and redundancies in the research activities to ensure each activity is contributing to the ABF's mission. In addition to analyzing ABF activities, the program also agrees that there are opportunities to more clearly measure and communicate improvements in the DBTL cycle time. These metrics are currently tracked by the ABF on a project and consortium basis, and are recorded and reported internally. These cycle times are also reported during public-facing presentations. Careful development of these metrics and tracking therein have been a focus of the ABF management. One of the FY 2020 milestones, in fact, was to define DBTL cycle times with specific unit operations. The go/no-go milestone for FY 2021 was "5 metabolic pathways and/or tools transferred between hosts, with 2X improvements in second host, with metrics defined for each case." This go/no-go served to ensure that DBTL cycle times can be tracked effectively, and that the ABF is able to demonstrate quantifiable progress. Each of these milestones has been met, providing a foundation for future work to accelerate DBTL cycle times and effectively monitor progress.

One component of more clearly tracking progress is measuring DBTL cycle times, as described above. While that is one option, there may also be an opportunity to use other types of metrics. Another approach would be to better articulate advances in host development, such as how quickly hosts can reach tier 2, or creating a beachhead strain that would lead to achieving the overall goal to reduce development time by 50%. Some current activities have been working to reduce cycle time in concert with an external partner, showing that ABF internal advances can be translated to industry.

The feedback from this Peer Review indicates that communication regarding DBTL cycle time and progress toward strategic goals can be improved. It will be important to demonstrate the DBTL acceleration capacity of the ABF as a whole. Progress made on individual projects also indicates progress toward the ABF's goals. For example, in working with the ABF, Lygos was able to improve isobutyrate titers by 20-fold by leveraging the ABF's capabilities. The program will work with the ABF to identify areas to improve both tracking and effective communication of progress.

Recommendation 2: Partnership projects should be strategic, with a look at helping companies mature, advancing ABF capabilities, and the entire bioindustry.

The program thanks the reviewers for their recommendations to improve the impact of the DFOs and SPPs. The program agrees that effective partnerships will have reciprocal benefit, advancing the external partner's capacity and building ABF's own capabilities. Improved analysis of the outcomes of these projects will help provide lessons learned for future activities and ensure the benefit balance is struck between the ABF and the

external partner. The reviewers also highlighted that it will be important to track and record whether companies are concretely benefitting from the ABF partnerships. There have been clear instances where companies have made significant gains through partnership with the ABF (e.g., Lygos). The program notes that a formal process to record these benefits will be helpful in the future. Including this in reporting requirements for future funding solicitations would provide one mechanism to formalize this type of data collection.

The reviewers note that ABF partnerships that benefit one single company may have limited impact to advance the bioeconomy as a whole. Although the ABF focuses on efforts that have the potential to be broadly impactful, benefits to individual companies *may* broadly enable the bioeconomy by penetrating new markets, or de-risking a novel technology. Understanding and making publicly available the targeted barrier(s) for an external project will help to make clear the benefit to the biomanufacturing industry as a whole, as suggested below. The program appreciates this note, and future cataloging of outcomes may help future planning efforts to maximize impact from ABF partnerships.

Finally, the program appreciates the comment that the ABF has an opportunity to further analyze the biotechnology industry to identify barriers. Substantial effort has been undertaken by the ABF to solicit and incorporate industry feedback, through the official IAB, and through informal interviews with members from industry. Additional outreach could be performed as a part of a future industry listening day, as a special session to catalog and prioritize barriers. The program agrees that this work to understand gaps is an ongoing process, and should be informed by as many partners as possible. Improving diversity, equity, and inclusion is key to ensuring all stakeholders are included and are able to effectively share their perspectives. BETO has a commitment to incorporating principles of diversity, equity, and inclusion across all platform efforts. These principles will be applied to outreach activities in the future to ensure that all stakeholders' perspectives are captured.

One key aspect fundamental to all projects is the choice of feedstocks. The reviewers highlighted that hydrolysate and lignocellulosic feedstocks are mismatched with the needs and capabilities in industry. The program notes the concern and will work with stakeholders to incorporate appropriate feedstocks that are within our authorization and are appropriate to the role of government.

Finally, the program appreciates the reviewers' suggestion that the ABF can leverage partnerships to inform host onboarding decision-making. There are many opportunities to strengthen the ABF strategic planning through partnerships, and the program appreciates the reviewers' identification of this opportunity for input on host onboarding.

Recommendation 3: Increase dissemination of ABF capabilities and outreach to stakeholders.

The program thanks the reviewers for highlighting this opportunity to raise the visibility of the ABF. The ABF website has been recently revamped and is undergoing continual improvements to ensure that it is user-friendly, up-to-date, and informative. The capabilities page has been updated recently in response to these Peer Review comments, and now lists additional ABF capabilities across the DBTL teams. Additionally, ABF has a twitter account for informal networking and information sharing. The ABF's twitter account has 886 followers at the time of this report. The program will continue to encourage the ABF to improve the impact of their communication efforts. The program is motivated to improve the visibility of the ABF and access to ABF capabilities to the broader community. Publishing a "year in review" or similar report could provide additional exposure to the public and stakeholders on ABF's accomplishments, capabilities, and areas for engagement.

Further, the SPPs provide an additional mechanism for coordination with the ABF. Effort is underway to streamline this process and ensure that the process is amenable and accessible to stakeholders.

ACCELERATING ENGINEERED MICROBE OPTIMIZATION THROUGH MACHINE LEARNING AND MULTI-OMICS DATA SETS

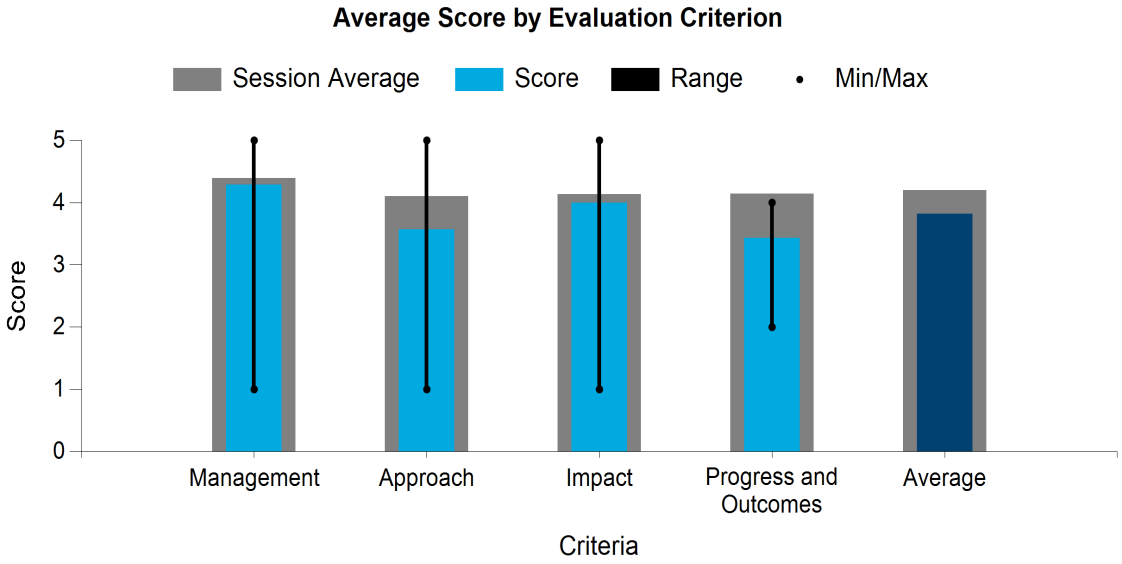
Lygos

PROJECT DESCRIPTION

WBS:	2.3.2.209
Presenter(s):	Mark Held
Project Start Date:	10/01/2018
Planned Project End Date:	06/30/2022
Total DOE Funding:	$2,857,142

The purpose of the proposed project is to demonstrate a high-throughput DBTL engineering cycle combining multi-omics analysis and machine learning with state-of-the-art strain production times. The host microbe will be *P. kudriavzevii*, and the target product malonic acid. The proposed DBTL cycle leverages the unique capabilities that both Lygos and the ABF bring to the table. Lygos will leverage its expertise in efficiently designing, building, and cultivating *P. kudriavzevii* strains. Multi-omics analysis in the test phase will be performed at the ABF labs. Lastly, machine learning techniques will be used in the learn phase to analyze the multi-omics data sets and make suggestions so as to increase malonic acid production in subsequent DBTL cycles; this work will be performed at Lawrence Berkeley National Laboratory (LBNL) and Lygos.

The anticipated significance of these data is truly monumental. To our knowledge, this is the largest data set of actual measured values that has been employed for this sort of machine learning. This work is expected to significantly improve Lygos's production metrics for malonic acid and will demonstrate the capabilities of the ABF to generate complex data sets such as this.



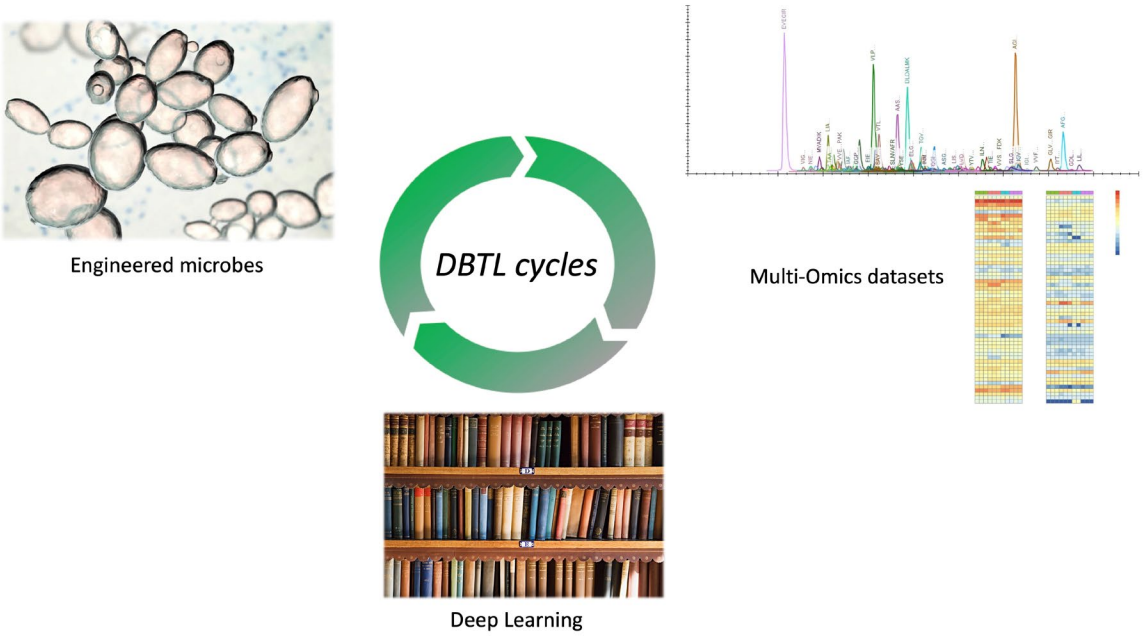


Photo courtesy of Lygos

COMMENTS

- Lygos and ABF seek to generate an extremely ambitious, large-scale DBTL experiment, collecting 80,000 omics and performance data points over six iterations. Beyond enhancing the production of a target-host pair of interest to ABF, they also seek to showcase big-data-driven machine learning to complement—or possibly even supplant—metabolic modeling in a nonstandard host. The idea is extremely attractive, and if the team can show that it works, it will have high impact. There are concerns as to whether the execution of the project will be able to match its intended scope, considering that only one of the six planned DBTL cycles has been completed. More importantly, it is concerning that there are still insufficient data to evaluate whether the magnitude of this approach is warranted. The initial recommendations have unknown design value. Further, it was mentioned that the approach can make thousands of recommendations, but there is no evidence yet whether their ranking is informative. Given the technical challenges and technical risks in scaling up to collecting thousands of data points per iteration, and because there is no prior evidence that says that all the transcriptomics, metabolomics, and proteomics data are of equal value, it may be better to instead focus on the most informative data and limit the scope to that—at least until there is evidence to support that more is better. This might mean limiting the number of time points, and/or focusing on only the omics type that was must useful in the first iteration. As a related comment, this is an expensive project. The justification for the scope (e.g., 80,000 data points) was not well conveyed, beyond saying that big data are necessary for machine learning.

- Strengths: Each partner of the team has complementary skill sets. The team managed to collect a large number of data points through multi-omics. Weaknesses/areas for improvement: The project/technical approach has a very long DBTL cycle. The entire project only aims to complete six DBTL cycles with a $2 MM total budget. This is not cost-effective in terms of current industrial standard or compared to other ABF projects. The project did not include titer, rate, and yield (TRY) metrics or fold of

enhancement in TRY metrics, which limits the impact of the project. It is not clear whether the machine learning-recommended modification can improve malonic acid production and to what extent it can be improved.

- The goal of this project is to obtain omics data sets and apply machine learning algorithms on those data sets to make nonintuitive predictions to engineer *P. kudriavzevii* for increased malonic acid production. The management of the project seems clear, with leads identified for different parts of the project. With respect to approach, the project tasks are well defined, with Lygos focused on the design and build of the strain, and ABF focused on the omics and machine learning tasks. The impact of this project is high for both Lygos and the ABF. On the Lygos side, this project has the potential to accelerate the engineering of *P. kudriavzevii* for malonic production. Potentially, the *P. kudriavzevii* data sets could be reused to optimize *P. kudriavzevii* for other on-pathway products. On the ABF side, the large data sets will drive the application and development of novel machine learning algorithms to generate ranked predictions. The impact of this project on the general bioindustrial field will depend on whether the algorithms will be sufficiently general to be quickly repurposed for other pathways, strains, and targets. The project is behind schedule, having experienced multiple delays, and the team proposes to recoup time by completing three DBTL cycles in a month, which may be an overly ambitious goal. At this point, there are no data to determine if the machine learning predictions are helping with strain improvement.

- Unfortunately, the management of this project is extremely poor and is causing serious problems in project process. This assessment is made because the management team should have already made the difficult decision to not move forward with this project. Sometimes the most difficult decision is the one that is in the best interest of all parties. In this case, the goals set by the management team are unattainable and unrealistic, thus earlier decisions to end this project should have been considered. The collaborator set out to conduct six DBTL cycles, with only a couple achieved and fully anticipating to complete the remainder in a very time-constrained environment. Mostly, ABF seems to be functioning as a fee-for-service, and if quantifiable performance metrics were available, a clear argument for how to proceed with the project could have been made much sooner. Although the data generated by this project could be impactful, poor management of this project failed to capitalize on that aspect. It almost seems that this project was too early to be integrated into ABF's program.

- The management structure and implementation strategy were summarized well, and it appears that the project is well managed. The approach is exciting and innovative, and is quite ambitious. Although many people have wanted to implement this type of approach or have done it in academia, on a smaller scale, or inside companies behind tall firewalls, this may be the first time it is being done as part of a private-public partnership, in service of a near-term commercialization target. The tools and approaches developed in this project have a clear and strong potential to be widely applicable and beneficial for many different companies and commercial products. Although the team has made good progress and was understandably delayed by the factors noted, there is a risk that the project will not be completed by the end of 2021 (which was noted as that endpoint), because so far there has only been one turn of the iterative DBTL cycle, and there are five remaining iterative DBTL cycles in the project plan. If these DBTL cycles are done sequentially, there will not be enough time to complete them by the end of 2021. Perhaps clever staggering can be done so that there are two parallel iteration paths—i.e., the odd and even DBTL cycles might need to be pulled into parallel efforts, such that DBTL 1, 3, and 5 are iterative with each other so that learning from a prior cycle informs the next cycle, and DBTL 2, 4, and 6 are another iterative path, but there is not cross-pollination between the two.

- The scope of work is ambitious and cutting edge; this FOA supports the mission and vision of the ABF. It is good to see in these later FOA projects the emphasis on DBTL and reducing that cycle time, or showing gains in what can be done in the same amount of time. There was discussion about the number of recommendations. This is a nonissue for me, as they are ranked, recommendations are free, and it is up to the end user how many to act on. It took two years to do one DBTL cycle. This speaks to the difficulty of the work involved (>13,000 data points per cycle for metabolomics and proteomics) and is not surprising. It is better to do the work well and get good-quality data rather than rush to meet a deadline. COVID-19 certainly slowed progress as well. The project is just about to assess the initial work and recommendations in the first DBTL cycle. Completing the next five DBTL cycles will be critical to understand and continue to develop the learn capabilities being developed in this project. It's highly unlikely the project is going to get five DBTL cycles in the next nine months, however. Even if they do, likely the learn component will be compromised, as the team will be rushing to move onto the next cycle and not giving as much time to really dive into the data and extract as much information as possible. The project should request a no-cost extension, beyond what was already given due to COVID, to finish the project without rushing through the last DBTL cycles. The progress/outcomes is a "4" because the progress has been slower than outlined for the FOA (but is still realistic) and also the outcome of the work hasn't been proven, as the results of the first DBTL (out of six) were still underway at the time of the presentation. Also, the presentation was a little vague that only one DBTL had been completed, and five had not yet been done. The impacts of the first DBTL were overstated; the theoretical impact of certain engineering was presented pretty strongly, and it looked like this was actual data, but it has yet to be shown to be as effective as modeled. Overall, in the next review, the presenters should be more straightforward with the status of the project. This is necessary to get an honest review of the work for DOE, and it also reflects poorly on the presenters if it looks like they are trying to overstate progress. Going forward, ABF and reviewers should be more skeptical of aggressive timelines for doing ambitious work like this. To be clear, the work being done in this proposal is very high quality and beneficial to both Lygos and ABF's goals. I'm not saying "don't fund projects like this in the future," but be more realistic about what can get done within the FOA time frame.

- This project seemed well managed, with monthly meetings, coordination meetings as needed, quarterly progress, and financial reports. They prioritized frequent communications to combat the complexity of the workflows and utilized Gantt charts. The approach was not shared in extensive detail, but the aim of completing six DBTL cycles was ambitious to the point that the cycles were likely rushed. It seems unlikely that significant time was spent on learn, and the prioritization of creating large data sets and large numbers of recommendations (I think it was 2,500) seems less important that focusing on quality. However, the distribution of responsibilities between Lygos and the labs was a nice demonstration of leveraging complementary skill sets/capacities. The impact is high: *P. kudriavzevii* is an important organism commercially, and for the transition away from petrochemicals, malonic acid is an important target. It is also good that they intend to publish a paper. The progress is good: They have made a mutant library as planned, developed an omics pipeline, and done a training set. It seems that they had some setbacks related to a fire but have managed to stay on their intended timeline.

PI RESPONSE TO REVIEWER COMMENTS

- The project team would like to thank the reviewers for their feedback, questions, and comments. We recognize and apologize for leaving some detail out of the presentation, which would have improved clarity around the goals and our progress on this project. This was done to protect confidential information. Several reviewers made note of the aggressive timelines and cycle times. These are very valid points. That being said, the project team feels that we have the tools and skill sets to achieve those goals (as long as we do not encounter unforeseen challenges such as COVID-19). We also feel that pushing toward those sorts of goals will be of value for the industry. If achieved, this will illustrate that we can, in fact, iterate on DBTL cycles rapidly and efficiently to improve strain performance. Thank you again for your input and guidance in this regard.

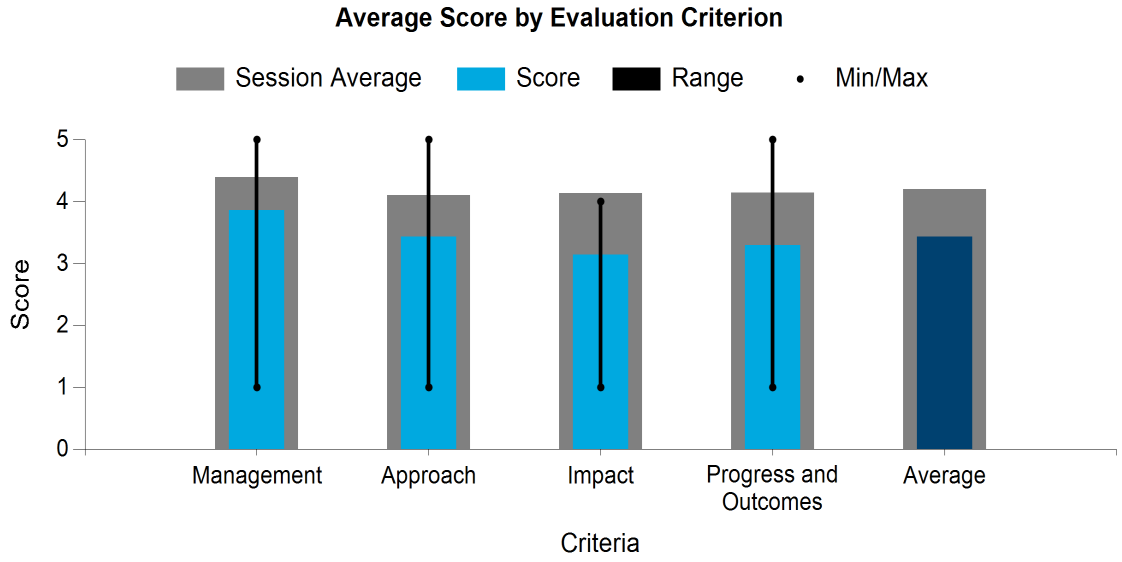
DEVELOPMENT OF BACILLUS AS AN INDUSTRIAL HOST FOR THE MICROBIAL PRODUCTION OF BIOPOLYMERS

ZymoChem

PROJECT DESCRIPTION

WBS:	2.3.2.213
Presenter(s):	Harshal Chokhawala
Project Start Date:	10/01/2018
Planned Project End Date:	09/30/2022
Total DOE Funding:	$1,666,908

The overall goal of this project is to develop a *B. licheniformis*-based bioprocess for the production of >20 g/L γ-PGA from C5 and/or C6 sugars. Objective 1 – Validate the utility of the *B. licheniformis* strain for production of γ-PGA. Objective 2 – Develop and implement genetic and metabolic engineering tools for optimizing native and non-native pathways in *B. licheniformis* strains. Objective 3 – Develop methods for both scaling γ-PGA production to 300 L and subsequently recovering pure γ-PGA.



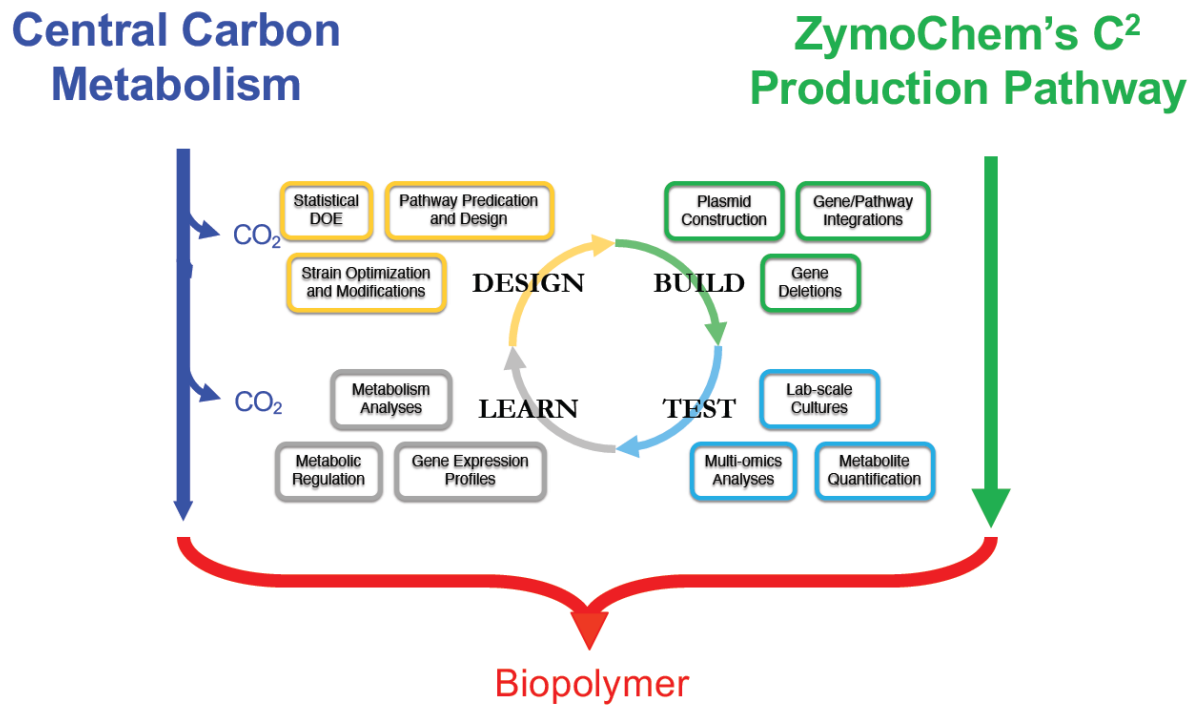


Photo courtesy of ZymoChem

COMMENTS

- Strengths: The project seems to be efficiently managed. The team was able to identify overlapping skills during the project and can adapt its management structure quickly to use team members' expertise. On the flip side, why were these issues not identified before the project started? The team managed to reach their 2x improvement in titer and rate ahead of schedule.

- Weaknesses/areas for improvement: Given the presented information, it is not clear how much this project has benefited from ABF's involvement. It is also not clear how this project can improve ABF's capability. The presentation showed very little data on their results. The amount of progress was not comparable to other projects in this review. Scale up to 300-L fermentation was performed, with no data to show how it was regarded as successful.

- The goal of this project with ZymoChem is to use a strain of *Bacillus* to produce biopolymers. The management plan is not clear, as several tasks were at some point redistributed from ZymoChem to the ABF. Also unclear is the nature of ZymoChem's contribution to this project. The ABF is tasked with generating synthetic biology tools for the organism, stain engineering, and scale-up. The impact on ZymoChem is high, as they are leveraging the ABF's capabilities to move forward with the company's objectives. The impact on the ABF is less clear. The ABF has onboarded and generated tools for a new *Bacillus* strain, but it is not clear how different this strain is when compared to other *Bacillus* strains already in the ABF portfolio. However, this is the only project presented that takes full advantage of the scale-up capabilities at the ABF (300-L reactor). Thus, this project could, potentially, serve as a blueprint for the evaluation of future DFO scale-up collaborations.

- The management is good: They established clear ownership, set clear expectations but allow owners of tasks freedom to pick their strategy, and are in frequent communications. The approach is good: The goal of improving titers by 4x would be very impactful, and demonstrating 2x improvements and establishing a non-model host and scalable processes are good steps to achieve that. The potential impact is good: *Bacillus* strains that produce biopolymers from diverse feedstocks with reduced CO_2 loss would be impactful on the economy. However, the presenter did not clarify how the results would be shared publicly, if at all, so that is a weakness. The progress is excellent: they have demonstrated genetic tools, completed a metabolite characterization, are ahead of their 2x milestone, and ran a scale-up fermentation and process development of a strain at pilot level four months ahead of schedule.

- The management structure and approach are both sound and well-conceived. The approach is solid and has merit to advance the state of the art and generate progress toward the BETO goals, and has the benefit of spanning all the way through the DBTL cycle, including pilot-scale fermentation and downstream processing. However, the technology is innovative mostly in its new combination of fairly straightforward approaches previously developed and demonstrated for other projects. The impact of the project, if successfully completed, will be to create a novel host organism making novel biopolymers with lower CO_2 emissions and higher TRY. However, the four-fold targeted improvement in titer and rate metrics over benchmarking values seems modest, unless the benchmarking values were for a process that is close to commercialization. The team has made good progress and delivered good outcomes to date, somewhat past the halfway mark of the project duration.

- There was a lack of information presented on this project, which skews the ability to objectively evaluate. The presentation lacked detailed information to such an extent that it was very challenging to see the value of this project. Such limited information sharing will endanger the project instead of benefitting it. From the presentation, it appears that there is no tangible benefit of this project to ABF. The project is not very well planned, and there are no clear benefits to ABF.

- This project is a good example of the national laboratories helping a small company do work it couldn't have done otherwise. Due to limitations on sharing data, it was difficult to evaluate the technical progress of this effort so far. Mention was made of establishing metabolomics, genome editing, and scale-up, but the presenter could not share what technical challenges were overcome nor any performance data (e.g., of the scale-up). It was not demonstrated whether the engineered strain enables carbon lossless conversion from feedstock. It also was not clear what specific ABF capabilities were invoked to enable these developments, so the impact for ABF technology demonstration was also hard to judge.

- This project uses an undefined species of *Bacillus* that is not on the ABF list of hosts to onboard. The only *Bacillus* species on the list is *B. coagulans*, which is a thermophile (optimum temperature is 50°C), and it's not clear how relevant work done in the *Bacillus* species is relevant to ABF host onboarding. The response to this question is that many of the tools developed for this *Bacillus* are applicable to other *Firmicutes*, and therefore relevant (e.g., *Clostridium* species). Going forward, DOE/BETO should prioritize work in the hosts identified by the ABF for onboarding as first priority, and second priority to other hosts that are related and could offer cross-development of tools. Even if some of the genetic tools are applicable across hosts, it is still better to focus resources on the defined host list to maximize the speed of developing those hosts. It wasn't clear from the presentation if this *Bacillus* species has already been worked on at ZymoChem or was something new just started on this project. The presenter said that this species was screened and chosen before applying for the FOA. Is this their main chassis? It is

relevant to the review because after talking up their C2 technology in the slide deck, including the summary and impact slides, it only came out in questioning that the project team has not yet been able to get the C2 technology to work in *Bacillus*. Presumably then, this isn't the main chassis of the company? This should have been addressed in the presentation, both why this strain was chosen for this project if it's not the main chassis, and the fact that the C2 technology has not yet been successfully implemented in the species. Keep in mind that the reviewers only have the information presented in the slides. Was this strain chosen for reasons that help product formation or tolerance, or was it chosen because it can eat lignocellulosic feedstocks? Does the latter matter to the company? (There was no mention of lignocellulosic feedstocks on the ZymoChem website). As stated to the ABF/BETO in other reviews, the emphasis on requiring companies to use lignocellulosic feedstocks needs to be reevaluated. These feedstocks are not going to be available for at least a decade, and making companies use them only diverts resources from work that would be deployed more quickly if they could use standard feedstocks. Compromises and alternatives to satisfy BETO's need to develop biomass feedstocks is discussed in other ABF reviews. The exact progress on tasks 1–4 was not clearly stated. There was a vague mention of "successful development of genetic tools and recombinant protein expression within the host" and "successfully ran scale-up of fermentation and process development of the strain at pilot level" as the only information on progress and outcomes. I work in industry, and I understand you can't talk about everything and need to keep information private. That said, ZymoChem needs to learn from other companies' examples of how to give enough data during required review periods and enough information to evaluate progress. For example, graphs can be unitless or names of genes labeled gene 1, gene 2, etc. Other presenters during this session should be good examples. The score of "3" reflects more the lack of information to enable an objective review than a poor prediction of the eventual outcome of the project. Although other companies/projects did this too, this particular presentation was the most egregious at being vague and also obscuring information and glossing over the work that wasn't going well or wasn't done yet. Not only do the reviewers need that information to make a fair assessment, but also it reduces trust in the company presenting. This project felt more like a DFO, where the company gained more benefit than the ABF, which should not be the case for a FOA.

PI RESPONSE TO REVIEWER COMMENTS

- Thank you to all reviewers for the comments. It is well understood by ZymoChem that we need to talk about the data and what was achieved in more detail. We have done so for other projects and have seen other companies do the same for Peer Review projects. We always strive to do that. However, please understand that the strain, target product, and product's end application are all currently business/intellectual property (IP) sensitive information, making it harder than usual to share details leading to the progress presented. Regarding reviewers' comments on C2 technology demonstration in current strain, this is an upcoming milestone toward which we are working. As described in the Peer Review, we recently developed capabilities toward genetic manipulation in the strain—this is crucial to establish the C2 technology in this strain. We are actively working toward demonstrating this milestone. Based on discussions with BETO management, the team is looking into applying more sophisticated learn approaches to understand the moderately large amount of omics data (transcriptomics, as well as metabolomics) generated through this project, and subsequently guiding strain engineering strategies to improve strain performance. In terms of result sharing, we expect to publish results in leading journals once IP sensitive information is protected.

ADVANCED ALGAL BIOFOUNDRIES FOR THE PRODUCTION OF POLYURETHANE PRECURSORS

UCSD

PROJECT DESCRIPTION

WBS:	2.3.2.216
Presenter(s):	Stephen Mayfield
Project Start Date:	10/01/2018
Planned Project End Date:	06/30/2022
Total DOE Funding:	$2,570,000

The primary goal of this project is to develop algae as a platform for the production of polyurethane precursors. In collaboration with ABF partners Pacific Northwest National Laboratory (PNNL) and LBNL, we will advance our algae platforms from their present baseline levels as laboratory-scale research projects (TRL2) to pilot-scale production at the University of California San Diego (UCSD) algae facility (TRL5). To achieve these advances, an initial project focus will be to develop the genetic tools and high-throughput screening technologies necessary for the production of polymer precursors in algae and cyanobacteria. In parallel, using metabolic modeling we will identify potential production bottlenecks, and then use genetic engineering to increase the production of key polyurethane precursors. Multiple rounds of DBTL will be used to achieve these goals. The most promising candidate production strains will be evaluated under industrially relevant production conditions, and data from these experiments will serve as inputs for TEA and LCA evaluations. Because algae can be grown heterotrophically or phototrophically, we will directly compare the costs of chemical production under these two industrially relevant conditions. To date, the project has achieved good successes creating new genetic tools for algae, and enhanced production of succinic acid using metabolic modeling and genetic engineering; we have reached our go/no-go milestone of 1 gm/L succinic acid production in algae. We have a way to go to achieve the FOA milestone of 20 gm/L, but our early success suggests that significant progress toward this goal can be met under this award.

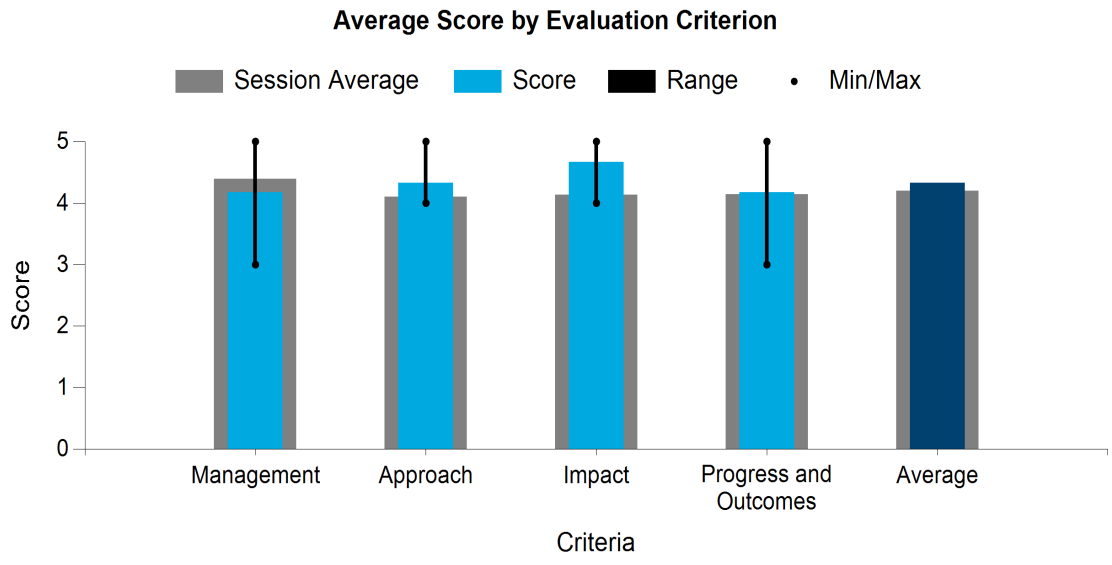


Photo courtesy of UCSD

COMMENTS

- Overall, this is extremely high-quality work, with ambitious scope and cutting-edge synthetic biology; all this supports the ABF's goals and mission. The scope is appropriate for a FOA, and sharing between academic labs and ABF/national labs is appropriate. This work will absolutely advance the goal of

making renewable, biodegradable polyurethane in algae. This is in line with the goals of ABF. The biosensors and mass spectrometry-based high-throughput screening work was not presented. The presenter said that work was "not quite ready" to present, but if any work had been done, an update should have been given. If the work is behind schedule, that should have been brought up. The work on synthetic promoters and metabolic modeling for increased flux is well thought out, well executed, and the outcomes are promising. The project reached 1 g/L and is a "go" for further scale-up work. The presenter noted that they still have a long way to go to get to 20 g/L. The honesty in this simple statement is noted and appreciated; a lot of other FOA presentations purposefully glossed over or obfuscated information that was not positive. 20g/L should be the target, as you need to push to high titers to be industrially relevant (the presenter said likely would need 40 g/L to be industrially relevant). The project still has time to get to this goal and lots of leads with the promoters and modeling work. As stated in other reviews, the requirement to have the project use lignocellulosic biomass is not a good use of ABF/BETO resources and will slow down progress on successful commercialization. Biomass sugars are not going to be available for at least 10 years. There are ways for ABF to support BETO's mission to bring biomass feedstocks to reality without requiring DFO and FOA projects to develop their platforms on hydrolysate. (Testing on hydrolysate to understand and benchmark differences is a good idea, but not develop the hosts to produce on biomass sugars.) An important question, which was debated after the presentation, is how the host organism fits within the ABF list of alternative hosts to onboard. This species is not on the list, and yet significant resources are being spent on developing it. The system fits into BETO's algae work, but does it fit in ABF? This is a question for ABF/BETO to decide. Going forward, how much should ABF focus on onboarding and developing only organisms on the list? My recommendation is to focus resourcing as much as possible on the list; otherwise those efforts are going to slow down. However, there always needs to be discretion to consider off-list projects, if they bring other significant value to the ABF. However, it appeared there was a lot of benefit to the ABF with the metabolic flux modeling, and I'm giving the benefit of the doubt regarding biosensor work and mass spectrometry-based high-throughput screening; the latter should be highly applicable to other projects. I'd like to hear more from ABF about what benefit this project has brought to the teams. Lastly, the presenter said how much he appreciated the BioFoundry aspect of the ABF, and what a huge benefit it was to their team. ABF said they gained valuable experience running the cycles to improve the BioFoundry. This again brings up the question, posed in other ABF reviews, of how the ABF sees itself contracting these services to companies and academic labs in the future. Consider how to bring in more business, especially by making the contracting quick and easy with short turnaround times.

- Strengths: The project has a clear management structure based on each party's complementary skill sets. The project has characterized a large set of promoters in algae that will add to ABF's new capability in engineering photosynthetic organisms. Further characterizing this library may result in new knowledge to our understanding of gene expression in algae, so the scientific impact is clear. The team has achieved their milestone of 1 g/L succinic acid production.

- Weaknesses/areas for improvement: The final target of 20 g/L was set too high and will be challenging to reach because photosynthetic organisms in general have weak flux in tricarboxylic acid cycles. A large metabolic engineering effort may be needed to achieve this target. A plan to collaborate with or transfer the developed technology to industry is needed to improve this project's impact.

- The management of the project was not very evident to me from the presentation, but it seemed as if PNNL, LBNL, and UCSD were coordinating successfully. The approach of focusing on creating synthetic promoters utilizing machine learning and metabolic engineering tools that predict productivity

was sound. It was interesting to see LBNL utilized as basically a stand-alone build service for this project; it seemed like there may have been opportunities to better utilize the labs' expertise, but that it was also valuable for the labs to test their capacity/high-throughput workflows. The impact was high: the project was directly connected to commercial potential by the intent to take the research to parent companies like Reef, and the researchers have been contacted by many shoe companies, demonstrating demand. The progress was somewhat mixed, with a few unexpected issues that came up that seemed only partly addressed, but strong results at least on the promoter front. It seems as if substantial optimization will be required in the next phase of this project.

- The management structure was not entirely clear from the presentation, especially in terms of how progress is tracked and course-corrected with the very large number of participant organizations and stakeholders. However, the management appears to be working fairly well because the project has made good progress on many different parallel and interconnected tasks. The approach is well-conceived and comprehensive, going all the way from molecular biology tool development to mid-sized laboratory fermentations and on to TEA/LCA analyses. If successful, the project will clearly and substantially contribute to advancing the state of the art and making progress toward BETO goals, and the project includes several innovative approaches along with more traditional or straightforward approaches. In about 16 months, the project appears to have made substantial progress and is on track to achieve the end of project milestone and deliver good progress toward the overall goal of developing an algae-based production platform for polyurethane precursors that can be commercialized.

- This is another good example of how the work benefits ABF overall. The project is distinct from others, by looking at algae as a model production organism, and ABF is able to learn from the activities and improve its own resource capabilities. ABF members play a pivotal role in the work, and they will benefit from the outcomes with new data, new tools, and a comparative capability across a multitude of chassis for production. The danger with this project is that ABF may quickly fall into a fee-for-service role, rather than helping drive technology development. ABF certainly leverages their expertise to inform the collaborator, but it is unclear what quantifiable metrics help ascertain success.

- This project seeks to develop a new system for bioproduction of precursors for biodegradable polyurethanes. To do this, it uses the ABF's DBTL capabilities for promoter mining and metabolic engineering, and pairs them with sensors, screening, and scale-up capabilities offered by academic collaborators, which notably includes TEA. Significantly, cost-share comes from companies a bit closer to end users than many other ABF collaborations. For the promoter mining, it appears that the empirical discovery was helpful, but it was not clear whether the machine learning that is applied to the results is going to be predictive. Deep learning methods are susceptible to adversarial samples, wherein random sequences can get high scores by chance (false positives), so the utility of the system for producing new promoters will need to be demonstrated. The new OptTilt approach appears to predict knock-outs that increase productivity, which is encouraging. This aspect would be strengthened by highlighting predictions that would not have been predicted via conventional optimization approaches. At this stage in the project, the path toward combining the improvements into a commercially viable strain still appears challenging, as productivities are still ~40 times lower than needed. As such, perhaps the 1 gm/L go/no-go should have had a higher threshold. The equations on slides 12, 13, and 15 were not presented in an informative way.

PI RESPONSE TO REVIEWER COMMENTS

- Part of the reason that we are applying machine learning through multiple design-build-test cycles is to determine if this process is indeed predictive and useful for generating better promoters for use in algae technology. At the end of the project, we will do more targeted experiments on the best few hits from our promoter libraries and confirm that we did, indeed, develop improved genetic tools that can be used by other groups and in future projects. The risk of adversarial samples disrupting a useful generation of promoters is recognized. In published literature, there are examples of GAGA and GCGC repeats in mammalian enhancer sequences being scored as important by machine learning approaches. They are real DNA elements associated with enhancers, but they are structural and generally not regulatory. The machine learning found this pattern, but from a biological point of view, the results are not very useful. Overall, the reviewer is correct to say that deep learning is susceptible to adversarial examples, but adversarial examples are not the same as false positives or false negatives. The kind of work we're doing with deep learning will definitely be susceptible to false positives/negatives because the amount and quality of the data is not great (relatively speaking). One way that we have overcome this is through an ensemble approach—using many mediocre predictors and, basically, only taking things that do well in all of them. We expect more false negatives this way, but it should screen out a lot of the false positives, which is what we care about most. We have also intentionally included positive and negative predictions in the synthetic promoter constructs that did not pass our sanity checks, so that the expression of these "edge cases" can be used to improve algorithm robustness. Collectively, especially when we compare the newly generated promoters to what is known about the basic biology and general sequence-function relationships of promoters, we are not observing concerning behavior so far. As for the productivities, we have passed the 1g/L go/no-go goal, which was initially set to be an order of magnitude less than the stated 20 g/L of the FOA and two orders of magnitude above our starting baseline productivity (50 mg/L). Although 20 g/L is still two-fold less than what is likely market competitive, we think that a 20-fold increase in productivity with the potential through demonstrated single improvements of an additional four- to eight-fold improvements over the course of two years is quite impressive and puts us within reach of the FOA goal.

- The target of 20g/L was in the FOA and original project requirements. Given that we have reached 5 g/L already (technically 12 g/L accounting for perfusion), and this is without the additional genetic modification demonstrated for photosynthetic production that has provided two- to six-fold improvements over prior productivities, we optimistically believe that the 20g/L target is within range when our organism is grown mixotrophically, as also required by the FOA.

- Yeast biosensor work for indicating chemical concentrations in the media was not presented, in part, because of delays related to COVID and time allotted for the presentation. Preliminary succinate biosensors have been generated and are undergoing optimization to provide linear responses in the current working range (0.5–20 g/L) while minimizing the impact of endogenous succinate. We understand why the reviewer brings up the topic of the requirements for ABF DFO and FOA projects to develop their platforms on cellulosic hydrolysate, a substrate that is not presently commercially available. However, if we are able to produce our product on this crude sugar stream, then we should easily be able to utilize the more pure sugar streams from corn starch or sugar cane sugars. We agree with the reviewer that it is an important question regarding whether or not to bring the host organisms used in this project (cyanobacteria and green algae) into the ABF. For this particular project, the scope of ABF tasks does not include working with these organisms directly (i.e., physically). However, the fact that these organisms are being actively developed by an ABF collaborator supports their relevance, and

this project is contributing to (at least design and fabrication of) genetic tools for these organisms (e.g., synthetic promoter libraries). The ABF regularly and periodically evaluates and prioritizes—with input from BETO and other BETO-supported consortia and programs—additional organisms to onboard and further develop, and the host organisms used in this project will be among those discussed in future prioritization meetings. In terms of benefits to the ABF, this project certainly has added to (as well as tested/evaluated) our capabilities regarding the iterative design, fabrication, assessment, and model refinement for synthetic genetic libraries (promoters in this particular case). These capabilities will surely be useful and applicable to future ABF projects. The reviewer's comments regarding contractual issues and accessibility of ABF capabilities are addressed in the ABF's responses to the reviewer comments for the ABF's Overview, Industry Engagement & Outreach, and DFO Introduction presentations.

- We recognize that there are many moving parts and many groups involved in our project. The key participants hold biweekly Zoom calls and actively coordinate by email as needed. Many of the objectives are well contained within each group's expertise and abilities, so the work is more chunked out and clearly delegated than may have been obvious in the presentation. Additionally, monthly calls with Daniel Fishman at BETO and quarterly reports are also used to ensure progress is tracked. It's unclear as to what extra levels of organization and coordination are expected or if they would obviously improve project progress.

- There are situations when projects with small and focused scope are appropriate for the ABF, under an SPP mechanism that could be compared in some ways to a fee-for-service model. These services/capabilities provided by the ABF must not be available elsewhere (i.e., not available from a commercial vendor), and are often key enablers for small companies that lack the resources to otherwise obtain/recruit/operate capital-intensive expertise or infrastructure. However, most collaborative projects (medium to large in scope) with the ABF differ substantially from a fee-for-service model, and use a collaborative research and development agreement (CRADA) mechanism, in which both the ABF and the collaborator co-invent and co-develop. In our opinion, the ABF is in no danger of falling into a fee-for-service role, as we are (and will continue to be) predominantly driving technology development with our collaborators.

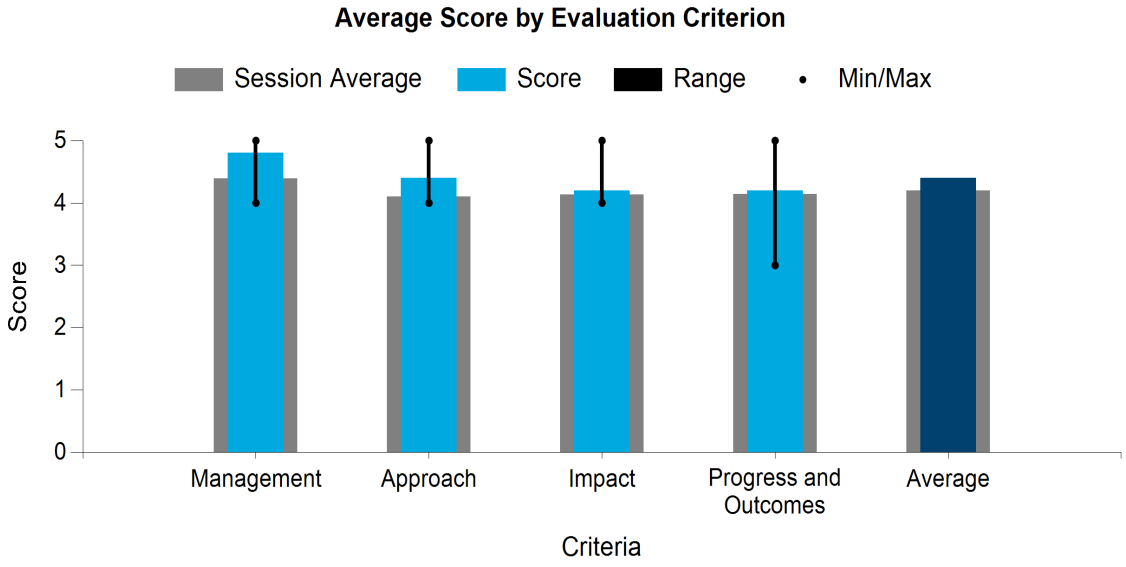
ACCELERATING POLYKETIDE SYNTHASE ENGINEERING FOR HIGH TRY PRODUCTION OF BIOFUELS AND BIOPRODUCTS

UC Berkeley

PROJECT DESCRIPTION

WBS:	2.5.3.207
Presenter(s):	Jay Keasling
Project Start Date:	10/01/2019
Planned Project End Date:	06/30/2023
Total DOE Funding:	$3,125,741

Polyketide synthase (PKS) enzymes have a modular, deterministic logic that holds the potential to act as a flexible chemical factory for the biological production of a huge diversity of valuable small molecule compounds. However, engineering a custom PKS to produce a specific desired product currently requires years of trial and error, for reasons that remain poorly understood. The goal of this project is to develop a rapid, high-throughput, DBTL cycle for PKSs and demonstrate its utility for production of materials precursors. The objectives are (1) to develop a rapid, high-throughput DBTL cycle for PKSs that will enable production of a large number of unnatural, organic molecules on demand at high TRY; (2) to demonstrate the utility of the PKS DBTL cycle to produce three molecules: one commodity chemical (caprolactam) and two novel materials precursors (2-allylcaprolactam and 2-benzylcaprolactam); and (3) to demonstrate the utility of the PKS DBTL cycle to increase the TRY of one molecule (caprolactam).



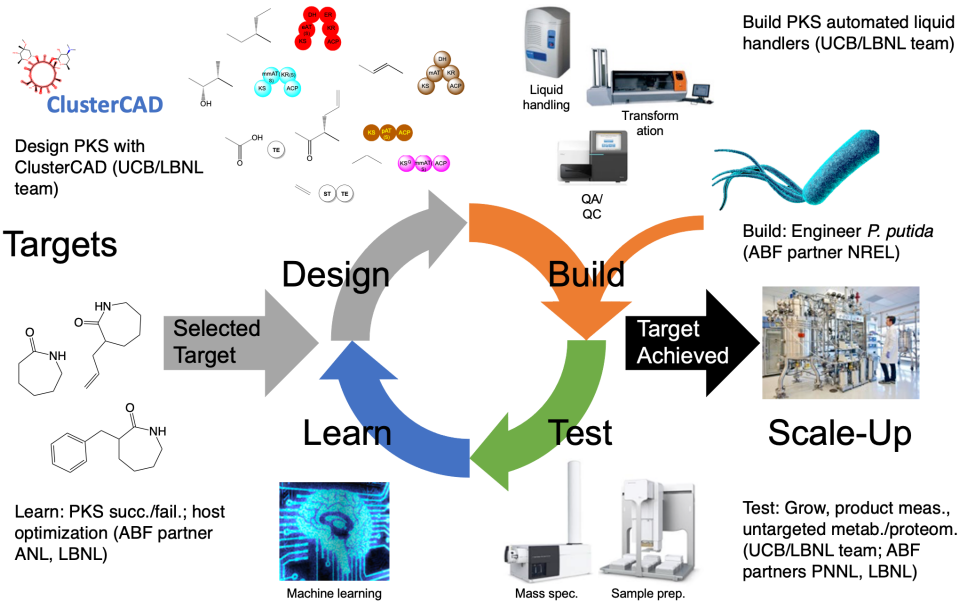


Photo courtesy of UC Berkeley

COMMENTS

- Overall, this is a good project. The challenge is that ABF needs better metrics to measure such success stories where higher research results in greater results.

- Overall, the project is highly ambitious with development of cutting-edge synthetic biology tools. This project is fully in line with the ABF mission and vision, and the scope is in line with a FOA. It is good to see an emphasis on DBTL cycle and cycle time reduction. Slide 20 explains risks and mitigations. All the mitigations are essentially "build and test a lot of stuff." Often, projects offer complicated risk mitigation strategies, but in reality, the idea of just increasing "shots on goal" is empirically the best way to succeed. The project has mechanisms to learn from the repeated cycles and what works and doesn't work; but, just ensuring that a lot of different designs will be built and tested is a proven strategy for success. It's good to see seamless interface with what is developed and the public ABF databases ("We will make our platform available for the Agile BioFoundry and others to engineer PKSs rapidly"; "We will release software under open-source licenses"). There is not private/public conflict like in other FOAs reviewed. Some tasks are completed on time, with a few very soon deadlines only 20% done. It's too early to really judge the overall progress and outcomes of the project. For now, it's tracking fairly typically for such a large and ambitious project. The ABF said so far they are getting lots of valuable information, training, and instrument expertise on the build aspect of the project.

- The management of the program seemed excellent—each facet of the project was clearly assigned and played to each participant's strengths, and they were all in frequent (at least every 2 weeks) communication, plus regular subteam meetings. The approach is good, thoughtfully leveraging technologies including ClusterCAD software, liquid handling robots, and machine learning throughout. The impact is good—engineering hybrid PKSs could have a substantial impact on both the healthcare sector and on the production of small molecules more generally. Although this research is fairly early stage, if successful, it could fuel significant commercialization in later years. Additionally, the intention

to make all results open source greatly increases the potential impact. The progress seemed good for the stage of the program—they built a build pipeline and tested it, and are making machine learning algorithms as planned.

- The management plan and structure are clear and well-designed, with frequent communication and risk mitigation plans. The approach is well-conceived, innovative, and broad in scope as well as potential impact and applicability. The go/no-go decision points are thoughtfully chosen and will serve as valuable checks on the approach, management, and progress. The project leaders are commended for their end-of-project milestones that include clear targets for accelerating the DBTL cycle time and increasing DBTL throughput, as well as including TRY targets for three products from cellulosic biomass. One thing that was not clear with regard to the impact of this project is the extent to which this future manufacturing platform can be used for novel commercialization of commodity chemicals; it is clear that it will be extremely useful for novel specialty chemicals, high-value materials, or pharmaceuticals, but the pathway's maximum theoretical yield is likely to be very low, and thus the minimum production cost per kg is likely to be too high for commodity chemicals like nylon (which was stated to be one of the potential targets). It would be good to have at least a preliminary TEA to guide the choice of targets to pursue with this technology. The progress and outcomes are on track, and have already delivered some value, although there is quite a distance remaining to achieve the final milestone.

- This effort seeks to use ABF's capabilities to aid in enzyme mining to discover and develop PKS enzymes for bioproduction of caprolactam. The effort is organized in terms of developing an automated discovery pipeline, and then applying it to the specific target-host pair. As a side benefit, the discovery pipeline will be transferred to ABF once developed. The project has high potential impact, in that it could provide access to new classes of important molecules. However, it also incurs some risk because PKS can be difficult to find and to express in functional forms. In large part, the team is addressing these risks by brute force, by building hundreds of candidate systems. Unfortunately, they report that so far that only a very small fraction of the engineered PKSs function, so this approach may be insufficient. They mention that machine learning models will be built to predict successful PDK designs, but it was not possible to evaluate this progress. Indeed, noting that this is consistent with the intended scheduled, most of the key technical accomplishments to date fall on the side of software: designing constructs, pathways, and metabolic engineering targets. Experimental validation of these tools will be critical. As such, go/no-go gates should also include much earlier demonstrations of PKS activities and actual production of target end molecules. Because there has been no test or learn described so far, it would be helpful to get more clarity on the contributions that the ABF has made the project so far.

PI RESPONSE TO REVIEWER COMMENTS

- Indeed, engineering PKSs has proven extremely challenging, which is a key issue that our project aims to help resolve. In addition to using a brute force approach, we are informing our designs with some prior knowledge, such as the idea that chemical similarity can be predictive of PKS activity. Thus far the ABF has begun developing *P. putida* as a host for engineered PKSs, including engineering the precursor substrate pathways that will be necessary for the PKSs in this project. Additionally, the ABF has been developing machine learning pipelines and models that will be ready to use when the first experimental data from this project become available.

- Thank you for the positive feedback on our project and mitigation strategy. Indeed, we experienced some laboratory equipment failures, but we were able to develop strategies to keep moving forward despite this.

- We agree that integration of TEA into our target selection process would be extremely valuable for choosing new targets in the future once the system is developed.
- Thank you for your enthusiastic comments about our project and its progress.
- We thank you for the positive feedback on our project.

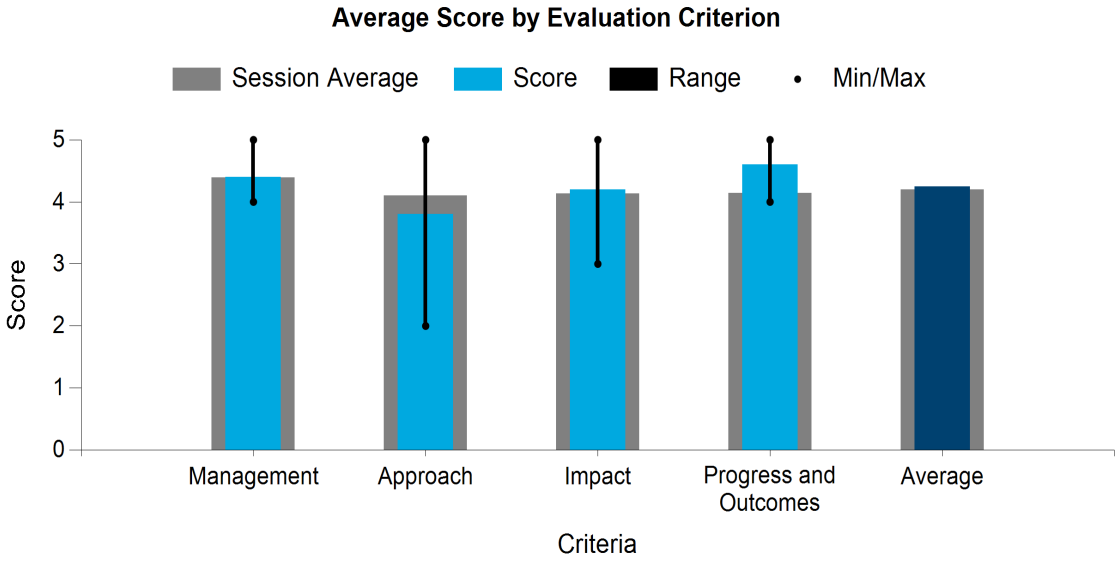
DEVELOPING MULTI-GENE CRISPRa/i PROGRAMS TO ACCELERATE DBTL CYCLES IN ABF HOSTS ENGINEERED FOR CHEMICAL PRODUCTION

U Washington

PROJECT DESCRIPTION

WBS:	2.5.3.212
Presenter(s):	James Carothers
Project Start Date:	10/01/2019
Planned Project End Date:	12/31/2022
Total DOE Funding:	$2,269,966

For industrially promising microorganisms in early stages of development, creating technologies for rapidly engineering complex multi-gene programs will be transformative for accelerating data-driven strain design. We are developing CRISPR expression technologies to create new abilities to activate bacterial gene expression, and to create platforms for combinatorial multi-gene expression tuning. To enable accelerated DBTL cycles, we are combining these new CRISPR technologies with advanced ABF capabilities for multi-omics analysis and machine learning. We are demonstrating the immediate applicability of these tools by rapidly improving the production of bio-based industrial aromatics in multiple ABF organisms. Specifically, we are (1) developing effective CRISPR gene activation (CRISPRa) tools, (2) engineering multi-gene, mixed CRISPRa/i programs, (3) integrating machine learning and computational evolutionary strategies to infer mechanistic models to drive the design of CRISPRa/i programs, and (4) optimizing industrial aromatic production through DBTL cycles at least 30% more efficient than the current state of the art. By supporting the development of new CRISPR gene expression tools and accelerated data-driven workflows, DOE funding is dramatically improving our ability to engineer bacteria for industrial chemical production.



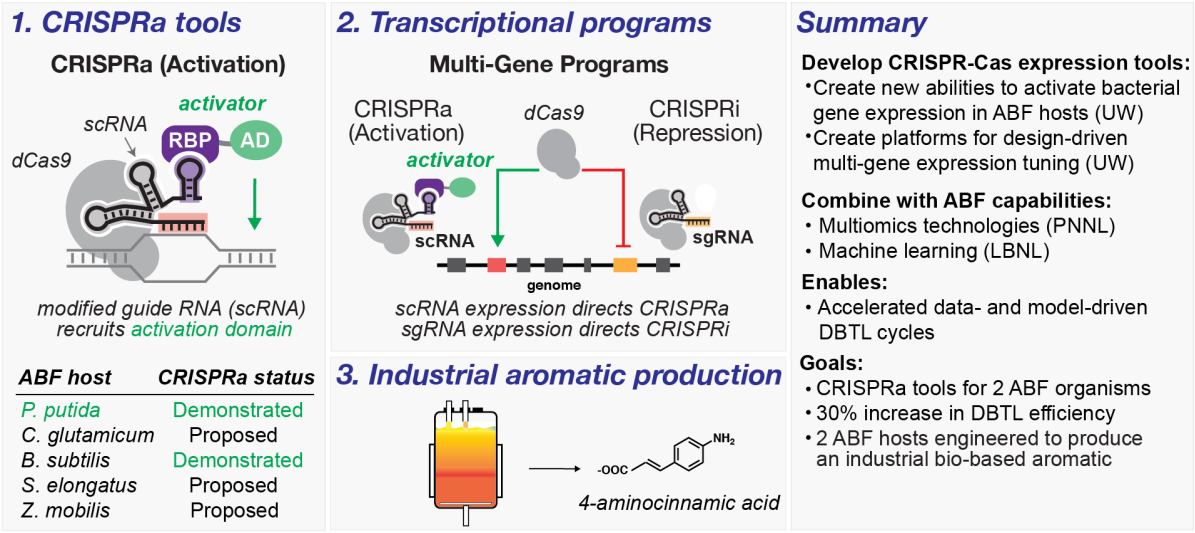


Photo courtesy of U Washington

COMMENTS

- The management of the program was very good: The division of labor among participants was logical, biweekly calls were conducted, and monthly tracking was underway. The approach seemed highly complex, and simplicity was not prioritized in the design of the program, reducing the likelihood of success. However, the potential impact is good despite this, especially because the tools are being developed for microbial hosts that ABF selected, ensuring that the commercial relevance ABF has considered in their selection of hosts is carried to the tools developed in this program. The additional emphasis on DBTL efficiency, including a measure of titer/time, makes these results generalizable. The progress was very good: they passed CRISPRa tools into *P. putida*, and demonstrated a 30% increase in DBTL efficiency.

- The management plan appears to be well-conceived with respect to frequent video calls and written communication among participants. However, it was not entirely clear the extent to which the management had thought carefully about identification of risks in the technical execution and strategies for mitigation or course correction, or about routes for integration and feedback from related projects or more distant stakeholders. The go/no-go criteria and rationales help alleviate any concerns about the management plan. The ambitious project approach has a high level of innovation, has substantial potential to advance state of the art with respect to BETO goals, and is rigorously conceived. This approach is likely to be successful (eventually) for many of the ABF host organisms, provided sufficient time and resources to optimize the technology, given the gene expression modulation demonstration in other organisms and some prior demonstrations of the modeling approaches. The impact of success of this project is potentially enormous in terms of accelerating the tailoring of production strains to make particular products with the fermentation performance required for commercial viability. The successful demonstration of a multi-genic pathway balance or expression tuning for optimal performance via this project's modeling and compact DNA engineering approach would be a significant advance that would shorten timelines and reduce development costs, despite the potential subsequent need to recapitulate the expression profile in a more stable, Cas9-free manner for the final manufacturing strain. The project

appears to have made good progress in the eight months since its inception, and the outcomes appear to be promising.

- This is a good example of how ABF is leveraging newer technologies and innovative approaches to address biomanufacturing. The program benefits both ABF and the academic performer, and it creates new tools for ABF's repertoire. The ABF management is doing a good job of organizing this project and tying in the appropriate groups within ABF to maximize benefit. The challenge is that ABF needs better metrics to measure such success stories where higher research results in greater results. More projects should be like this project—innovative, technology drivers.

- This project is a high-risk/high-reward academic project with likely a long time before it could be fully implemented across many hosts. It is good to have a few projects like this in any portfolio, and it is appropriate for the ABF to fund more basic research like this, which could have a big impact. The impact, if successful, would dramatically reduce the DBTL cycle by simultaneously affecting many genes at once in a strain (over express or knock-down at the same time). This certainly would be a major accomplishment for any organism, which currently is limited to a single genetic change per transformation; the list of organisms for which multiple gene edits can be done per transformation is exceedingly small. The progress is mostly on time and tracking as expected for an earlier stage project with a lot of work still to do. My concerns for this project are the following: (1) It will require a deep knowledge of transcription factors for each new host in order to get the over expression constructs to work. That will be a tremendous amount of work, assuming you're trying to develop organisms that are not closely related. For all the effort, time, resources, why not just work on getting a standard CRISPR system to work with multiple targets in a single transformation? Model genetic organisms can routinely alter three to five sites at once through CRISPR engineering. This project is one way to reach such a goal, but I see restrictions to the amount of overexpression you can achieve through this system, as well as questions of the degree of inhibiting gene expression, as opposed to a true knock-out. Again, it's good to see this project as a risk mitigation to achieving multi-locus engineering directly with CRISPR, as long the direct CRISPR work will be resources as part of host onboarding. (2) There was a question if this would be used for prototyping or for manufacturing. A few thoughts on that: Any strain engineered for manufacturing is going to need a lot of stabile, genetic changes, way more than could be realistically achieved though this system. Even if this system could be optimized to routinely affect five to six loci at once, that is not going to be enough to engineer a strain for manufacturing. A manufacturing strain usually needs dozens of genetic changes. That means this system would be good for prototyping. However, once the gene expression space has been explored using this system, and you find the optimum, then you'll need to make the changes permanent so you can do another round of CRISPR a/i exploration. Again, this speaks to the need to still develop tools to do multi-locus engineering directly on the chromosome for any host. However, this tool could certainly speed up the pace of finding expression levels that enhance production. (3) Finally, with regard to manufacturing, any plasmid-based system is almost doomed to fail in manufacturing. Selective pressure to lose the plasmid to shut down pathway expression is intense, and it is so easy for a cell to lose a plasmid, even with selection. (As an additional note, antibiotic selection is often prohibitively expensive at scale, let alone often prohibited by regulatory concerns.) Having a mobile element (i.e., a plasmid) with gene editing tools on it will be nearly impossible to get approved from regulatory agencies. It's too easy for a selective marker to get integrated into the chromosome to allow the cell to kick out a plasmid, and thus the system would be highly unstable.

- This project seeks to develop CRISPR-based transcriptional activators and repressors for new hosts, which is complementary to ABF's host-onboarding program. The aim is to facilitate breadboarding to test changes in gene expression levels in a host without requiring from-scratch strain construction to explore each new hypothesis. As such, I think the end-of-project milestone of a 30% DBTL efficiency increase could be made more aggressive. The project has been running for less than a year, but the team has already demonstrated activation of five orthogonal promoters. The expression levels are represented relative to off-target activation, which is important, but it should also be made clear whether absolute activation levels are high enough to be useful for testing production scenarios. There are some early hints of this presented in a mevalonate production context. Activation of endogenous genes appears to be successful in some cases and not in others. Looking forward, there is mention of a number of learn goals, including defining rules to de-risk CRISPRa designs, using the Automated Recommendation Tool to survey the combinatorial space, and multi-omic analysis, but no progress was reported in these areas yet. They may be hampered by technical limitations, say, if they require a larger dynamic range than is accessible via the CRISPR tools or if they are easily confounded by off-target regulation. As such, early go/no-go decisions on these analyses will help focus efforts on the highest impact aspects of the project.

PI RESPONSE TO REVIEWER COMMENTS

- We agree that it will be important to use data obtained early in the project to focus later efforts on aspects of the work expected to have the most impact. The end-of-project milestone of a 30% DBTL efficiency increase was set in accordance with the DE-FOA-0002029 AOI 7b project metrics. As noted by the review, our recent, newly published work (https://doi.org/10.1016/j.ymben.2021.04.002) shows that CRISPR-based tools can produce high dynamic ranges and suggests that levels of gene expression necessary for metabolic engineering applications can be achieved in the ABF host Pseudomonas putida KT2440.

- We appreciate the enthusiastic review of our project. We are gratified that the review recognized the high level of innovation in the project, found strong alignment with BETO goals, and approved of the overall approach. Regarding risks and mitigation, management has crafted plans to identify risks and pursue alternative strategies; these are detailed in the project Technical Volume and the Statement of Project Objectives (SOPO). We apologize if these were not made sufficiently clear in the 2021 Peer Review and will bring these to the fore in future presentations.

- We appreciate the time and care the reviewer has taken in offering suggestions for maximizing the industrial impact of the work. In particular, these will be immensely helpful as we develop future research directions that build on the current project. We thank the reviewer for the positive review of the project. As noted in several of the reviews, we agree that this is a high-risk/high-reward project. Nonetheless, the rapid progress we have made thus far makes us optimistic about the overall chances for project success.

ABF—Overview and Infrastructure

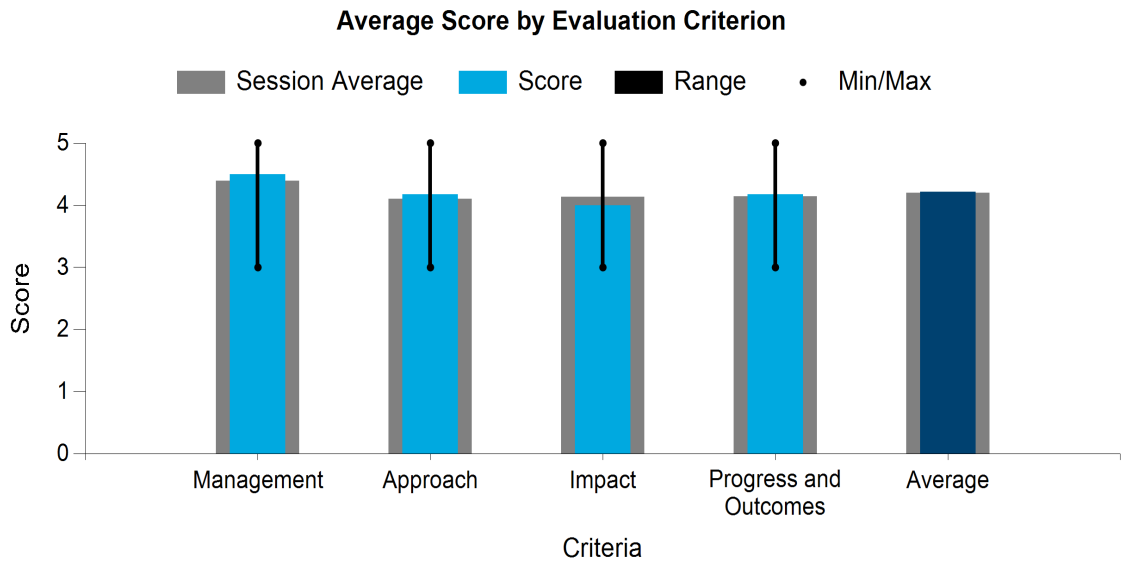
ABF

PROJECT DESCRIPTION

The ABF is a public biomanufacturing infrastructure capability that increases U.S. industrial competitiveness in the production of biofuels and bioproducts and enables new opportunities for U.S. private sector growth in the biomanufacturing sector with the potential for significant commercial deployment and job creation.

WBS:	ABF1
Presenter(s):	Nathan Hillson
Project Start Date:	10/01/2019
Planned Project End Date:	09/30/2022
Total DOE Funding:	$18,187,000

The Agile BioFoundry's overall goal is to enable biorefineries to achieve 50% reductions in time to bioprocess scale-up compared to the current average of around 10 years. Its outcomes will include the development and deployment of technologies enabling commercially relevant biomanufacturing of a wide range of bioproducts by both new and established industrial hosts.



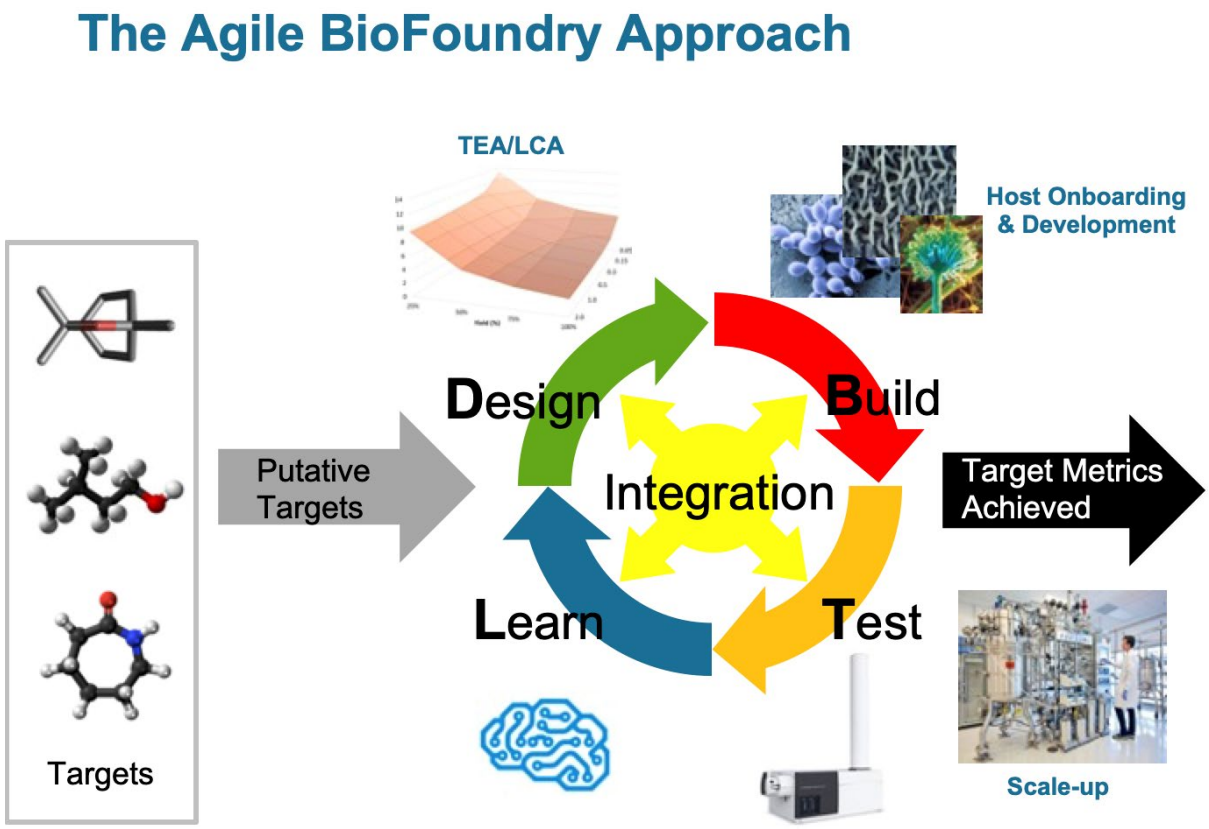


Photo courtesy of ABF

COMMENTS

- Management of the program appears to be well thought out and executed. Communications across ABF seem transparent and frequent, which must be challenging given the number of participants and the geographic distribution, and utilization of multiple communications tools seemed strong. On approach, the chosen metrics for DBTL efficiency seem inadequate for capturing the complexity of the challenge, because wall time or capacity time can both (as I understood them) fail to identify things like resource dedication. Pairing those metrics with more qualitative measurements or orthogonal quantitative measurements would better ensure that gains are practically useful. Potential impact of improving DBTL efficiency is large. The beachhead concept is particularly strong in terms of approach and potential impact: It is a thorough model that should create lasting benefits for applications across biotech, regardless of trends in targets or changing markets. Progress is promising, particularly in terms of microbial hosts onboarded/brought up to tier 1.

- Management: Organization and management appears clear and well organized. The various groups involved, despite being spread over many locations and national labs, appear to be working well together with good communication. Risks are clearly stated. Mitigation strategies have been implemented as best they can. Probably the risk that is the hardest to mitigate is predictive scale-up, and method transferability/reproducibility. Predictive scale-up is very hard. Make sure to focus predictive scale-up to classes of molecules. This might limit the current scope, but expecting predictive scale-up across a wide

range of molecule types is expecting too much. For example, even with a class of molecules like terpenes, how to ferment and purify a liquid terpene (at room temperature) is very different from the approaches you take to a solid terpene. How you separate and purify a polar molecule is very different from a hydrophobic one. Do not get too ambitious in expectations of easy transferability/reproducibility for a wide range of molecules. Also, because the available equipment and the location make such a huge impact on scale-up, it's not hugely beneficial to the overall goals of the ABF to be highly focused on this. Getting really good fermentation reproducibility between two labs (one being NREL, which is at high elevation, for example) likely won't contribute much when the process is eventually transferred to the industrial site. It would be better for ABF to focus on identifying the parameters that have the biggest impact on a manufacturing process; this can be done at one national lab. Once the most impactful parameters are known, they can be top priority to be addressed (later) at the manufacturing site and the capabilities there. Finally, helping scale-up appears to be the mandate of the new BioMADE consortium. Be clear on goals between ABF and BioMADE and do not duplicate efforts. It is ok to reduce scope in ABF if work is now being handled by BioMADE.

- Approach: ABF is doing innovative, state-of-the-art work to advance the bioeconomy and synthetic biology, and is making America a strong leader in green tech/biotech for jobs and supporting the Biden/Harris administration goals. ABF is following BETO goals regarding biomass feedstocks, so this is a 5. However, following this directive is reducing the impact the ABF is making toward some of its stated goals, which lowers the impact category to 4.

- Impact: Currently, ABF is meeting the relevant BETO program goals by requiring industry-funded projects to use biomass-derived feedstocks, or at least demonstrate a minimum performance on biomass feedstock (hence the score of 5). However, industry is not using biomass feedstocks, and won't be for at least 10 years, at best. There is no commercially available biomass feedstock if anyone wanted to buy it. Price is always the problem, and apparently there are still major issues with process (e.g., the POET plant going from production back to demo/development). BETO should still be working on biomass feedstock, but does the ABF need to be requiring industry to use it in funded projects now? It seems counter to the goals of the ABF of reducing time and money to commercializing targets. Time and resources are being spent on activities that have no commercial relevance (for the foreseeable future). Making industry engineer microbes to use biomass feedstocks will almost certainly lead down a path of metabolism that makes using conventional sugar suboptimal, thereby further reducing the effectiveness of ABF's goals and wasting resources. ABF can continue to develop new microorganisms that efficiently use biomass feedstocks, independently of industry-collaborated projects, in service of longer-term BETO biomass feedstock goals. Additionally, ABF could be doing a better job with industry outreach to make sure the entire field of synthetic biology is aware of the extensive resources offered by ABF (especially for smaller and new companies), as well as making clear that it's possible to access ABF resources outside of FOAs and DFOs, which frankly do not operate on the time scales of most industries. I do not know how much money/work is brought in under the SPPs. This should be a significant revenue stream for the ABF and the national labs, which could in turn be used to fund more academic outreach funding. Otherwise, the impact factor is very high. The development of new microorganisms, offering a high-throughput pipeline to the community, open-use software, and regular access to complicated and expensive capabilities like proteomics and metabolomics are hugely beneficial to biotech and the bioeconomy.

- Progress and outcomes: Much was made during the presentation of the beachhead idea, yet no projects presented actually addressed this or really made use of it. Dr. Keasling explained that the beachhead

concept was developed in response to the previous review, and work based on those more recent funding opportunities that emphasized beachhead work were too new for review. Therefore, still a 5 score on this topic. Going forward, beachheads either need to be used in most partnered projects or the concept needs to be redefined/abandoned. Could create a public-facing document that explains WHY each host is important, and why it has been paired with that particular beachhead molecule: tricarboxylic acid, LCA, metabolic reasons, host reasons (e.g., known resistance to the downstream or upstream molecules' toxicity).

- Overall, the approach is very well thought out, and the management approach has several checks and balances to ensure tasks progress in a timely manner. Good communication channels have been established, and regular progress reporting keeps DOE abreast of ongoing activities. That said, the tasks set forth do not carry sufficient metrics to ensure that the products of this effort have a demonstrable impact on the biomanufacturing industry. Task 1 DBTL needs to improve metric reporting of just demonstrating use of the DBTL infrastructure. This task requires a customer feedback system and metrics to quantitatively measure improvement for industry, other than just saying companies x, y, and z used this infrastructure. Indicating that the DBTL automation workflows have improved two-fold is qualitative assessment because no metrics were given as to how that number was reached. Industry would never accept such an assertion without specific metrics that inform that result. Similarly, an assessment of transfer between hosts led to two-fold improvements in the second host is an arbitrary evaluation that does not hit the heart of the problem, whether the team was able to inherently reduce the level of investment and labor needed to transition between hosts and create measurable improvements in financial investments for such a transition. Overall, the metrics are insufficient for this type of effort. The approach is very academic focused. While transition from TRL 1 through 4 still requires fundamental research investments to optimize products, there was no clear indication in the management approach to ensure that this effort is significantly informed and shaped by industry interest and application. Most of the workflows focus on creating a publicly accessible resource, which is more of an academic exercise instead of a platform technology development approach relevant to industry. For example, target and host selections should primarily be informed by industry, and technology off-ramping needs to include industry quality control requirements. Dr. Hillson presented that one of the metrics is a 50% reduction in bioprocess scale-up; however, there were no metrics presented to specifically address this deliverable in a quantitative way (a 20-fold improvement in titers is not a valid metric). In fact, the impact statement was a repeat summary of set goals, not an evaluation of metric indicators toward meeting these goals; this issue is especially pertinent for government to be able to show that the overall effort is delivering a capability to the bioeconomy that is commercially viable. For the DBTL task, it was not clear how the learn concept feeds back into existing efforts to improve current capabilities and makes them more industry friendly. It was also difficult to discern how the DBTL infrastructure interfaces to share data, protocols, and lessons learned to create an iterative learning process. Furthermore, it was not very clear how this learning process informs users (specifically industry) on how to effectively use the infrastructure and share feedback on lessons learned from using the DBTL infrastructure. This is one of the top three potential challenges identified, but concept for mitigation was effectively provided. The management team's focus on the Global Biofoundries Alliance is another example of an academic exercise that is more a distraction than a benefit to ABF. Although international partnerships are absolutely critical in leveraging existing resources, capabilities, and institutional knowledge, the Global Biofoundries Alliance is squarely focused on providing an academic resource. Most foundries are not financially viable and struggle with identifying a clear mission and vision justifying their existence. This reason is why the Global Biofoundries Alliance was created in the first place. ABF should not spend too

much time on Global Biofoundries Alliance and should instead focus on serving industry like it has been tasked with accomplishing.

- Strengths: (1) The project has clear management plans and has efficiently implemented these plans. (2) Technical risks were properly identified and mitigated. (3) Developing pathways for production of important beachheads is very useful to shorten industrial scale-up time. (4) Excellent progresses in expansion of beachhead/exemplar target and the rapid host transfer of bioproduction capability have been made.

- Weaknesses: (1) Better dissemination is needed of ABF's capability, including the DBTL tools, developed beachheads, and onboarded strains. It is important to make these tools and strains more easily accessible to the public to improve ABF's impact. (2) Actual use of beachheads by industrial partners needs to be seen in the coming years.

- The Overview and Infrastructure presentation provided an introduction to the ABF and its overall approach in a clear and compelling manner. The focus on host onboarding and beachhead exemplars to develop the foundational infrastructure, testbeds, and know-how is appropriate and has the potential for high impact on commercialization, the national bioeconomy, and the health of our planet. The overall goal of moving from a current state of needing roughly 10 years and $100 MM to develop a new bio-based manufacturing technology package to needing only 5 years and $25 MM is a terrific overall goal. What was not evident from the presentations is a connection between this overall goal and the tasks and activities undertaken. Although the tasks and activities seem generally well chosen, well defined, and likely to have high impact, there is a missing step in the reasoning (or at least in the presentations). It would be good to make the connection more apparent and rigorous. First, what are the barriers to shortening the timeline and cost of technology development for industrial fermentation-based manufacturing? For example, barriers may include things like (a) companies are choosing the wrong targets or the wrong microbial hosts, (b) companies are needing to develop by themselves the genetic tools for new hosts, (c) companies are not making use of high throughput or automation or machine learning sufficiently, (d) the throughput, quality, and cycle time of the DBTL cycles are inadequate, (e) not enough use is being made of omics data, (f) more modeling is needed to guide experiments, (g) downstream processing is generally done after strain development rather than in concert with it, (h) it is very difficult to predict strain performance or relative ranking in benchtop fermentation vessels based on measurement of performance in 96-well plates, etc. Second, once the key barriers are identified and prioritized, evaluate the extent to which the current portfolio of ABF activities addresses these barriers. Make a grid showing which internal ABF activities and which of the FOA/DFO awards are addressing each key barrier. Third, evaluate if there is good coverage throughout the grid, or if there are gaps where more effort is required. How can the grid be used to guide decisions on future FOA/DFO awards and internal ABF activities? On a more granular level, if a key barrier is insufficient functioning of the DBTL cycles, what aspects of the DBTL cycle improvements would give the greatest "juice for squeeze?" For example, DBTL cycles could be improved along several axes, including throughput/capacity per turn, cycle time, data quality and richness, experiment design that makes the data sets more well-crossed and less sparse, and greater automation to decrease headcount-related expenses. How is each internal activity and FOA/DFO contributing to solving one or more of the challenges? Is there a good match between the magnitude of the problem (or payoff expected from solving the problem) and the magnitude of effort?

- The team should be congratulated for executing the extraordinarily complex challenge of building a distributed capability and simultaneously seeding its adoption in industry and academia. However, specifically regarding the "infrastructure" aspect of the ABF, I felt that there was insufficient information presented to enable evaluation of ABF's DBTL infrastructure. Rather than a high-level overview, there needs to instead be a clear, comprehensive presentation at Peer Review of the state of each individual capability. This presentation should include a quantitative description of the current state of each capability, a quantitative description of how it has changed or improved since the previous Peer Review, and uptake numbers that track how much it is being utilized internally and externally. Without such descriptions, there is no way to evaluate progress and relevance of the DBTL infrastructure aspect. This presentation should also use straightforward metrics in a form that reviewers can directly compare to the state of the art in companies or in the literature, and it should clarify jargon and tool names, because it cannot be assumed that reviewers are familiar with all of the ABF's acronyms. Picking on "build" as an example, there was no quantitative summary of the ABF's current build capabilities (such as in terms of assemblies per day or strains per month), nor was there quantitative information available on the ABF's website. Moreover, in tabulating ABF "build" activities in FOAs and DFOs, the panel saw very few examples of utilization (<200 constructs), which very likely underrepresents its true uptake (and if it doesn't, then that would be good to know for strategic planning as well). Such a summary, spanning all ABF DBTL capabilities, would also have value well beyond Peer Review for industry engagement, as it would make the government's unique capabilities easy to evaluate and consider.

PI RESPONSE TO REVIEWER COMMENTS

- We agree with the reviewer that there was insufficient presentation time allotted for a comprehensive evaluation of the ABF's DBTL infrastructure. There was simply not enough time to do this given the prescriptive presentation formatting and mandatory content requirements for BETO Peer Review 2021. Regarding the quantitative metrics referenced by the reviewer, we are making a deliberate and concerted effort to better track the frequencies and efficiencies of our various unit operations (using, for example, our Design Implementation Verification Automation [DIVA] and Experiment Data Depot [EDD] software platforms). Although these data were not available for this Peer Review, we should be better positioned to present these metrics at the next Peer Review in 2023. That said, it would be difficult to compare these metrics apples-to-apples to those at companies or other institutions, as there are no community standards (that we are aware of) for these metrics, although there are emerging efforts (e.g., Tom Treynor's benchmarking initiative via SynBioBeta) in this space. Perhaps by the next Peer Review, this will be more feasible to accomplish. We continue to work on raising the community's awareness of ABF's capabilities, as described in the industry engagement and outreach (IEO) presentation, through for example our refreshed website, social media channels, scientific and technical conferences, and industry listening days. Awareness is but one aspect of accessibility, which also includes economic and contractual accessibility, which within the constraints of DOE national laboratories, we are also striving to improve through, for example, our DFOs and prospective collaborations between the National Science Foundation and DOE to specifically increase accessibility for academics who are often challenged by cost-share requirements.

- We agree that we would like to see and showcase collaborators leveraging our nascent beachhead efforts in the coming years. We agree with the reviewer that we must be very focused and strategic in our choices of molecules (whether for demonstration projects, beachheads, predictive scale-up, etc.) and not assume that knowledge or modeling performance will always transfer well between host organisms, molecule classes, or processes. It is an important and ongoing research and development effort for the

ABF to better understand how well and how reliably we can transfer knowledge (in different domains across the ABF).

- We do not agree with the reviewer's assertion that there is little value in demonstrating process transfer between ABF facilities. Our reasoning is that while any given ABF facility may not be exactly representative of a prospective industry site, the more we understand about what is required for any transfer between any two facilities in general (e.g., protocol details, minimum information standards) and where the transfer sensitivities lie, the better. If we cannot or do not endeavor to understand such transfers between ABF facilities, this will likely delay and make much more difficult prospective transfers to industry.

- We agree with the reviewer that understanding a given process' sensitivities (within a single facility) is also important. Regarding downstream processing, including separations and purifications, the ABF coordinates with BETO's Bioprocessing Separations Consortium to tackle process-oriented separations challenges. This allows the ABF to leverage access to experts in process design for improved industry relevance. We will continue to seek productive coordination and collaborations with BioMADE and are open to redirecting our efforts as appropriate. We do not currently anticipate down-scoping our efforts to understand process scale-up and transferability, as these are in the ABF's TRL sweet spot and many aspects of which are at a lower level of maturity than where BioMADE is positioned. We are optimistic that the ABF and BioMADE will have mutually beneficial cross-entity business development activities, in which we refer prospective collaborators to each other as appropriate.

- Regarding feedstock requirements, these are good considerations for our DOE EERE BETO Technology Managers and Leadership to deliberate. We are hopeful and optimistic that additional feedstocks (e.g., C1), which are demonstrably of interest to industry, can be added within an expanded scope for the ABF. As noted above in our response to another reviewer, we continue to apply effort to better disseminate the availability and improve the accessibility of ABF capabilities. As mentioned, contractual accessibility is one component, and SPP and CRADA mechanisms outside of the DFO process are important mechanisms. Although the ABF does not have many examples of fully industry-supported SPP projects, and (to-date) no fully industry-supported CRADA projects, we do think that these will be crucial to the continued sustainability and impact of the ABF. We agree with the reviewer that if collaboration partners do not leverage the ABF's beachhead efforts in the coming years (after they are established, of course), we will need to select different beachheads or discontinue these efforts. We are currently developing our Host Onboarding Tool (HObT) web application, in which we will have the opportunity to better publicly express why each microbial host has been selected, and with which molecule categories/processes it may be especially well suited.

- We agree with the reviewer that to-date, we do not have any direct support/evidence in terms of the ABF impacting the timeline and cost of the commercialization of a bioprocess. What we do have are leading indicators that we are positively impacting these commercialization efforts of our collaboration partners. The reviewer provides excellent examples of hypotheses (many of which we are also actively pursuing) of means through which the ABF can help accomplish this overall goal.

- We fully agree with the reviewer that doing a gaps/priorities analysis is important, and as described in the DFO presentation, we have been tracking which capabilities (e.g., machine learning) proposers (whether selected for support or not) have requested, and which barriers they are applying these capabilities to. We will also be looking at our frequency and efficiency metrics of our various unit

operations to identify those that would return the greatest return on investment for further improvement (e.g., automation) efforts, in line with the reviewer's suggestion. We are investigating, for example, how much value additional omics data sets or analysis methods add (i.e., marginal benefits analysis). As the reviewer mentions, we must always evaluate the (opportunity) cost/benefit relationship of prospective research and development efforts, and evaluate our next-best alternatives.

- We understand that we were not successful in clearly articulating/relaying to the reviewers our definition of efficiency. As presented, we are using an efficiency metric that is expressed as a number of samples (or equivalent) per wall time per resource (scaled by clock time/cost of human and instrumentation resources). There are many different ways to express efficiency (and as mentioned above in response to another reviewer, there are emerging community benchmarking efforts that will help out here), and we will continue to evaluate if there are alternative versions that enable us to maximize alignment with our overall goal for the ABF. For example, the above definition does not speak directly to the efficiencies gained by the establishment of a molecular beachhead, or being able to generate the same amount of knowledge/predictive power with fewer samples/cycles.

- Because the reviewer did not clearly understand what was presented in terms of our efficiency metrics, we understand but do not agree with statements concerning the insufficiency of the presented metrics. When presenting our transfer targets/tools as part of the go/no-go milestone, we did not divide out for the reviewers how much wall/clock time and resource requirement improvements contributed to the two-fold (or greater) efficiency improvement factors. We do have these numbers, which were included in our milestone completion report, but it is a good suggestion for future Peer Reviews that we present these breakdowns explicitly for the benefits of the reviewers.

- The ABF's efforts are informed and shaped by industry interest and application (see for example our IEO activities, including our IAB; as well as the many DFO and FOA collaboration project presentations); we strongly disagree with the reviewer's assertion to the contrary. As discussed above, we do agree that we do not have evidence/data supporting the ABF's contributions to reducing timelines and cost for bioprocess commercialization. As also stated above, what we do have are leading indicators that the ABF's capabilities are making positive impacts (e.g., in the leading indicator example of Lygos increasing a titer by 20-fold).

- We agree with the reviewer (as discussed above) that there was insufficient time to discuss and describe how past learnings feedback both scientifically and operationally to improve our future endeavors, and how these learnings are shared with our collaboration partners.

- We do not agree with the reviewer's assertion that the ABF should not spend time on participation on the Global Biofoundries Alliance. The reviewer appears to misunderstand, and as a consequence mischaracterizes, the Global Biofoundries Alliance, and we receive these comments accordingly. Although the Global Biofoundries Alliance is composed exclusively of nonprofit biofoundries, member biofoundries are not solely academic resources, and like the ABF, do routinely interact with and support industry. It is crucial that the ABF continue its participation in the Global Biofoundries Alliance, not only to demonstrate U.S. national leadership and have a seat at the table regarding international standardization efforts, but also to enhance our situational awareness of activities across the globe in biomanufacturing, and to leverage others' efforts and maximize the ABF's impact where possible.

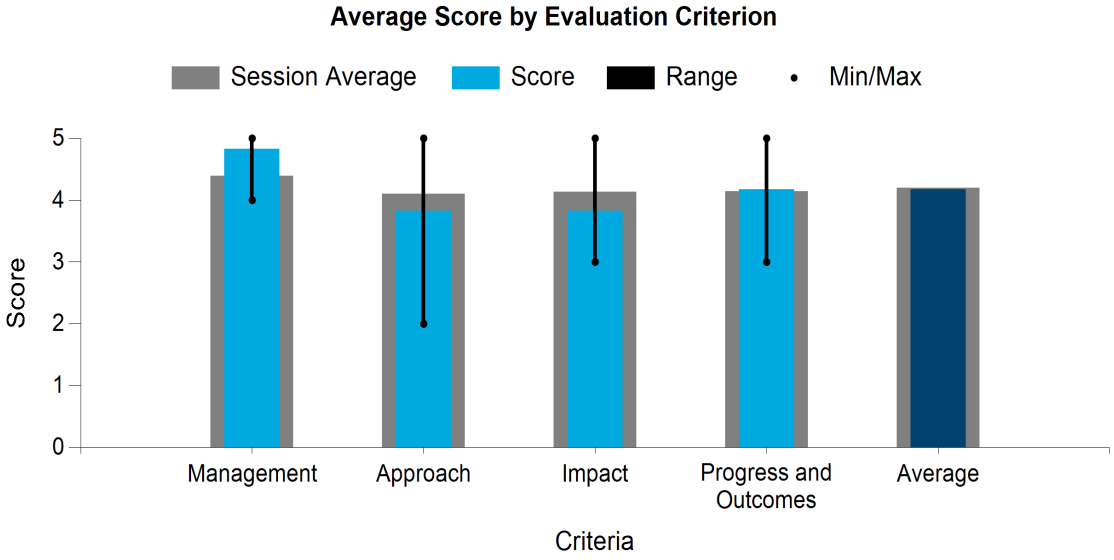
ABF Industry Engagement Lab Call—Neidle Lab

UGA

PROJECT DESCRIPTION

WBS:	ABF10
Presenter(s):	Ellen Neidle
Project Start Date:	07/25/2017
Planned Project End Date:	12/01/2020
Total DOE Funding:	$1,000,000

Metabolic engineering for biomanufacturing often involves genetic diversification followed by the selection of desired traits. This project sought to develop new methods to improve this approach. The key challenge is direct selection of bacteria producing maximal amounts of a target molecule rather than relying on selection for increased growth, which may not correlate with bioproduction. The unique genetic system of the bacterium *Acinetobacter baylyi* ADP1 provides a basis for accelerated laboratory evolution ("Evolution by Amplification and Synthetic Biology," or EASy). Biosensors provide a basis for high-throughput screening. The chosen target molecule, terephthalic acid (TPA), is a commodity chemical used to make polyethylene terephthalic acid, a polymer used in fibers, bottles, and packaging. Because of a large commercial demand for polyethylene terephthalic acid and its problematic accumulation as waste, there is strong interest in the production and degradation of TPA. To facilitate engineering anabolic and catabolic pathways, a TPA biosensor was developed in ADP1 by modifying a transcription factor from another bacterium. Furthermore, the EASy method was used to generate ADP1-derived strains that consume TPA, revealing novel TPA transport proteins, as published in Metabolic Engineering in 2020. These advances lay the foundation for TPA to be degraded from plastic waste and/or synthesized from renewable feedstock to increase growth of the bioeconomy.



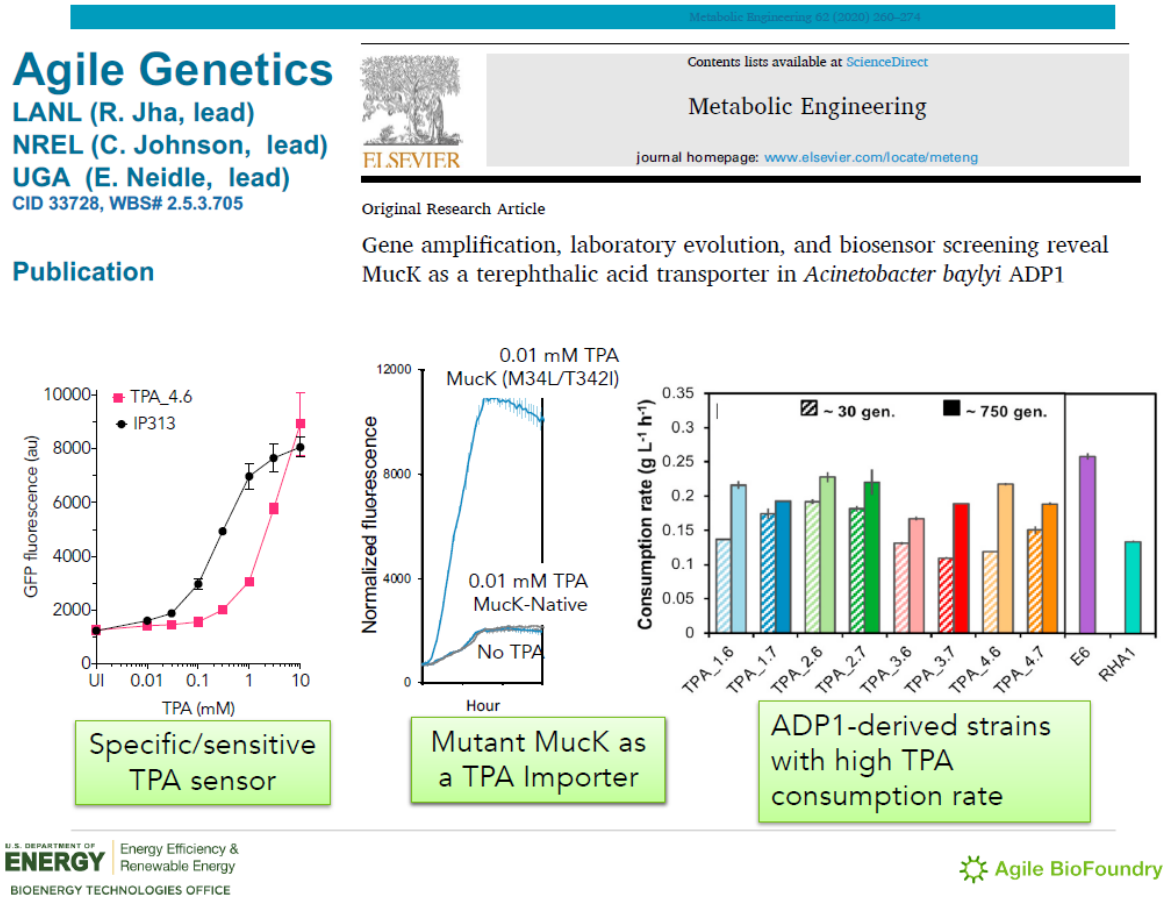


Photo courtesy of UGA

COMMENTS

- Management: Structure, collaboration, and communication among groups look good. The presenter stressed how the embedding of her student at a national laboratory was amazing training and very helpful for communication. ABF definitely needs to figure out how to help academic labs with cost-share requirements and simplify the CRADA process. ABF/DOE is working on this.

- Approach: From the perspective of the ABF, it is not clear why they should be investing in development of *Acinetobacter baylyi* when it is not on the current list of hosts to develop. I'm guessing this project was funded before that list was generated. It's convenient that the strain is naturally competent, but that does not necessarily make it an organism that should be developed. The presenter and the ABF should decide if there are other metabolic/physiological reasons to make this an official host target. As recommended in the review of the host onboarding presentation, and the overall review of the ABF, the ABF should focus on developing ~10–15 organisms to tier 3–4 to make them highly useful for synthetic biology and not do work in other organisms without a highly compelling reason. In other words, it is better to have 10–15 really tractable organisms than 50 organisms at tier 1 or 2. Natural competence makes transformation easier, but the reliance on random integration and lack of ability to target specific regions makes it not desirable for synthetic biology work. For these reasons, natural competence alone is not persuasive as an argument to continue work in *A. baylyi*. Frankly, I don't see the approach of gene

duplication prior to mutagenesis as important at all. Evolutionarily speaking, yes, gene duplication has led to many new diverse activities. However, for synthetic biology, it is a red herring. If your goal is enzyme improvement, doing that by random whole-genome mutagenesis is highly inefficient. The ABF should focus on developing tools for highly efficient, targeted gene insertion and tools for high-throughput gene (enzyme) mutagenesis and screening. The big synthetic biology companies all have capabilities to do site saturation mutagenesis on every single amino acid position in a gene of reasonable size (approximately 500 amino acids) and screen such a library in the relevant host strain in a matter of a few weeks. Then, follow that first round of screening by combining single improvements and testing combinations. This is what the ABF should be working toward achieving.

- The presenter is absolutely correct that growth is not a good indicator of product production. That's actually an understatement, as usually growth is competing with product formation, and when you select a strain to grow better, you usually just select for reduction in product formation. It's very rare when you can link product formation to growth; in those cases, you have a very powerful tool in growth selection. Without that link, it's almost always counterproductive to select for growth. The presenter acknowledged this and moved to the biosensor to screen for more product formation. The biosensor work was very well done and highly successful. The dual florescent read out for gene copy number and activity is very important. You need to know that higher activity is not just due to extra copies. This work has been a big benefit to ABF and also demonstrates a very powerful tool for the scientific community.

- There was much discussion during the review of the high interest in biosensors to industry, and here the ABF can play a huge role in having the expertise to develop this technology as a resource, or spin out a company based on their expertise to supply biosensors to customers. As discussed among the ABF folks, it is important to develop external biosensors and sensors with a large dynamic range (or easily adjustable dynamic range) for industry; that often starts off making mg/L of product but eventually ends up making 50 g/L or even 100 g/L of product. It's great ABF is working on external biosensors. Keep the biosensor work a high priority.

- Impact: It's clear why TPA is a significantly important molecule for industry and economy. As discussed above, the approach is not going to be as efficient at delivering impact compared to a focused enzyme mutagenesis and screening strategy. It's not clear from the presentation why this host would be the best choice for making this molecule (score of 3). Impact of development of the biosensor was very clear and highly relevant to synthetic biology and furthering rapid synthetic biology development (score of 5).

- Progress and outcomes: I'd give this a 4.5 if I could. Although I don't see the development of *A. baylyi* as well justified, the development work on the organisms was completed as outlined in the original contract. The development of the biosensor was done well and resulted in papers, patents, and many additional requests from other lab. That is all 5. However, one of the main goals of the project, to develop an enzyme capable of carboxylation of benzoate to TPA, was not achieved (milestones 3 and 4). Creating an enzyme function where there is currently no known function is highly ambitious and risky, and this is in alignment with ABF's goal to advance synthetic biology and the biomanufacturing of molecules. Failure in creating de novo enzyme function is common. There are whole companies dedicated to doing this with little proven success. However, again, the overly simplified approach (random whole-genome mutagenesis of multiple copies of a gene) is probably the least likely to succeed in such a high-risk endeavor.

- Strengths: The team has successfully developed a TPA biosensor, which will facilitate the screening of the decarboxylase for its reverse reaction and bioproduction. The combination of EASy and biosensor for selection using green fluorescent/red fluorescent protein ratio is a smart idea and a novel approach. This project also developed ADP1 as a robust microbial host for bioproduction. Satisfactory progress has been made by the team. This project will have higher impact than some industry teams with bigger grant size. Weakness/area for improvement: It will be great to see transfer of the developed technology to industry.

- The goal of this project with the Neidle academic laboratory is to apply directed evolution to metabolically engineer *Acinetobacter baylyi* for the production of terephthalic acid. The project is well managed, with frequent calls and acknowledgement for the need to pivot as results become available. With respect to approach, much of the work was done by the ABF with the Neidle laboratory mostly providing expertise on *A. baylyi* genetics. This is potentially a missed opportunity as university collaborations could serve as a launch pad for workforce development. The impact of this project to the ABF has been high. The ABF has onboarded *A. baylyi*, and developed workflows for biosensor development and protein-based sensor/fluorescence activated cell sorting that are being reused within the ABF to generate other biosensors for other targets and made to work in other organisms besides *A. baylyi*. The project seems to have had a lower impact for the Neidle laboratory. The project was terminated due to go/no-go milestones not being met. Given that this is the only university-led DFO presented, and the fact that this was the only DFO terminated due to not meeting go/no-go milestones, it raises questions as to whether the current DFO model is equally serving industry and universities. This project may present an opportunity to conceive a different DFO strategy for universities that do not have staff scientists and rely on students to meet the go/no-go milestones. University-led projects could help the ABF fulfill BETO's mission with regard to workforce development and inclusion goals.

- The management of this program seems excellent: The university, NREL, and Los Alamos National Laboratory (LANL) are in frequent communication, the experimental plan is broken up into parts to reduce risk, alternatives are set up, and they are able to rapidly pivot if needed. The approach seems strong: They articulated the existing shortfalls of directed/adaptive evolution strategies, identified potential biological solutions, and prioritized efficiency and ease of use. They utilized newer tools, like biosensors, for biggest impact. The impact was good: The target molecule is of high commercial relevance, and the chassis choice, while less explicitly tied to commercial objectives, also seemed sound. The progress was good: They ran into some pitfalls with production, but had excellent success at degradation.

- The management structure slides described most of the essential components of a good management structure. What was missing is a way to communicate and consult with (and learn from) those who had previously worked on similar types of projects, both those who have previously engineered strains to couple growth with product formation (e.g., Bernhard Palsson) and enzymologists who can provide expert insights into the key reaction in the pathway (i.e., decarboxylation and carboxylation reactions). The approach seems to be unnecessarily baroque. While some may consider this to reflect a high level of innovation, others could reasonably argue that the overall high-level goal (i.e., accelerating the improvement of target molecule production via harnessing evolution) can be addressed and has been addressed more powerfully and successfully using a simpler approach.

- There are three concerns with this project's approach. First, the choice of enzymatic pathway could have been better. Enzymatic decarboxylation is highly energetically favorable and generally considered to be

an irreversible reaction, and the reverse reaction (one-step enzymatic carboxylation) is extremely rare in nature; therefore, it is highly unlikely that an enzyme that catalyzes the reverse of the desired reaction (decarboxylation) will be able to catalyze the desired reaction (carboxylation); the PIs should have chosen a different pathway for this proof-of-concept work, where it would be easier to evolve the desired enzymatic function. Second, it is not clear that the DNA direct repeat expansion/contraction approach is necessary—it may be just a red herring, a difficult to enact trick that is unnecessary to evolve the desired phenotype. Why not just add two copies of each gene in different locations? Also, the instability of the tandem repeats has as much peril as virtue; it is not clear that evolution will often select for mutations if multiple (unstable) copies can provide the needed function, but if multiple copies are beneficial, one cannot use this as the manufacturing strain because direct repeats are unstable without continuous selection. Also, a hypothetical mutation that conferred a 15% improvement in activity from one of three copies of the same gene would overall result in only a 5% increase in total enzymatic activity in the cell, which may be hard to detect reliably. Third, it is not clear why the project members did not make use of the biosensor to select directly and continuously for higher growth in cells with higher product formation. Instead, the project used discontinuous selection via FACS, which is time-consuming and requires an expensive instrument. Why not couple the sensor to a gene that is necessary to grow, and whose expression level correlates positively with growth rate? Such a growth-promoting gene reporter would permit continuous and inexpensive selection for higher product formation.

- If successful in achieving the overall high-level goal of improving bio-based TPA production, the project would have moderate impact. The TEA, LCA, and market pull for bio-based TPA production was not presented, so it is hard to evaluate the benefits and commercial traction of such manufacturing. Some of the intermediate goals and outputs will likely have beneficial impact on other activities; for example, the biosensors developed and perhaps the onboarding of *Acinetobacter baylyi*, although it was not described why this organism was chosen or how it provides nicely complementary advantages to the other species being onboarded at the ABF. Also, the insights into TPA degradation are potentially useful for material recycling or bioremediation, although this seemed not to be the primary motivation of the project.

- The progress and outcome were lower than expected and projected. Two of the milestones were met, one was partially met, and the final one was not met. The major barrier to progress was the carboxylation reaction, which is a type of enzymatic reaction very rare in nature and thermodynamically unfavorable. Prior consultation with an enzymologist or someone with knowledge of thermodynamics would have dissuaded them from choosing this pathway for this technology development project; if another pathway had been used, the work would likely have been more successful.

- This project seeks to leverage ABF's ability to develop biosensors to improve a directed evolution approach that modulates the numbers of chromosomal copies of a gene. The original target of the approach was TPA production, but efforts were redirected to its degradation instead. In this pursuit, the collaboration was quite successful: Degradation was successfully addressed, the function of a new gene (the transporter) was identified, and the two-color/ratiometric enhancement of the approach was demonstrated. From an ABF perspective, the key importance of this adaptive laboratory evolution work is that it showcases the potential utility of its ability to develop novel biosensors with high specificity. It was not clear whether ABF intends to adopt the evolution method (EASy), as it appears that it may be specific to a particular host (*A. baylyi*) that is not immediately relevant for onboarding in a production context. It would also be good to understand where EASy may be more or less appropriate to employ than droplet adaptive laboratory evolution.

PI RESPONSE TO REVIEWER COMMENTS

- We thank the reviewers for helpful and thoughtful comments. We appreciate the acknowledgement of our success in developing biosensors, in engineering TPA degradation, and in discoveries related to TPA transport. We share enthusiasm for the importance of biosensors and will build on our success and the constructive suggestions provided. From the reviewer comments, it appears that we were not sufficiently clear in conveying the importance of the EASy methodology, of using *Acinetobacter baylyi* ADP1 as a host, and in explaining some aspects of the project. The importance of using this organism for synthetic biology and biomanufacturing was not the focus of our project presentation, but these topics are explained in publications of Neidle and colleagues (https://doi.org/10.1093/nar/gkaa167; https://doi.org/10.1042/EBC20200136). Similarly, the significance of the EASy methodology is more clearly explained in a publication from the UGA-NREL team members (https://doi.org/10.1073/pnas.1803745115). Briefly, while natural competence underlies many of the advantages of using ADP1 as a host, the benefits to synthetic biology are not solely from DNA uptake per se. Small linear DNA fragments appear not to be readily degraded but can serve as platforms for recombination with utility in high-throughput applications (https://doi.org/10.1021/acssynbio.0c00240). Continued development of such methodology is important both for using ADP1 as a platform organism and for generating portable and novel enzymes and pathways for use in other organisms. There was confusion as to the mutational approach we planned, and some reviewer comments inaccurately described our methodology. Miscommunication may have resulted from our inability to reach the stage of the project where mutations were to be introduced by linear DNA. Overall, this was a very ambitious project. With limits in time and funding, we chose to pivot and accomplish important results that could be completed in the allocated time frame. This pivot led to an incorrect assumption that the project was canceled due to not reaching milestones. The project extended for the full duration, and as noted in a review, “the impact to the ABF has been high.” We disagree that the project had a low impact for the Neidle lab; however, as the first academic project of the DFO, problematic aspects of this type of arrangement did become evident. Specifically, without any funding provided to the academic partner, the resources for cost-share and the ability to involve academic researchers and students is constrained. Nevertheless, all members of the team benefited from conducting this project, and the foundations have been laid for fruitful, ongoing collaborations.

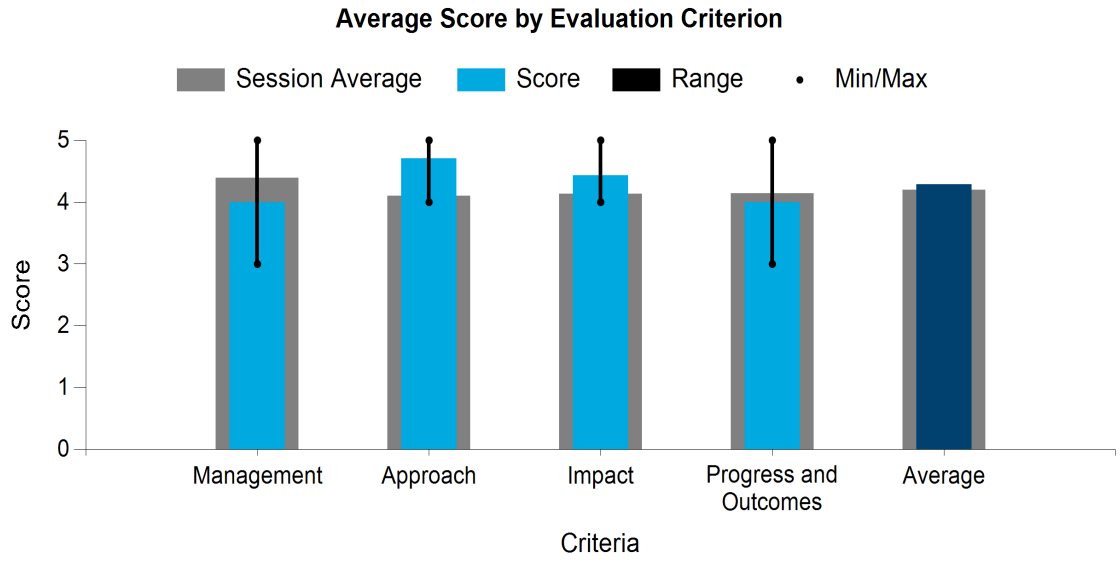
ABF Industry Engagement Lab Call—LanzaTech

LanzaTech

PROJECT DESCRIPTION

WBS:	ABF11
Presenter(s):	Wayne Mitchell
Project Start Date:	07/25/2017
Planned Project End Date:	04/30/2021
Total DOE Funding:	$1,405,000

LanzaTech is a leading gas fermentation company with active commercial projects under development in Europe, Asia, and the United States. A partnership among LanzaTech, Argonne National Laboratory (ANL), and NREL proposes to develop a first-of-a-kind analytic pipeline to support the DBTL cycle for continuous microbial gas fermentations. LanzaTech brings a truly unique historical and still growing collection of data on acetogenic organisms and gas fermentation, coupled to a unique experimental capability. This project will leverage statistical methods, metabolic modeling, and deep learning approaches that have been applied successfully in the analysis of large data sets from a variety of fields. These approaches will be used to fully exploit and optimize the robust industrial hosts developed at LanzaTech as a bioproducts platform. We will integrate these modeling, omics, and fermentation data into a data warehouse, and then use deep learning expertise from ANL to develop an "in-line" artificial-intelligence- (AI)-guided process that will monitor and adjust industrial fermentation conditions in real time to optimize fermentation output. Model output will be integrated with ABF EDD resources. The AI system will be validated in silico in this project on both LanzaTech and NREL fermentation data and implemented in laboratory-scale fermentation systems in future collaborations.



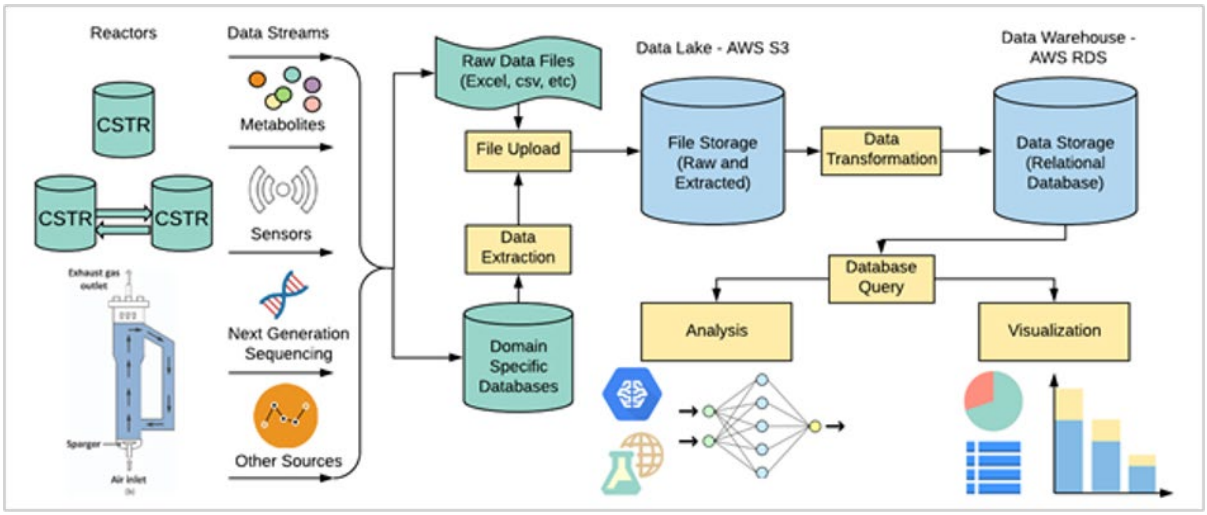


Photo courtesy of LanzaTech

COMMENTS

- LanzaTech and the ABF have worked together to generate models that can recognize different fermentation states and recommend adjustments to nudge transitions to desired states. The concept is sound, and it addresses a challenge that needs to be addressed to help ensure the viability of gas fermentation processes. In addition, it is significant that this project was made possible by the willingness of a company to share large data sets with the ABF that it could not have accessed otherwise. Despite this promise, it was difficult to evaluate the technical success and practical impact of the project, as neither error rates nor precision-recall curves were presented. Although cross-validation was employed, a much more useful demonstration would have been to deploy the model during fermentation in real time and demonstrate state control. If successful by these metrics, this effort could set the stage for ABF to develop AI-based fermentation automation for other processes as well.

- Strengths: The project goals are well-aligned with ABF's mission. This project also contributes to ABF's capability in machine learning and fermentation. The use of AI to provide fermentation surveillance and operation guidance is a novel and efficient approach. The team has solved multiple challenges in their project.

- Weaknesses/Areas for improvement: The project lacks fundamental quantitative metrics to evaluate the outcome. To what percent is the current AI module able to replace human in bioreactor monitoring? How much has the current AI module improved the fermentation outcomes? Without these metrics, the impact is unknown and can be limited. The role of NREL in this project is not clear.

- The goal of this project with LanzaTech is to develop an AI algorithm to monitor an existing industrial bioprocess. The management of this project is clearly defined, with identified leads as well as potential risks and mitigations. With respect to approach, LanzaTech provides the data while the ABF provides the machine learning and AI expertise. The impact of this project for LanzaTech is high, as it will eventually allow them to replace human-based process monitoring with AI algorithms, enabling them to reduce costs and optimize their bioreactor uptime. The impact of this project on the ABF is high as well,

as it allows them to improve their AI algorithms using industrial data, an important part of the learn component of the DBTL cycle. The impact of this project on the general bioindustrial field will depend on how general the algorithms are and whether they could be applied to other bioprocesses to optimize their reactor uptime. This project also allowed the ABF to work with a medium-sized company, and lessons learned from this interaction could potentially increase ABF collaborations with other medium-sized companies. Progress on this project is good, and the AI is identifying trends previously identified only by a human operator.

- The management of this progress described in the presentation was a bit vague, but it seemed that LanzaTech, ANL, and NREL are well aligned. The approach is sound: starting with 10 years of data, identifying a high-quality data set, applying expertise in statistical learning, and using that to address data infrastructure. The potential impact is high: creating an AI system that would increase the up-time of a bioreactor that can handle variable/chaotic off-gas inputs would help unlock a critical component of the circular economy. However, the impact would be significantly higher if papers or patents were coming out of the work, which they have not yet done and it sounds like they may not do at all, so that there would be more widespread commercial benefit. This downside seemed like it was outweighed by the benefit the labs said they got from working with a mature machine learning company. On progress, it was difficult to discern based on the level of detail presented, but they hit several of their milestones. However, they required an extension due to COVID, which is somewhat understandable, but it was not obvious whether that might have been avoided given the remote nature of the work.

- There is a clear benefit to both LanzaTech and ABF. ABF got to test and refine machine learning and AI capabilities with very large, high-quality data sets with real-world applications. LanzaTech improved data capture and data storage to enable machine learning, and will benefit from the results of the work. When asked if the work was very specific to LanzaTech, the response was “no, the work is generalized and could be applicable to many other projects.” Overall, the project appears on time to finish all tasks. It’s not clear if the lofty ambition that the project, as it stands as of this review period, will actually result in clear action items for LanzaTech to simplify running the complex fermentations or reduce down time. Machine learning usually is harder and takes longer than anyone thinks. Still, the work was well executed and has made good progress. Final impact appears to be seen. ABF should look to do more projects like this going forward. There is not any conflict in results or impact between the private company and ABF; it’s a clear win for both parties.

- This project appears to be a good example of how the interface with industry can help create new capabilities within ABF and allow for a pressure testing of those capabilities based on the volume of data generated. The approach is not novel; however, it helps ABF remain agile in developing new tools for data analytics. The machine learning approach can help with large data sets, especially when industry begins to head to larger-scale production with increased fermentation capacity. Overall, this is a good project, but quantifiable metrics were not clearly described. Measuring success toward reaching ABF’s mission will only be honestly evaluated if there are quantifiable metrics that can help interpret progress made toward ABF’s goals.

- While the management approach was hard to judge, because it was not described in any detail, the approach is very innovative, exciting, and potentially high impact. The project has clear and immediate potential to positively affect current and future commercial products made by LanzaTech, and is crafted in a way to provide benefit to ABF/BETO/DOE by testing the hypothesis that such artificial neural networks can be beneficial in guiding process and genetic changes for bio-based manufacturing, and

depositing the computational tools and models in the ABF's repositories. The potential impact of this project, if successful, is highly beneficial, and the approach may be generally applicable to many other fermentation-based product manufacturing needs—resulting in both short-term and long-term beneficial impacts. The progress to date has been on track, and intermediate benefits were described. The final results are greatly anticipated in the next few months to determine the overall outcome. It is not yet known whether the model will be able to successfully predict process and genetic modifications that improve fermentation performance. Even if the approach is not yet refined enough to guide performance improvements, the project has made good progress toward that overall goal.

PI RESPONSE TO REVIEWER COMMENTS

- We appreciate the reviewer's positive assessment of the project's concept, the challenges we address, and appreciation of the importance of partnerships like these enabling sharing of large data to achieve impactful results. Because of time constraints in the presentation, we were unable to elaborate on agreement of the automated approaches for steady-state fermentation states versus those identified by bioreactor operators. The error or "disagreement" rates in this analysis are completely under control of the modeler. We have judiciously chosen weightings and variability parameters in data from fermenter analytics to result in settings that identify 95% of the steady states indicated by subject matter experts. These same settings have identified additional steady states of the bioreactor that are currently being curated by fermentation specialists. Some were more short-lived and, thus, ignored initially. Once this validation is complete and we have collectively decided upon settings for the automated analyses, then we will be able to discuss our true error rates in more detail (when many false positives currently were just missed in manual evaluation) and generate precision-recall curves. Regarding the reviewer's comments on reactor performance, such tasks are beyond the scope of this project, and measuring process improvements with specific, quantifiable metrics will be the focus of subsequent work.

- We thank the reviewer for their positive assessment of our goal to use AI to guide gas fermentation, and of our progress to reach these goals while handling challenges in our project. Regarding the reviewer's comments on reactor performance, such tasks are beyond the scope of this project and measuring process improvements with specific, quantifiable metrics will be the focus of subsequent work. The NREL scientists working on this project have many years of industrial fermentation experience and have scaled many processes from lab to bench to pilot operations. They engaged with modeling efforts and scientists at LanzaTech to understand and downselect the most important questions that could be addressed by AI/machine learning approaches and helped identify parameters from different scales of production that would be most influential in the automated identification of bioreactor states that signaled stable, maximized production. In addition, they were instrumental in understanding of transitions between non-steady-state conditions to identify biological and process relevance. Even though NREL had the smallest amount of resources directed toward activities on this project, the results would not have been as extensive or impactful in their absence.

- We appreciate the reviewer's assessment of our progress and the potential impact of using large, high-quality data sets to develop AI and ML. The reviewer sees the value of ABF/industry relationships. Regarding the reviewer's critical comments, the data infrastructure work was internal to LanzaTech, and the model construction was generalizable. As previously stated, measuring improved reactor performance improvements informed by AI will be the focus of subsequent work.

- We appreciate the reviewer's favorable comments on the project's clear and immediate potential to influence how essential products can be made with LanzaTech technology. The reviewer also pointed

out our progress toward our goals to predict process and genetic modification to improve fermentation performance.

- We appreciate the reviewer's positive assessment of our approach of utilizing high-quality data sets, teamwork for creating AI, and potential impact for gas fermentation to plug into the circular economy. To clarify the structure of the team, LanzaTech was responsible for data collection, curation, and clean-up, ANL was responsible for model construction. Regarding the sharing of information gained through this DOE-funded research project, these protected data were produced under agreement no. DE-EE0008500 with the U.S. Department of Energy and may not be published, disseminated, or disclosed to others outside the Government until five years from the date the data were produced, unless express written authorization is obtained from the recipient. Upon expiration of the period of protection set forth in this Notice, the Government shall have unlimited rights in this data. This Notice shall be marked on any reproduction of this data, in whole or in part.

- We appreciate the reviewer's favorable review of our management structure, approach with data sharing and AI development, and potential high impact results to reduce costs and optimize time by replacing human-based processes with AI algorithms.

- We appreciate the reviewer's comments on the productive partnering of industry and ABF to take advantage of large data sets and develop machine learning. Regarding the comments on quantifiable metrics, measuring the actual improved performance of reactors guided by AI will be the focus of subsequent work. Regarding the reviewer's comments on quantifiable metrics, we agree these are necessary to interpret the improvement made toward ABF's goals; improved reactor performance due to AI guidance will be measured in subsequent work.

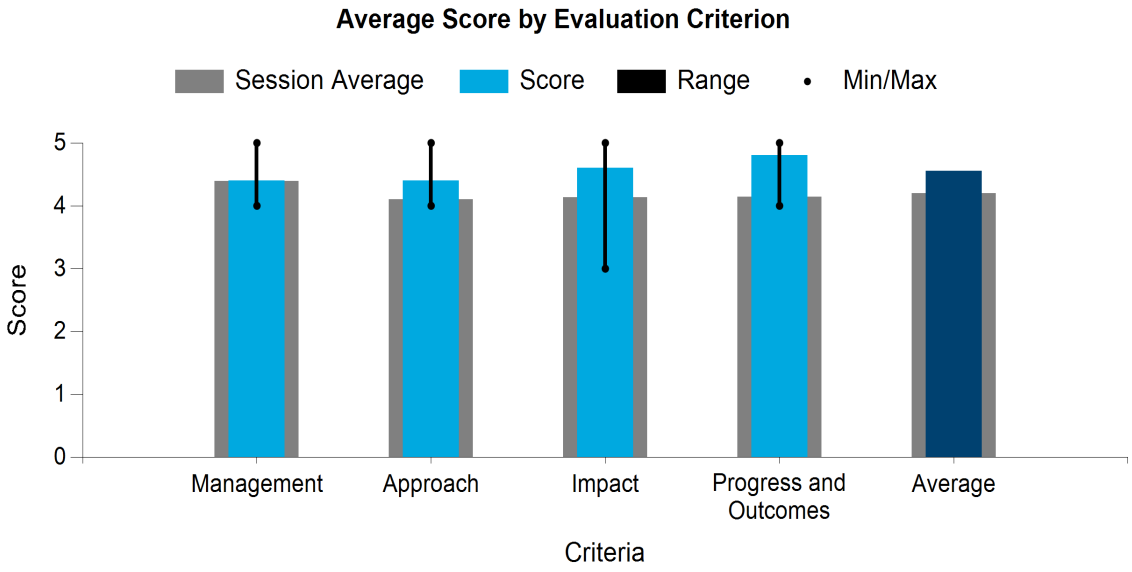
ABF Industry Engagement Lab Call—Visolis

Visolis

PROJECT DESCRIPTION

WBS:	ABF12
Presenter(s):	Deepak Dugar
Project Start Date:	06/03/2018
Planned Project End Date:	01/11/2021
Total DOE Funding:	$543,000

The Visolis project aims to develop an economic and sustainable route toward the production of mevalonolactone (MVL) in two novel microbes using waste biomass as the feedstock (sugar and syngas/waste gas). The two microbes will be engineered with proprietary genes from Visolis along with genetic tools optimization and the employment of a DBTL cycle to afford the MVL pathway. The integrated process will realize reductions in both CapEx and OpEx due to tolerance of low pH during fermentation, which eliminates the costly base addition and alleviates contamination, and improved carbon-conversion efficiency, an important objective for economic production of bioproducts.



COMMENTS

- Both of the strains used in this project are on the list of hosts to onboard, so all the work to make the strains genetically tractable are fully in line with ABF goals. The work also benefited Visolis, which is a small company, and the additional resources and support to engineer the nonstandard strains was mutually beneficial. For this project and all future projects, DBTL cycle time should be reported, and success should be partly judged on the reduction of DBTL cycle length.

- In this DFO, the ABF's capacity for onboarding new hosts is used to help Visolis produce a platform molecule that is key to its business. By screening several promoters, these efforts increased production in

a bacterial strain nine-fold, and through a copy number edit and a gene deletion, they enabled production in a yeast strain at three-fold higher than target. From the Visolis perspective, this effort was very successful, producing a strain that could have commercial viability. From an ABF perspective, the utilization of its core capabilities was not well showcased, as the highlighted work came across as standard metabolic engineering practice that might be accessible via a typical research laboratory.

- Strengths: The team has successfully onboarded two microbial strains *Clostridium ljungdahlii* and *Pichia kudriavzeveii*. A nine-fold enhancement in target molecule production was achieved in engineered *C. ljungdahlii* strain. Production in *P. kudriavzeveii* has resulted in three-fold higher titer than project target. These results demonstrate the impact of the project. Visolis efficiently used ABF's capacity in strain development and metabolic engineering through this project.

- Weakness/area for improvement: The benefit of using two strains to produce one product is not clear. If two different processes are needed (both aerobic and anaerobic fermentation), this approach will require higher capital and operation costs.

- The goal of this project with Visolis is to engineer microbes to perform syngas fermentation for chemical production. With respect to approach, Visolis performs the bioreactor experiments, while the ABF develops tools for *Clostridium ljungdahlii* and engineers *C. ljungdahlii* and *Pichia kudriavzeveii*. The impact for Visolis is high, as this collaboration will help accelerate their time to market. The impact on the ABF is also high. Visolis is providing expertise in working with *C. ljungdahlii* and *P. kudriavzeveii*, and while the ABF is onboarding these organisms without clear delineation of whether they fit within the ABF's strategic plan for organism onboarding and development (physiology, metabolism, phylogeny), both are non-model hosts known to be of interest to the bioindustrial community. As such, the tools developed by ABF for these organisms will potentially be of use for other applications. Progress on this project has been good, with the set-out milestones met.

- The Visolis-ABF project appears to have been successful in achieving all of the progress and outcomes that were planned at the outset of the project. Successful development of genetic engineering and metabolic engineering tools in two historically non-model organisms will help open up and de-risk these organisms as future hosts for other projects, and the presentation described well the merits of these two organisms as hosts for particular target products. In addition, there is clear commercialization potential for these organisms for Visolis, and for other companies in the longer term. The project also successfully demonstrated proof-of-concept product formation in both organisms. While the approach is valuable and will enable advancements in the bio-based production arena, as described above, and is clearly relevant to BETO goals, it was also relatively straightforward in conception, so it doesn't have an enormously high potential for innovation. Because the project appears to have been successfully completed, it can be inferred that the management plan and implementation strategy were well-conceived, but it is otherwise hard to judge the management of this project.

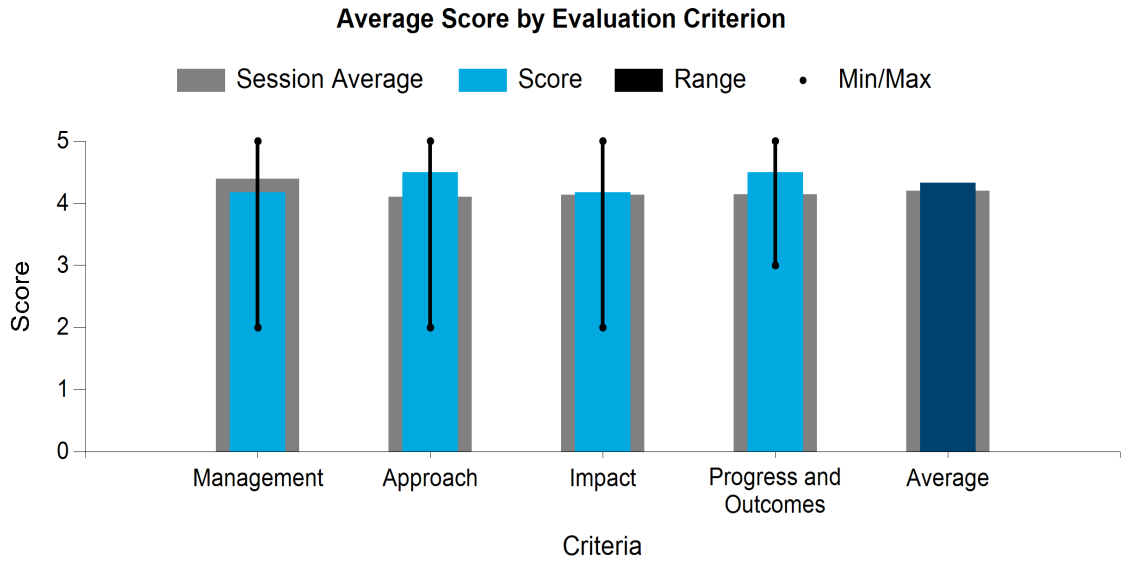
ABF—Target and Host Engineering

ABF

PROJECT DESCRIPTION

WBS:	ABF2
Presenter(s):	Gregg Beckham, John Gladden, Jon Magnuson
Project Start Date:	10/01/2019
Planned Project End Date:	09/30/2022
Total DOE Funding:	$19,805,000

The Target and Host Engineering teams apply the DBTL tool suite available within the ABF to specific hosts and exemplar target molecules to improve TRY. Although this task focuses on host optimization, it also supports and leverages many other tasks—such as IEO, DBTL infrastructure, integrated analysis, HOD, and process integration and scale-up—and milestones (DBTL tracking, automation, beachheads, etc.) within the ABF. In pursuit of the TRY goals, we have further refined existing DBTL tools and developed new tools in cooperation with the DBTL Infrastructure team, which is essential for meeting our TRY-related milestones. The main hosts in this task are well-developed bacteria and fungi that cover a wide swath of biological diversity that have desirable industrially relevant properties and established genetic engineering tools. These hosts are *Pseudomonas putida, Rhodosporidium toruloides, Aspergillus niger,* and *A. pseudoterreus*. A dozen additional hosts are currently being brought into the ABF by the HOD team for future deployment in this task. In our main hosts, we have made significant advances in TRY improvements for several beachhead molecules (metabolic nodes leading to many potential bioproducts) and their exemplar target molecules. We focus on improvements in TRY, as reflected in our Annual Milestones, with the intent of developing broadly applicable tools that meet the variety of issues encountered in developing hosts from initial pathway insertion to optimization of higher TRY processes. These TRY improvements are achieved by applying existing DBTL tools to strain engineering and parallel bioprocess development. The integrated analysis and process integration and scale-up teams are crucial for focusing the research of the target/host team on the most impactful aspects of host development and for bioprocess development, respectively; thus, the work of those teams is also represented within this presentation.



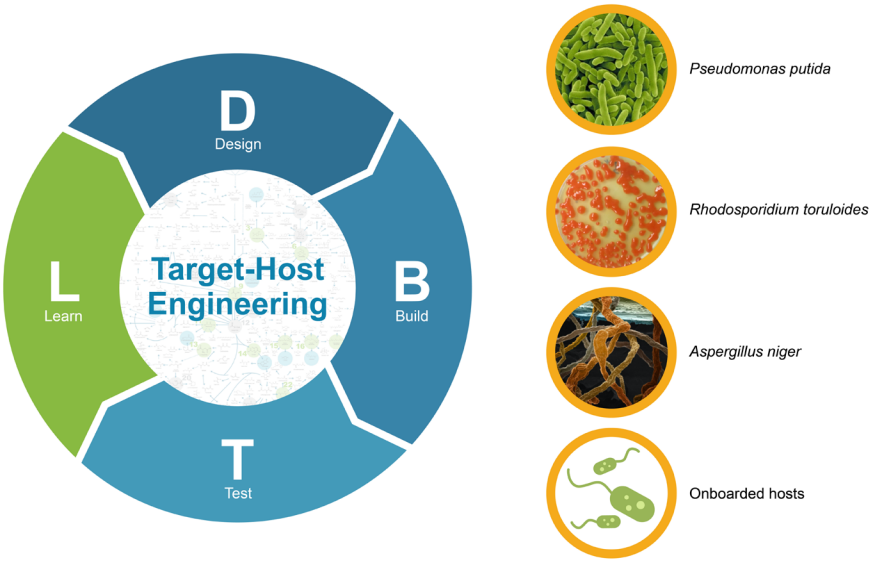


Photo courtesy of ABF

COMMENTS

- 60% and then 70% theoretical yield goals are highly ambitious. I agree with these goals as they will force progress and development faster. However, I wouldn't see it as a failure if these were not achieved in the timelines. As long as cycle time is being reduced by significant margins, and steady progress is being made on TRY goals, that is what matters. A lot of this talk was focused on beachhead ideology, but there was no mention of this in further talk. Dr. Keasling explained the beachhead concept is newer and those projects are just underway. This is something to monitor going forward. It's a good idea; let's see how it plays out in practice. Be honest, and if in a year or two it's not working for some reason, then switch gears. The emphasis on TEA and LCA is one of the highlights of the ABF and so important. I'm glad LCF and reducing greenhouse gas is "approved" again as a metric from DOE. As I mention in all other ABF reviews, the emphasis on hydrolysate as a feedstock should be part of the ABF, but not a sole defining requirement for all projects, microbes, and molecules. Industry doesn't use hydrolysate and won't for many years (at least a decade). Also keep in mind what is relevant and impactful to synthetic biology and industry today and the next 5 to 10 years. As I also write in the host-onboarding review: Do you need to develop filamentous fungi? That is a very difficult question. Why have people used filamentous fungi in industrial applications in the past? Because they already made and secreted molecules people wanted, so they were used to make that molecule. Within the idea of ABF/synthetic bio/HTP engineering, a multinucleate, multicellular organism is a nightmare and highly impractical for automation. Take a very objective and honest assessment of the need of filamentous fungi in the ABF. Do they offer something totally unique you can't get from single-celled organisms? If yes, do the advantages still outweigh all the negatives of culturing and genetics? If not, then there is no strong justification for continuing under the ABF. I know there are labs, expertise, people, history, projects, and a lot of investment in filamentous fungi already. It's so hard (nearly impossible) to cut something after so

much time and investment; however, it's important to not spend valuable resources on something that is not in line with the ultimate goals of the ABF. This work can continue under other projects in the national labs, but not in the ABF.

- *P. putida* work is highly impressive. The approach, impact, and progress are all very clear and exactly in line with ABF goals. Work on *R. toruloides* was also very solid with good progress. This organism isn't as genetically advanced as *P. putida*, so progress will be a little slower. The biosensor work is of huge potential impact to the whole industry. There was much discussion during the review of the high interest in biosensors to industry, and here the ABF can play a huge role in having the expertise to develop this technology as a resource, or spin out a company based on their expertise to supply biosensors to customers. Overall, the approach and impact of the work presented is very strong. Going forward, there should be more clear data on the state of DBTL metrics. What was DBTL at the start of review period and at the end? How does this compare to goals?

- Strengths: The TEA/LCA-guided engineering approach made the identified target/host pairs highly industrially relevant. Excellent technical achievements have been made on the bacteria and fungi projects. Satisfactory TRY metrics have been obtained, and the project is on the right track toward final milestones. There seem to be a large number of researchers involved in this project. Although the management structure is not clear, the team remains highly productive during the years.

- Weakness: Utilization of the developed pathway or host by industry has not been demonstrated. Broader impacts beyond publication are expected.

- The ABF target-host engineering team presents an extremely impressive set of accomplishments, utilizing a combination of their expertise and data-driven insight to optimize production of commercially relevant products in a number of nonstandard hosts. The presentation of the technical approach, management plan, and progress is extremely encouraging across target-host pairs. Aside from the individual successes, the nature of how they were achieved raises some questions about the tie-in to the ABF strategy. One question surrounds the selection and pursuit of beachheads. It is really good to see that the rationale for beachhead selection is backed up by case-specific TEA, process, modeling, and LCA, but it is unclear whether these selections are also vetted by potential industry partners. In fairness, some of the beachheads appear to be selected opportunistically based on projects already in flight (e.g., muconic acid). This bias is not intrinsically problematic, but it was not clearly presented, which gives the impression that the selection may be less data-driven than represented. Moreover, other than selection of the host, little of the reported engineering for these target-host pairs will carry over to other products utilizing these beachheads. The exception is the xylose-based beachhead, for which the current engineering optimizes the beachhead, rather than downstream biology. The second question concerns the connection to ABF DBTL infrastructure. The term "DBTL" is used throughout the presentation to mean very different operations that are often isolated, and ranging from directed evolution to strain construction to proteomics. This usage makes it unclear what was done to achieve these gains. More importantly, it causes an (unintentional) blurring between what was accomplished via unique ABF DBTL infrastructure versus via skilled metabolic engineering. To pick two extreme examples: First, in the case of aconitic acid, DBTL was used at one point to refer to a single gene deletion (cad) that resulted in a five-fold titer improvement. Second, no machine learning-based study was described that has completed its evaluation for any of these target-host pairs. (3HP is close, but from the data, it looks like just upregulating panD, bapad, hpdh gives the best performance.) To be sure, many insights have been learned from prototyping and omics data, but these insights have been gleaned using ABF's

expertise, and not its learn infrastructure (e.g., ART or other tools). For project elements that are successful because of ABF's unique expertise, that is laudable, and the ABF should proudly describe it as such. And where the successes invoke core DBTL infrastructure, such as massively parallel strain construction, ART, etc., then that should be highlighted differently, thereby showcasing a direct connection to the capability. Otherwise, it gives the incorrect impression that the ABF is using its expertise to cover gaps in infrastructure.

- The goal of the target-host engineering section is to validate accelerated DBTL approaches by applying them to the engineering of three hosts (bacteria, yeast, filamentous fungi) to increase the carbon flux through key metabolic nodes (beachheads) to produce platform chemicals (exemplars). The premise of this section is that industry will use a host that has a high carbon flux to beachheads for further engineering toward a value-added chemical. The team has a clear management plan that features frequent communication among the personnel at the three main sites, and risk mitigation strategies that include collaboration with other BETO projects and industry to obtain input on beachheads. The impact of the host-engineering section is high, in particular the selection of host-beachhead-exemplar systems based on TEA data for a specific exemplar. This is a significant innovation in the field and likely to be adopted by groups outside the ABF to select host-target pairs. Using TEA data to guide the host-target selection will focus efforts on pairs with the potential to be commercially significant, with the goal of reducing the time required to bring a bioproduct to market. With respect to the approach, although the team currently has arbitrary biosynthetic performance (titers, rate, yields) targets for host-exemplar pairs, it will be incorporating TEA information to determine when to stop optimizing a host-target pair. This will increase the relevance of the host-target engineering goals. From the data presented, it was difficult to glean how much DBTL cycles have accelerated over the last two years, or the quantifiable impact of accelerated DBTL on achieving a specific biosynthetic performance metric for host-target pairs. Parameterizing current ABF DBTL workflows for different hosts would allow better tracking of the DBTL impact over time. Increased dissemination of DBTL workflows would allow industry to incorporate insights acquired by the ABF into their strain engineering strategies and enable the wider community to implement the DBTL workflows on hosts outside the ABF purview, and potentially, identify new DBTL challenges. Nonetheless, progress on the target-host engineering section has been tremendous, with multiple publications showing significant advances in the three selected target-host pairs.

- The work and accomplishments have been tremendous. It was not clear who informs beachhead selection (i.e., industry or ABF members). There is mention that TEA/LCA, commercial/DFO interest influence candidate evaluation; however, this approach may cause results to provide only incremental improvements rather than a step-change in molecule development. The latter would be much more attractive to industry. Because industry often does not pursue biologically available molecules (e.g., due to poor TEA/LCA, level of risk, limited availability of amenable chassis), it does not mean ABF should discount the molecules. Instead, ABF should take more inherent risk in the technology development and look for more outliers, which could provide a revolutionary change in bioavailable molecules that would be attractive to many types of industries. ABF may also consider looking at chemically interesting structures, which the petrochemical/synthetic chemistry industry cannot feasibly reach. The approach, once again, taken by the group is heavily influenced by an academic approach to create accessibility to molecules. The currently employed process is labor and energy intensive and will not readily translate to access other molecules or exploit chassis for improved commercial viability. The critical risks that do not seem to be addressed are that transfer across hosts, lessons learned, and molecule production capability are not effectively measured. Currently it is a qualitative assessment; instead, the work stream requires

processes closer to industry standards with clear quantitative metrics and benchmarks. The group presented how moving from *Pseudomonas* to *Rhodosporidium* was relatively successful; however, it required additional engineering investments. It was thus difficult to discern due to lack of metrics how well the transition worked toward the overall mission of ABF to reduce engineering and production time. Furthermore, it would be of great value to provide feedback in reverse order for host transition to establish whether any lessons learned from the feedforward transition created any lessons for improving the originating strain engineering. There were, furthermore, not clear metrics on the second critical risk data sharing and information management. Especially with a distributed system, there need to be metrics for how effective this process is across facilities and staff (especially new staff). The key is that neither critical metric is being accurately measured, and thus it becomes inherently difficult to ascertain whether the overall mission of ABF to reduce production times by 50% is being met. This session had concrete opportunities for documenting efficiencies; however, the efforts were more academic and focused on showing that the transfer is possible. The tremendous effort in engineering three distinct chassis will certainly be helpful; however, there is a lot of overlap with other activities (e.g., Department of Defense Synthetic Biology for Military Environments) to create a library of chassis for production application. The key aspect missing from this work is to create generalizable work structures that allow the engineered tools to be applied across all 20 strains being documented in the HObT. There needs to be more effort in an industrial perspective of project management and workflow process organization to have greater utility for the tools developed in this process. In relationship to previous reviews, it does not seem that sufficient effort has been put into addressing these concerns.

- The work described in this session is going well and making good progress, by which it can be inferred that the management approach is working well. However, the management appears to be distributed among several stakeholders, resource providers, and researchers, without a single point of accountability who is responsible for making the tough choices and hard prioritization decisions when they arise. This management by committee can lead to indecision and drift, and thus it is important to consider if there should be one person who is ultimately the decider or the tiebreaker for key decisions. The beachhead-exemplar approach is a valuable way to prioritize and leverage limited development bandwidth, and it makes sense to match a given beachhead with a subset of the host species and understand how "portable" the advancements in one host are to the other hosts within the subset. The use of TEA/LCA to guide the choice of exemplars (and beachheads) is commendable, although as noted, it has some caveats. Some mention was made of including purification (post-fermentation) unit operations in the TEA, which is very important because sometimes the downstream processing costs represent a small fraction of the overall costs, and other times downstream processing accounts for up to 80% of the cost of production via fermentation; thus, it is critical to consider the likely or estimated downstream process when choosing molecule targets and setting TRY goals. Another positive feature of the approach is that the project teams appear to be choosing wisely from among a menu of tricks and tools for each particular host-target pair, rather than using a rote one-size-fits-all path. This will aid an overall goal of learning which approaches and tools are valuable at which stage of development for which types of target molecule; not all approaches/tools will be useful at all times. In addition, it is critical to include the people responsible for "learn" in helping to design the design-build-test experiments to tailor "learn-friendly test experiments," as mentioned on slide 20. Ideally, the ABF will find rigorous ways to track which narrow technical approaches work and which don't (either never work or don't work in some cases), and then identify, refine, and disseminate recommendations to guide the choice of technical approach at each phase of development across a wide array of host-target projects. This hypothetical recommendation engine (e.g., when to use ALE vs. biosensor vs. transporter overexpression vs. pathway gene expression rebalancing vs. media optimization) would, if successful, greatly accelerate strain

development and thus contribute greatly to the shortening of development timelines. The progress and outcomes illustrated by the four target-host engineering vignettes described in the presentation are excellent, and bode well for achieving the subsequent goals and milestones for the ABF as a whole.

PI RESPONSE TO REVIEWER COMMENTS

- The beachhead-target selection and implementation process was conducted using a very intentional and structured process involving multiple inputs, including input from industrial stakeholders. Please refer to our previous response for beachheads above for more details. Some beachheads, like xylonic acid, can be directly produced and optimized, but many others like pyrophosphate beachheads for terpene production (e.g., geranyl pyrophosphate) cannot be produced directly because they are toxic and therefore must be connected to a downstream product. However, all the beachhead strains are developed with the expectation that they can be modified to produce other products. Much of the TRY optimization occurs upstream of the beachhead, and that knowledge and specific strains can be used for development of any product downstream of the beachhead. Not all of our DBTL efforts are an "all caps" DBTL that use the full complement of the ABF capabilities, like some of our more advanced test and learn tools. We have many different capabilities that we can rely on, from the metabolic engineers' knowledge, to advanced multi-omic and visualization in sophisticated genome-scale models, to machine learning. The specific capability we choose depends on the state of target-host development, the extent of knowledge we have of the host's metabolism and specific dynamics of the beached-target pathway, the number of engineered variants we have developed, the specific complement of host-specific engineering tools that are available, the current level of TRY, and the resources allocated to specific target engineering effort. For example, within "test," it may be appropriate to utilize routine high-performance liquid chromatography for screening of target molecule production early in strain development, then move on to individual omic analysis, like proteomics, to acquire basic knowledge around pathway performance before leveraging the full suite of multi-omic test analysis. We rely on the low-resource-intensity approaches first, then advanced resource-intensive approaches as strain optimization matures and the need arises. Machine learning is being used in multiple engineering efforts and will become even more prominent as we push upward on the TRY metrics in more advanced engineered strains where the need to identify new engineering targets that we have not been able to identify by traditional approaches is high. We are certain more success stories from machine learning will emerge soon as we push into the increasingly difficult end of the TRY spectrum.

- We are fortunate to have a large, diverse, and deep team, where more than one person is an expert in each of the crucial skills. The introduction presented the management structure where the three ABF PIs (in order of presentation) are the leads of the bacterial, filamentous fungal, and yeast components of this work. We do interact intentionally and regularly across teams due to the many aspects of DBTL that translate well across these teams, and we think that is actually an indication of strong management structure for a distributed foundry. Regarding the comment: "utilization of the developed pathway or host by industry has not been demonstrated," we note that we are still fairly early in the development of these target-host pairs but do in fact have an industry partner that is using a pathway developed in one of our ABF hosts (see DFO comment below). We are capturing intellectual property that would be appealing to industrial partners. Our publications are an aspect of the public mission of the ABF in terms of openly disseminating and enabling precompetitive scientific information. We are also forming strong collaborative partnerships with industry using the annual DFO, BETO FOAs, and other mechanisms to ensure technology transfer to industrial partners. One recently announced partnership is with C16 Biosciences, of New York, NY, who will work with ABF labs to engineer fatty acid synthesis in the

ABF host *Rhodosporidium toruloides* to produce sustainable replacements for palm oil. This project will leverage knowledge, engineering tools, and the specific strain developed by the ABF for production of lipid-based bioproducts.

- We agree that 60% and 70% theoretical yield goals are ambitious, and we also agree that not hitting them, but getting close (and getting to within goals set by TEA and LCA) is sufficient to still be deemed a success. In addition, we are considering developing beachhead-target specific TRY metrics that reflect the level where a specific beachhead or target may become industrially viable. We acknowledge that filamentous fungi are much more challenging from an automation perspective, but automation of high-density plate cultures isn't a criterion for inclusion of an organism in the ABF. In the 2019 Peer Review, reviewers commented: "The selection of a filamentous fungus, especially *Aspergillus*, is an excellent inclusion in the host-target development programs due to industrial relevance and as an opportunity to optimize a DBTL cycle." These organisms have a huge potential, as exemplified by their successful use in industry to produce a number of bioproducts, a potential that we feel is worthy of investment within the ABF. Two filamentous fungal *Aspergillus* species are used by industry for production of commodity chemicals (citric and itaconic acids) because: they are the most productive organisms for the bioprocess (e.g., ~200 g/L for citric acid), and they are highly acid tolerant, thus producing the free acid at a final bioprocess pH below 1.0. This is very important for downstream processing as it saves an equal weight of off-spec gypsum by negating the need for neutralization during the bioprocess and acidification during downstream processing. The conidia and septate cells are mononucleate and haploid in the industrial base strains that we utilize, so they are quite capable of being genetically manipulated, as we presented. Beyond the small molecules, filamentous fungi are used for the production of many, if not most, of the enzymes utilized in the biomass processing area. They are extraordinarily important in the BETO mission area of commodity chemicals and biomass enzymes and worthy of the time investment despite their recalcitrance to growth in high density culture plates. In terms of the DBTL metrics, we agree that more quantitative metrics should be presented. We will correct this going forward, and these data will be a key part of our peer-reviewed publications as well, such that these data will ultimately be available for the community at large.

- In terms of the management efforts, there are seven national laboratory PIs in the ABF, and three of those PIs (the presenters) are the ultimate decision makers leading the bacterial, filamentous fungal, and yeast target-host teams. Therefore, there is a single person to make hard decisions on those teams, in consultation with the ABF PI Nathan Hillson and the BETO Technology Manager Gayle Bentley. In terms of TEA and LCA, downstream processing is always considered in the overall costs, and indeed, downstream considerations inform host selection (e.g., low-pH compatible fungi for producing diacids). The TEA/LCAs are inherently modular, allowing substitution of different downstream processes (as well as other unit operations) where appropriate for the needs of different target/exemplar molecules. In addition, we are actively collaborating with the BETO Bioprocessing Separations Consortium; for example, one of our past milestones was sharing broth from the ABF bioprocesses for separations with one of the Separations Consortium teams. In terms of test and learn, we have members of the test and learn teams embedded in the target-host meetings to ensure that experimental design can be most impactful for learn and for broader DBTL activities. The suggestion of a host engineering recommendation engine is intriguing and something we do within the target-host teams as part of our planning and management practices. The DBTL tracking we are already doing will help provide the data needed to establish a more rigorous way of identifying which technical approaches are most effective at a particular phase of development and will enable us to more formally develop this recommendation engine.

- We thank the reviewers for the positive comments and constructive feedback. As discussed during the overview session of the ABF, metricizing the DBTL workflows is a core part of our milestones and overall management plan for the ABF. These DBTL metrics are being actively gathered by a point person on the three target-host teams, and we are increasing the automation of the metric gathering. The metrics are reported to central ABF management via monthly data gathering and meetings with each of the three main target-host teams (bacteria, filamentous fungi, yeasts). We will be reporting these measurement methods and DBTL improvements in peer-reviewed publications such that industry and others can adopt our learnings. Regarding arbitrary biosynthetic performance (titers, rate, yields) targets for host-exemplar pairs, we have discussed target-host specific TRY metrics and are in the process of determining the best approach to establish these metrics in a systematic manner.

- In terms of who informs beachhead selection, as noted during the presentation, we rely on a variety of inputs from industry, the ABF technical advisory board, those gathered from the synthetic biology and biomass conversion communities at large (including other BETO projects, e.g., the Performance-Advantaged Bioproducts Consortium), as well as internal input from the target-host and HOD teams considering the unique metabolic features of each onboarded host. There is not a single entity that we rely on for informing these decisions, but rather a collection of inputs from as many stakeholders as we can access. Once we have collected this input, we develop a justification for selecting a specific beachhead and exemplar molecule, which is then reviewed and approved by BETO. In terms of transfer of molecules between hosts, lessons learned, and molecule production capability, these are benchmarked quantitatively in our "transfer targets" efforts in the ABF, and at least two were noted in the presentation. We did spend more time describing the feedforward than the feedback information generated in the transfer targets work but both occurred. We will communicate these in future peer-reviewed publications so that the community can access these learnings. The critical data sharing and information management was better covered in the infrastructure presentation than it was in the target-host presentation. We agree that is a very important aspect of any foundry, especially a distributed foundry. The EDD forms a central hub for ABF data sharing and information management. Perhaps not conveyed sufficiently strongly during these talks, as these topics were covered elsewhere, but we stress that we are using industrial metrics and active project management principles to guide our work—the main thing we wished to convey in this particular session was the scientific progress that has been made on target-exemplar pairs. Design-test-learn aspects are the most translatable portions of the DBTL cycle across very diverse hosts. As noted in the comments here and in the host onboarding presentation, new organisms are being added, in part to help test and strengthen the overall DBTL workflows, especially with regard to the less universal aspects of build (e.g., transformation, antibiotic susceptibility, function of certain parts).

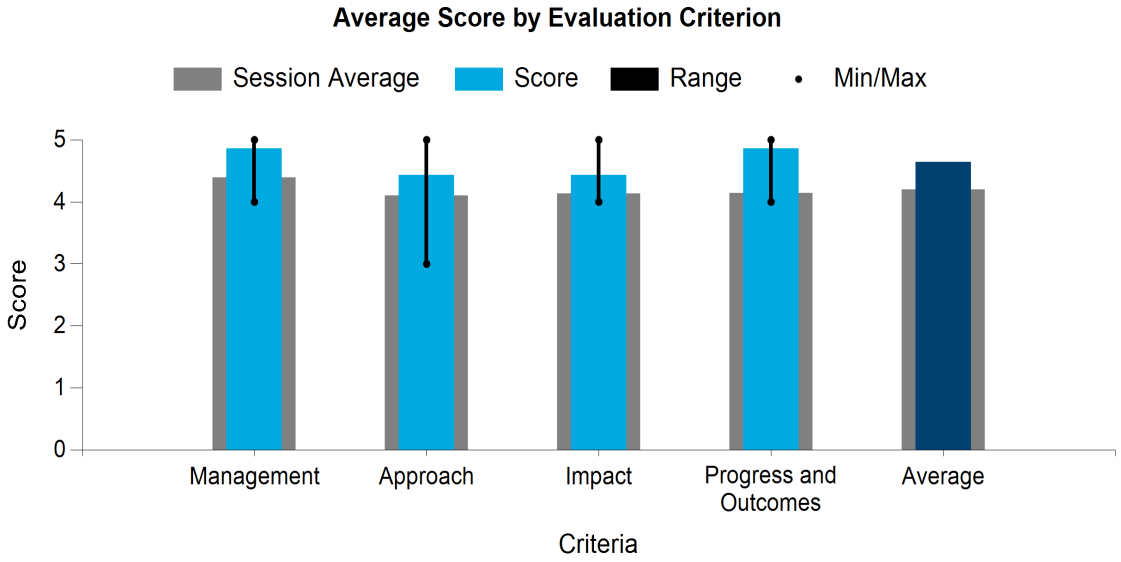
ABF—Host Onboarding and Development

ABF

PROJECT DESCRIPTION

WBS:	ABF3
Presenter(s):	Adam Guss; Taraka Dale
Project Start Date:	10/01/2019
Planned Project End Date:	09/30/2022
Total DOE Funding:	$5,928,000

Non-model microorganisms often have advantageous physiological traits that could be leveraged for advanced biomanufacturing, such as the ability to thrive at low pH or to utilize uncommon feedstocks such as syngas or oligomeric sugars. However, a lack of genetic tools and fundamental knowledge about these organisms hinders strain development. The role of the HOD team is four-fold: (1) evaluate and select proposed hosts for use within the ABF, (2) develop genetic tools and data sets that allow new hosts to be used for DBTL cycles within the ABF and by outside stakeholders, (3) improve genetic tools for BETO "state of technology" organisms to increase DBTL cycle efficiency across the BETO portfolio, and (4) develop a web interface with information and data on each ABF organism for internal and external stakeholders. In this talk, the HOD team will discuss our strain selection approach and our "tier system" framework for evaluating the readiness and guiding the development of an organism for DBTL cycles. We will further discuss progress in host development, detailing advances in the development of genetic tools, synthetic biology parts, biosensors, and physiological characterization for diverse organisms for both ABF and "state-of-technology" organisms. Finally, we describe progress on the web portal for sharing host-specific information within the ABF and with the community at large.



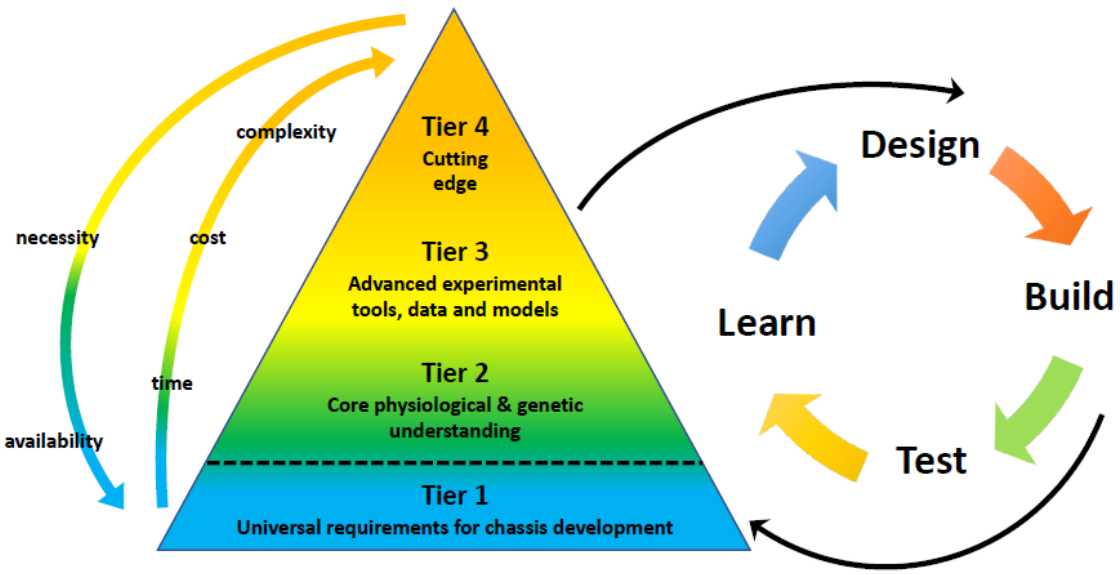


Photo courtesy of ABF

COMMENTS

- Management of the program seemed good, in that the technical coordination between the contributors was evident, though I think industry advisors should be better engaged, because the evidence of that was not very strong. On approach, the actual technical onboarding approach seemed strong. The selection of the hosts to onboard, however, was not strategic enough. Having academic laboratories vote means that these choices are subject to personal interests and does not ensure that the selections are of sufficient commercial relevance, particularly on the 10-year time scale. This seems like a missed opportunity to engage the IAB. A similar issue is true of the website: industry advisors should be more involved to make sure this is being built to help advance commercial objectives; the presented version seemed far more appropriate for academic audiences, especially because the presenter said they are not currently tracking use of the tool. On impact, the potential impact of positive results is obvious and enormous, even if it should be better informed by commercial potential. On results, having 10 hosts onboarded and five more in the process put the program ahead of the Q4 2021 milestone, which is particularly impressive given the pandemic.

- Of all the activities in the ABF, new host development is likely one of the most important and impactful activities, if not the most impactful, that will come out of ABF to transform synthetic biology. It's so important, yet it's also very time-consuming, expensive, unglamorous (in eyes of investors), and hard. This is exactly what DOE and the national labs should be investing in. This type of infrastructure development is key to opening up synthetic biology and the bio-based economy to produce a huge range of new molecules that currently cannot be made in standard organisms. However, most companies are not going to invest the time and money, mostly because they cannot, even if they want to. I wish I could pour money and resources into this group. Overall, the project is going very well, seems well organized, and on track with the current goals. It's a very ambitious, cutting-edge body of work that is being executed well. My comments below all fall into challenging the current approach and near-term goals, in the hope of strengthening this work to make it as impactful as possible.

- (1) My first big concern is the number of hosts and rate of tier elevation. The goals are 20 hosts chosen soon (15 already are) and getting them to tier 1, with slow tier raising across the board after that (2–3 hosts elevated by one tier). It will be much better and more effective to focus on a small number of organisms and advance them as rapidly as possible. Industry needs tier 4. Until you get to that level, the organisms won't be suitable for HTP screening. If you can't do a library screen in the host, it's not particularly useful. Having 15–20 organisms at tier 1–2 is not really helpful in any meaningful way. Having 3 new hosts at tier 4 is a huge win. Cover as much phenotypic space as possible in the few selected microorganisms, or better yet, engage a wide range of industries to ask what on the list would be their top 3–5 organisms that they'd be interested in using if they were at tier 4. Then focus on 1–2 new hosts, getting them to tier 4. As tools are developed and expertise made, ABF can either increase the number of strains raised to tier 4 at the same time, or keep the same number and cycle time, but tackle much more difficult organisms.

- (2) I have a little concern about how phenotypes were chosen and the criteria by which microbes were chosen. Overall, the process and selection criteria were done well. Recommendations going forward: I see the ability to use hydrolysate feedstocks was a high criteria. Absolutely this should be a priority, as we'll need such microbes to make hydrolysate feedstocks a feasible reality. However, by making this such a strong criteria, were other valuable phenotypes or microbes overlooked? Maybe only 30%–50% of the microbes need to be able to use hydrolysate, so there should be strong emphasis on traits industry sees as valuable as well. ABF should not overlook a really useful microbe because it can't use hydrolysate sugars well. I can't tell from the presentation how much industry had input on microbe traits for selection. How were traits chosen? How much was industry engaged to list traits important to them? Is there more than what is shown on slide 26? Can reviewers see the full list if that is the case for review purposes? For example, it's very expensive to cool large fermentations. Organisms that operate at higher temperatures (35°C–40°C) have a big cost savings in industry. I don't see that as a criterion for selection. What else could be important and missing from this list? A strong recommendation, if it wasn't done, is to survey a wide range of industries that do industrial fermentation and ask them what traits are important to them, then reevaluate the list. It might be too late to change the 15 already chosen, but if five more need to be added, get more input, and expand the criteria list. As (a few) microbes are raised in tier levels, choose some that further DOE/BETO goals for hydrolysate consumption, and some that fulfill industry's top criteria.

- (3) I can't wait for the host tool website to go live; in the meantime, however, there is no way to find out on the ABF website which organisms the ABF has chosen, or their tier level. Post the table on slide 31 with a column of the tier level. That will really help with industry engagement and showing what the ABF is offering.

- (4) The emphasis on portability of tools and transfer of tools among organisms is a good goal, but I would not emphasize this over the goal of quickly raising hosts in tier level. Of course, you don't want to be reinventing the wheel every time. Instead, accept that initially there will be a lot of tool development, and if you focus on hosts with very different phenotypes (and therefore likely not closely related) you aren't going to have much redundancy at first. As the ABF grows and develops the tools, skill, experience, etc., the synergy will come. It's far more impactful to develop a smaller number of microbes that cover a wide diversity of phenotypes than to show you can port a tool from one organism into a similar organism.

- (5) Do you need to develop filamentous fungi? That is a very difficult question. Why have people used filamentous fungi in industrial applications in the past? Because they already made and secreted molecules people wanted, so they were used to make that molecule. Within the idea of ABF/synthetic bio/HTP engineering, a multinucleate, multicellular organism is a nightmare and highly impractical for automation. Take a very objective and honest assessment of the need of filamentous fungi in the ABF. Do they offer something totally unique you can't get from single-celled organisms? If yes, do the advantages still outweigh all the negatives of culturing and genetics? If not, then there is no strong justification for continuing under the ABF. I know there are labs, expertise, people, history, projects, and a lot of investment in filamentous fungi already. It's so hard (nearly impossible) to cut something after so much time and investment; however, it's important to not spend valuable resources on something that is not in line with the ultimate goals of the ABF. This work can continue under other projects in the national labs, but not in ABF.

- Overall impressive progress.

- Strengths: The HOD team has made good progress on onboarding strains. The HObT website is going to be a major tool for ABF to deliver its products (onboarded strains, pathways, and genetic tools). This database will be a very important resource for the community.

- Weaknesses/areas for improvement: The HObT website should include information on where the onboarded strains, molecular tools (plasmids, promoters, pathways, etc.) can be found (e.g., Addgene). The website should also include protocols and experimental details to help users to obtain consistent results. Current efforts are mostly focusing on low tier development (transformation tools, promoters, etc.). The HOD team should start focusing on aggressive tier elevation for a few strains to improve industrial impact.

- The goal of the HOD section is to generate synthetic biology tools to enable the rapid engineering of non-model hosts for chemical production. The non-model hosts have been selected based on their metabolic, physiological, and phylogenetic characteristics. The premise for this work is that specific features of non-model hosts may help them outperform well-established biosynthetic hosts (e.g., *Escherichia coli*), resulting in higher chemical TRYs. The wider availability of synthetic biology tools for non-model hosts should increase the likelihood of industry adopting them, thus accelerating the overall time to industrial chemical production. The team has a clear management plan with leads for each of the onboarded hosts and frequent calls. With respect to approach, there is a clear criteria for host selection and onboarding based on both internal ABF expertise and external stakeholder feedback (industry). The team has shown that some of the synthetic biology tools are portable across organisms, which should (1) accelerate the onboarding of subsequent hosts and (2) reduce the cost of onboarding new hosts. However, it is not clear if the team has seen this trend over time. Additionally, it is not clear if the HOD team is taking advantage of DBTL workflows developed for other parts of the ABF, such as target-host engineering. The team's milestones are centered around onboarding as many hosts as possible and moving a portion of them through the different development tiers, about 20 by 2022. It is worth analyzing the impact the already onboarded and developed non-model hosts are having on the community (i.e., how many hosts have been adopted by industry/academic labs outside the ABF). This information may provide feedback on whether the team should refocus their milestones on a narrower set of hosts to onboard, take particular hosts to a higher than planned developmental tier, or expand a subset of non-model hosts. The impact of this section is very high with a significant potential to accelerate the bioproduction of chemicals at industrial scale. Tracking the usage of non-model hosts outside the ABF

will help quantify this impact. The HObT website should help disseminate these findings even further and potentially leverage the wider community to develop additional synthetic biology tools for ABF hosts. Additionally, the HOBT website could provide protocols and workflows to develop tools for non-model hosts that the community could replicate in order to develop tools for hosts outside the current scope of the ABF. Finally, progress on this section has been high, with the team onboarding more than 10 hosts to date.

- The HOD task is proceeding quite well and has the potential to substantially expand and accelerate the reach of fermentation-based bioproduct manufacturing. This is a clear example of the type of precompetitive technology development that government funding should support. The management structure seems reasonable but complex with many stakeholders and participants; while it appears to be working fairly well, it is not clear how critical prioritization and resourcing decisions are made, because there seems not to be any single point of accountability. For example, if resources are available to do only seven of 10 proposed tasks (where each task may be to advance capabilities in a given host species), how is it decided which three to cut or postpone? The approach seems well-conceived and is reasonably well-integrated with other parts of the ABF and BETO. Being able to have tools for genetic manipulation and foundational functional genetics and modeling for about 20 microbial species is potentially a game-changer for industrial biotechnology, and will at least greatly accelerate and de-risk the expansion of commercially relevant feedstocks from glucose and sucrose to a wide variety of other feedstocks. In addition, it may be that some species function better for making certain metabolic products right "out of the box," but that hypothesis has not been widely tested, and (assuming it is true) there is not a pre-existing knowledge base to help guide the choice of host organism for a given target molecule of interest. The ABF will start making a map of the landscape of interactions between an array of substrates, and array of host species, and an array of potential fermentation products, which is very exciting. One concern is that 20 species may be enough, and there may be more value (post-2022) in expanding the tool sets and know-how for these 20 rather than expanding to 50 host species in years 7–9 (as was suggested on slide 6 in Hillson's presentation). As noted above, the potential impact of this HOD task is enormous, and paves the way for accelerating commercialization opportunities across the country's bioeconomy. There was no mention of patenting the genetic malleability tools or otherwise restricting access to the knowledge and protocols created by the HOD task, which is commendable. The impact of this work will be increased if it is not placed behind a paywall. It is a shame that some other organisms have greatly restricted use for industrial biotechnology because the institutions that developed tools like plasmids or otherwise adapted common molecular biology tools for a particular organism walled off the organism via broad patents. The progress and outcomes of HOD to date have been impressive and appropriate. The HObT online portal is a terrific idea, and it will be good to see the public unveiling later in 2021. It is assumed that information like that shown in the grid on slide 26 will be included in the HObT portal. In addition to the columns in slide 26, it would be good to include classification of species based on features like maximum sugar uptake rate, maximum growth rate, temperature/pH/oxygen dependence of growth rate, phage susceptibility, biomass yield, natively produced byproducts, susceptibility to overflow metabolism (like Crabtree effect), tolerance to various chemical and other stresses, putative or measured oxidative phosphorylation ratio, preferred glycolysis pathway (EMP vs. ED), metabolic source of cytosolic acetyl-CoA (e.g., pyruvate dehydrogenase PDH, PDH bypass, ATP citrate lyase ACL), etc. In addition, the beneficial impact of the HObT portal and the onboarded hosts can be expanded by also including some complex multi-objective search capabilities (e.g., "give me a list of species that can grow above 35°C and below pH 4, using xylose, and tolerant to monoterpenes"); even

better, but harder to execute, would be a decision tree that guided users to the top recommendations for host strains to consider for their application, based on the cumulative learnings of the ABF.

- The HOD team describes a clear strategy for selection, onboarding, and communication of novel production hosts with high potential impact for DOE and external stakeholders. Their progress is impressive across the board, including their detailed tier-based organizational strategy, initial successes in tier 1 to tier 2 transitions, and their web-based tool for tracking. My only comment concerns ensuring industrial relevance. Though it may have been covered, it was not clear how extensively industrial feedback was used to select hosts. For example, it appears that some hosts are selected because of interest from a single company, and it is not clear whether they are of general interest. Other important drivers from industry might include picking hosts that use industrially relevant feedstocks, and starting with higher-tier organisms (e.g., starting with a tier 3 organism relevant to industry and bringing it to tier 4).

PI RESPONSE TO REVIEWER COMMENTS

- We thank all the reviewers for the positive comments. Applying the concept of DBTL across different organisms is not exactly the same as applying it iteratively in a single organism, but there are certainly parallels. For instance, libraries of plasmid origins of replication and antibiotic resistance genes that needed to be developed for one *Bacillus* species can be rapidly tested in a different *Bacillus* (and often *Clostridium*) species, making tool development in the second species more time- and cost-efficient. This contributed in part to our go/no-go milestone accomplishment. As we adapt tools like site-specific DNA integration systems from one organism to another, learnings from previous successes and failures directly inform how we go about adapting the tool to the new host. As we continue expanding genetic tools in different hosts, we anticipate these synergies becoming more common and apparent. Tracking the speed and efficiency of this process over time is an excellent suggestion. The HObT website has been developed to track host onboarding progress within the ABF using the tier system. However, we have not (yet) built in methods to determine whether or not we are achieving an acceleration factor over time. To that end, we will explore approaches in HObT for HOD, much as we are doing for build with the DIVA platform and soon for "test" in the EDD, to metricize the time and cost required to onboard and advance new hosts up the tier system. We also agree with the suggestion to track usage of ABF-developed hosts outside of the ABF. How or whether this information is presented publicly will need to be discussed with stakeholders. The HOD team (and the ABF team overall) has often discussed the relative merits for breadth (lots of organisms, generally less developed) versus depth (fewer hosts, more development for the chosen hosts). Based on reviewer feedback and continuing internal discussions, we will explore reducing the emphasis on the number of new hosts to onboard and focus more on rapid tier elevation for a more limited number of hosts. In this scenario, we would likely still onboard new hosts, but the focus would only be adding phenotypic or phylogenetic diversity, and the exact number of hosts might cease being a milestone. Also, while industry would obviously prefer all the organisms to be tier 4, we believe there is still great value in getting new organisms to tier 1. For example, in one particular collaboration with a company, they wanted to engineer a specific organism with their favorite pathway, but the strain was not transformable. By developing transformation methods and gene deletion/insertion tools to get the strain to tier 1, we have enabled rational metabolic engineering so they can now try to increase TRY of their product. As pointed out by this reviewer, metrics on usage of ABF hosts by industry/academia will help guide HOD activity in the future.

- Monthly HOD team meetings are held, with representatives from each of participating national labs, where prioritization and resourcing issues are discussed. Based on these dialogues and consultation with

other ABF team members, the HOD co-leads, Taraka Dale and Adam Guss, jointly make major decisions on project direction. In the event that a consensus could not be reached on some issue, the overall project PI Nathan Hillson would make the final decision, in consultation with the BETO Technology Manager Gayle Bentley. We agree that there is value both in having both breadth of organisms and depth of tools for a subset of organisms. As discussed in greater depth in the response to reviewer 1, going forward we will explore placing a larger emphasis on rapidly advancing a subset of organisms up in tier levels, and we will consider changing the target number of hosts. We have been populating the HObT with basic strain attributes such as gross phenotype, Bio Safety Level 1 or GRAS status, substrate range, etc. However, the specific suggestions listed above are excellent and greatly expand the scope and value of information that we could provide in this section, some of which we have previously captured in our "user stories" that we are using to guide HObT feature development. We will actively pursue adopting these suggestions. We envision this tool will become a major resource for labs that work with, or want to work with, any of the ABF organisms, having a collection of protocols, sequences of validated genetic parts, strains, links to data sets, etc. We also plan track internal and external usage of HObT as one way of quantifying impact.

- Plans to greatly expand the information available within HObT are underway, including locations for existing tools, sequences, protocols, etc. We also plan to link HObT to the ABF's EDD, the repository of experimental data and metadata for the ABF. As discussed in the responses to reviewers 1 and 2, we are reevaluating the relative merits of onboarding many organisms to tier 1 versus advancing fewer, select organisms further up the tier ladder. Consistent with the reviewer suggestions, we may shift to a greater focus on tier elevation to levels 3 and 4.

- Thank you for the strong words of encouragement; it is nice to know that our work is appreciated. As discussed in our responses to reviewers 1–3, we will explore decreasing emphasis on onboarding a specific number of new hosts, in favor of prioritizing tier elevation for a smaller number of select hosts. For host selection, especially at the beginning of the project, one major criterion was the ability to use hydrolysate sugars. However, we have also onboarded organisms that can grow on alternate feedstocks such as $H_2 + CO_2$ aerobically and anaerobically, and we are also onboarding organisms capable of growth on C1 compounds. One organism is a moderate thermophile, growing up to 55°C. Other traits we have prioritized (or plan to prioritize) include tolerance to pH extremes and salinity. For industrial engagement, we engage with industry in multiple ways. First, we collaborate with industry via FOAs and DFOs. Many of these joint projects have host onboarding components, where the company tells us specifically what organism(s) they would like developed, and we work together to develop the host and advance in tiers. Frequently, these hosts and tools are then made available for use within the ABF, or the genetic parts can be used in related organisms. We also engage with industry via the IEO team, the IAB, and through informal conversations. Company representatives have typically been reluctant to give direct answers on the organisms that they would like to see developed, and only occasionally shared phenotypes that they might like to see developed (e.g., use of C1 feedstocks or robust protein secretion). Having said that, we are often asked to provide more detail on host selection criteria and how, and to what extent, industry consultation influenced this process. Based on your suggestions, we will explore ways of condensing and presenting the results of industry input into the host selection process. We will continue to engage with industry to develop the best hosts for biomanufacturing. For filamentous fungi, they have indeed been used in industry due to their native abilities, such as the ability to secrete citric acid. However, they also have useful phenotypes beyond natural production. They are able to tolerate low pH, making them strong candidates for producing organic acids. Their filamentous nature, while cumbersome for HTP engineering, also provides advantages during cell retention for continuous

bioprocessing. But the point is still a good one—that we should be developing organisms because they are the most important organisms, and not only because we have worked with them historically. We will keep this in mind as we continue to evaluate our portfolio of organisms for use in the ABF.

- As noted primarily in the response to reviewer 4, we engage with industry in multiple ways and solicit feedback on what they would find most valuable. Primarily, we try to develop hosts that we think will be of broad interest to stakeholders. In addition, some hosts are being developed in direct collaboration with a particular company via competitively funded grant proposals. In these scenarios, we do still try to make the organism, or at least the developed tools, available to the community. Based on the suggestions of the reviewers, we will explore ways of more clearly presenting the results of industry input into the host selection process.

- As noted primarily in the response to reviewer 4, we routinely engage with the IAB and other industrial stakeholders seeking feedback on choices and progress. On the strategic choice of hosts, we did put considerable weight on industry input and believe that our current list of organisms will be impactful. We did select some organisms that we have previous experience with, but in those cases, the organisms were still selected for specific reasons related to industrial relevance, such as feedstock range, pH range, robustness, and/or production of beachhead molecules. Many other organisms used at the national labs were not selected because we do not think they have as much commercial potential as the ones we selected. As part of the host onboarding process, we also brought in several organisms with which we had little or no previous experience, again because they had traits that we thought were particularly relevant for industry. However, it is obvious from several reviewers' comments that we need to better communicate how industry input is utilized for host selection and development. We will explore ways of better presenting the results of industry input into the host selection process.

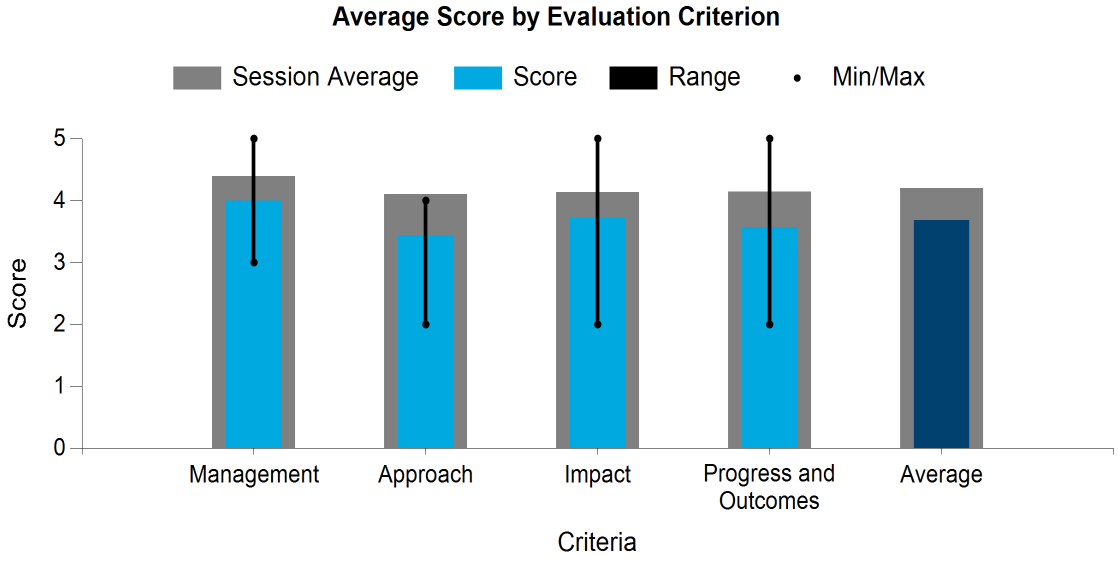
ABF—Industry Outreach

ABF

PROJECT DESCRIPTION

WBS:	ABF4
Presenter(s):	Christopher Johnson; Phil Laible
Project Start Date:	10/01/2019
Planned Project End Date:	09/30/2022
Total DOE Funding:	$1,080,000

The objective of the ABF is to develop infrastructure to support industrial biotechnology, and understanding the needs of the industry is critical to achieving this goal. In support of this, the ABF IEO team organizes and facilitates interactions with industry, providing feedback from the industry stakeholder community to the ABF and BETO that supports decision-making and project planning. The activities of the team also aim to increase the visibility of the ABF and attract collaborators from academic and industrial communities. The goals of this task are accomplished through the workings of three highly interwoven strategic focus areas (SFAs; Assessment, Outreach, and Interactions). For the Assessment SFA, an Energy I-Corps approach is used to understand the needs of the biomanufacturing industry by interviewing and surveying its members. In the Outreach SFA, members work to manage the ABF public profile and disseminate ABF information and resources to industrial, academic, governmental, and public stakeholders. For the Interactions SFA, the main goal is the coordination of community-building activities. This includes Industry Days and workshops to ensure research effectiveness and industry responsiveness. This SFA also facilitates interactions with the IAB. The IEO team collectively plans panels and sessions at key industry conferences. Overall, the IEO task contributes to the alignment of ABF activities with BETO's milestones and facilitates communication of the ABF value proposition to key stakeholders in industry, R&D organizations, and the public.



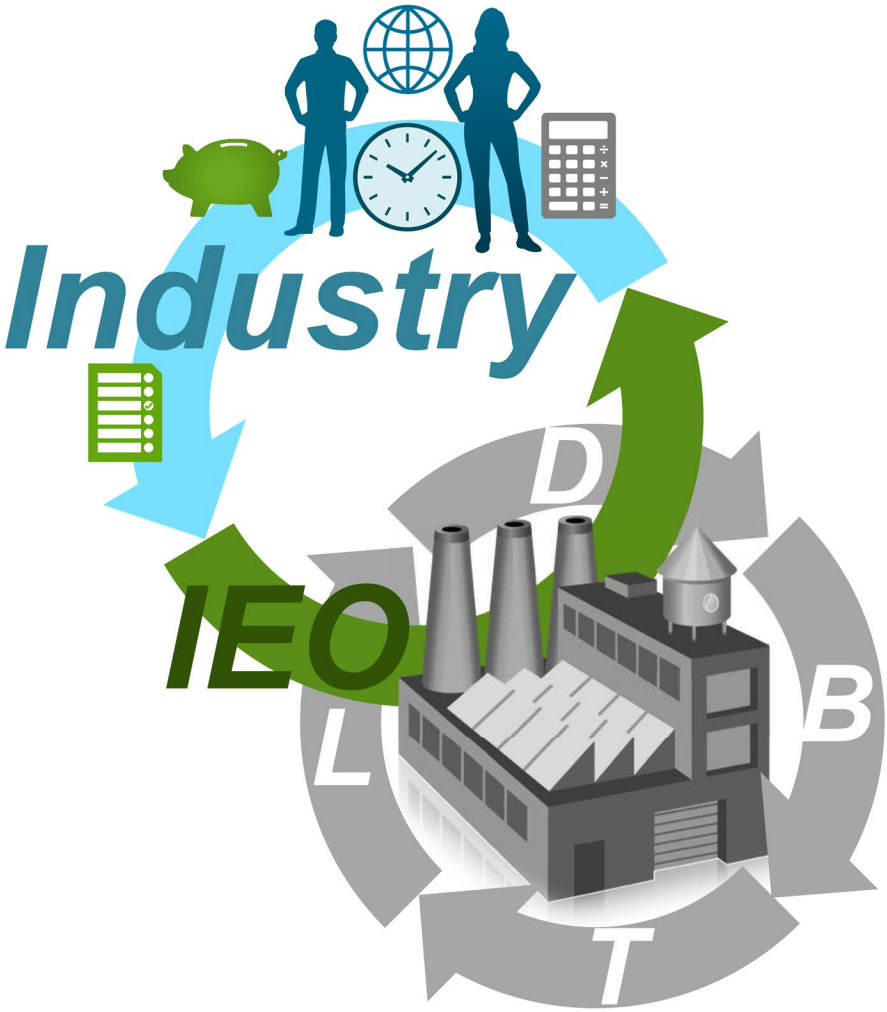


Photo courtesy of ABF

COMMENTS

- ABF has established a comprehensive program for IEO that comprises a portfolio of diverse forms of engagement and evaluation. These are the right types of activities for ABF to pursue to maintain awareness and to cultivate partnerships, and they appear to be progressing well. However, based on the presented material, as well as review of the public ABF website, funding activities should be shifted to place a much greater emphasis on ABF capabilities, from both an outbound and an inbound perspective. For outbound-oriented activities, more clarity is needed in communicating the portfolio of capabilities to industry in technical terms and with quantifiable metrics. (For example: If my company needs to build 100 yeast strains in the next two months, is this an appropriate project to work with the ABF? What if the number is 1,000 strains? What if I need to run proteomics on all of them? etc.) On the inbound side, IEO should solicit explicit feedback from industry on specific capabilities in order to guide the prioritization of ABF technology development, based on interest. This will help ensure that ABF's DBTL capabilities appropriately anticipate and serve industry's needs. One additional comment: For understandable reasons, IEO cannot share responses to its one-on-one interviews. However, an

appropriately anonymized public summary report would be of broad interest and would have substantial impact.

- As stated in intro slides: “Tremendous opportunity to learn how to increase success rate of relatively infant biomanufacturing industry” and “Foundry succeeds only as industry is engaged and adopts resulting technologies.” The importance of these two statements cannot be overstated. It’s clear there have been a lot more effort, resources, and organization put into industry outreach since the start of the ABF. The outreach efforts could be expanded/enhanced. Social media is a necessity, but I don’t see it reaching the right people to bring in industry partners. Conferences normally would be highly effective, but in COVID times it is really hurting outreach by not having in-person conferences. Interviews and surveys are also good tools, but still seem to be falling short. I would bet most biotech companies really don’t understand what the ABF is capable of providing. Are there regular newsletters/press releases sent to heads of departments for biotech companies? Is the distribution list only people who have signed up? I’d troll databases to come up with a long list of key people at a wide range of companies and spam a regular newsletter at them. The presentation listed a “fact sheet with active distribution.” I’d like to know more about this. There needs to be discussion and decisions made soon about whether ABF/DOE/national labs want to turn the capabilities being developed into a service for the community at large. What is the vision for the ABF to do more small, routine, higher-volume requests such as DNA design and synthesis? I see this as something a lot of labs and small companies would love to access, and that really serves a need that would dramatically speed learning and development across synthetic bio. The point of a BioFoundry is a service capability. It doesn’t make sense to limit the capabilities only to large FOA, DFO, and CRADA projects. How easy is it really for industry to access these capabilities (easy in time and red tape)? It says on the website six-week turnaround, but that is once work starts. How long does it usually take to get an SPP in place? That wasn’t clear in the presentation. Usually, you can get a contract in place with a contract research organization in a few weeks (~1 month). That should be the goal to sign up new industry contracts for small bodies of work. Presumably there is no cost share with a SPP? It is industry paying for a service? These feel the most straightforward to me, and likely the most attractive to industry (a contract research organization model), but they are hardly explained on the website. It’s really not clear to me how much the broader synthetic bio community is aware of all the capabilities being developed in the ABF. The proteomics and metabolomics work is also highly attractive, as most small companies and labs do not have access to that kind of infrastructure. Lastly, it is only fair that industry shares in responsibility for informing the ABF. The ABF outreach team is only as good as the industry partners they are engaging with. I know it is hard to find people to be on review panels and give their time. However, if ABF is not getting much feedback from their current industry review days, ditch the people who do not speak up and keep looking for people who do contribute. Probably 50% of the work of this team is finding people who will engage, and that is time well spent.
- It does not seem that the return on investment is benefitting ABF as a whole. For example, the response rate of 25% is quite low for interviews. It seems that not enough information is getting out about the benefits of ABF for industry, thus industry perceives this effort, the ABF, as redundant or excessive? This issue is a communication problem. It also does not seem that any feedback from industry engagement is feeding back into management or projects to help improve the customer experience. You will want to convince industry that a low-cost interaction with the communications team could result in financial benefits in more customer specific product development, industry-oriented tools to fill existing gaps, and disruptive research activities to develop step change technologies for industry to improve their commercial viability. The metrics for measuring success are very vague. The team may consider more detailed metrics that provide ABF specific customer information, demographics to understand what

ABF's customer base looks like. Customer metrics are everything! Although the product area appears niche, the application space expands well beyond the bioindustrial manufacturing and commercial energy sector, thus a broader audience should be considered for engagement. Leveraging Manufacturing USA, DoD Manufacturing Technology Network, etc., will help make that happen more effectively. Also, instead of seeking input, it might be worth exploring how to communicate better outwardly in addition to having an actively engaging website. Communicating the vision, the mission, and the benefits to industry would certainly get more engagement, if done appropriately.

- Overall, the IEO approach, plans, and progress have been well-conceived and successful. The updated website with videos (also posted on YouTube) is very helpful and clear. The regular postings on LinkedIn and Twitter have the ability to draw attention of new companies and individuals, along with the other industry outreach and conference attendance. The responses to previous reviewers' comments in the slide deck was very helpful to understand more about the structure, plans, milestones, and management of the IEO. One metric that could be used to judge the effectiveness of the IEO (although this is also affected by other factors outside IEO control) is to compare the number or total proposed cost of applications to FOA and DFO to the number or total cost of awarded applications to FOA and DFO. If outreach is effective, then the demand will far outstrip supply, which will enable the award committee to be very selective in choosing the applications with the very highest merit or very best fit to what the ABF needs to develop next to round out its offerings or shape them in the most beneficial way. In the DFO intro slides, it said that there was a four-fold oversubscription or overdemand relative to the award money available, which seems like a good number to target. One potential concern is that it seems that some companies are receiving multiple awards—it would be good to hear what ABF thinks about the relative merits of impacting a larger array of companies versus multiple awards to some companies.

- Strengths: The IEO team has made multiple efforts to deliver their outcomes. The "one-to-one" interview is an efficient approach to engage industry partners. The website has a clear introduction of ABF's capabilities.

- Weaknesses/areas for improvement: The new website should clearly list ABF's achievements in the past years (such as reduced DBTL cycles). It should also include resources that ABF has made available to industry and the scientific community, including available tools, strains, beachheads. If there is a separate webpage/database for strains and DNA, the link should be provided on the ABF's main webpage. The overall goal is to make ABF's products more accessible to enhance its impact. The IEO team should consider developing a method to evaluate the ABF's impact to both scientific community and industry—for example, performing some search to monitor how many time tools, strains, and beachheads developed by ABF have been used in both scientific research and industry.

- The goal of the IEO section is to (1) understand the needs of the biomanufacturing industry, (2) facilitate collaborations between industry and the ABF, and (3) disseminate ABF findings to external shareholders. Areas of needed improvement for the IEO section include development of a clear management plan, quantifiable metrics for success, and identification of clear risks and mitigation strategies. The comparative lack of refinement of the IEO section may be due to its late inception. However, the success of IEO is so important to the overall impact of the ABF that more planning, refinement, integration with other sections, and resources should be devoted to this section to achieve the full potential impact of the ABF. IEO needs specific goals and metrics to define success, without which it is difficult to judge its approach, impact, and progress. Evaluation was also hampered by a lack of clarity on the size of the companies interviewed by IEO, their location in the chemical supply chain, and

the industry segment they serve. Regardless, the number of companies interviewed per year (25) is far too low for such a large and expansive operation as ABF. With respect to approach, IEO is simultaneously interviewing industry about their needs and advertising ABF capabilities. De-bundling these two activities may reveal currently unidentified industry needs unrelated to current ABF capabilities, potentially opening doors for the ABF to provide new capabilities that are in line with industry needs. A more expansive IAB with representatives from a more diverse selection of the biomanufacturing industry in terms of company size, supply chain location, and industry segment would also be advisable. On a positive note, over the last two years, IEO has devised a non-negotiable CRADA to help accelerate contract negotiation time. This is of significant impact and should allow more companies to engage with ABF.

- The management of this program seemed adequate in that metrics were established but flexibility was maintained, exemplified by the pivot toward hosting webcasts with different themes when COVID thwarted other plans. The approach and its potential for impact were marginal at best. First, relying on website views and social media followers to indicate whether industry is engaged seem to be almost entirely inadequate and inappropriate goals given the fact that ABF's mission centers around easing commercialization of products. ABF needs to go to industry—there is no way for small companies to have ever heard of BETO, let alone ABF, and unless the program thinks creatively about how to reach these smaller organizations and other potential company founders, the people who find ABF's website and social media accounts are not the ones ABF needs to reach. Second, the program has made a very significant misstep in failing to prioritize diversity and inclusion in their outreach efforts. The networks of people who are familiar with ABF or its employees are the only ones you will reach via social media. The same is true of the IAB; better efforts need to be made to ensure a diversity of opinions. This is especially true with administration priorities in diversity and inclusion. The progress and outcomes this group presented were marginal, but certainly not enough to get the input needed to make sure ABF's structure/mission/approach will transform the commercial landscape.

PI RESPONSE TO REVIEWER COMMENTS

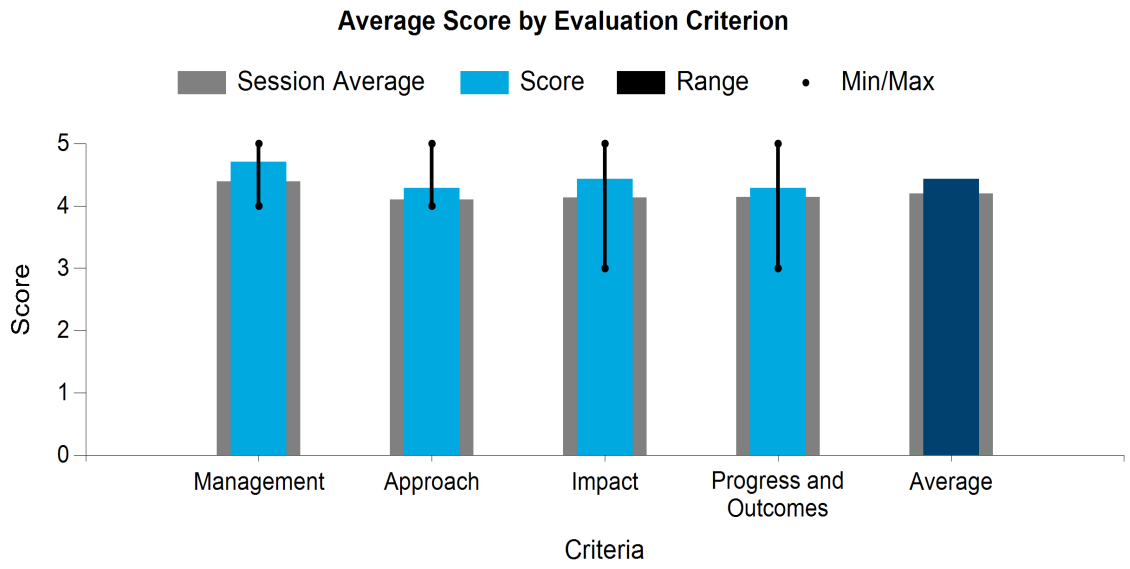
- The ABF IEO team thanks reviewers for their feedback and thoughtful comments. The IEO has expanded activities in recent years, and the amount of resources directed to this effort are evaluated yearly by the ABF and BETO. IEO communicates the vision of the ABF and will continue to do so, while the ABF Executive Committee, including the BETO Technology Manager, determine the vision and mission. The IEO team is not charged with creating the vision; it is responsible for engaging with industry to understand hurdles for the advancement of bioprocesses. The IEO regularly collects information from industry through interviews, surveys, and engagement with their IAB. This information is directly provided to ABF management and to BETO. Continuing to engage with the biomanufacturing industry in new ways, and expanding our interaction network, are ongoing priority activities of the IEO team. These industry interactions also lead to research partnerships and collaborations within the ABF. The IEO team frequently polls industry to understand how well our mechanisms for collaboration are working. We will continue to streamline and optimize the establishment of partnerships. The ABF DFO is a competitive process and has different foci every year. The competitive and peer-reviewed processes dictate who wins awards and how often. We will continue to explore innovative ways to discover and engage a broader industry base in outreach efforts. The ABF values diversity, equity, and inclusion and we do actively cultivate these values in the IEO activities that we have outlined. In addition, we are actively exploring ways of diversifying student involvement at ABF labs. For example, our IAB membership is reviewed yearly with a view to encouraging ongoing participation of all members and is

adjusted not only for science and mission but also for diversity (including areas of specialization, age/size of company, geographic location of operation(s), and gender—categories that will expand in the future to make it as inclusive and equitable as possible, while keeping the group to a number that can be productive). As the ABF has matured, the IEO task is pivoting from an emphasis on understanding industry needs and how the ABF can address them toward communicating our capabilities, successes, and opportunities to partner with us. This will be reflected in updates to the ABF website (https://agilebiofoundry.org/), such as the inclusion of research highlights, partnering success stories, informational webcasts, and other regular news items. We regularly update the capabilities on the ABF website to ensure they are up-to-date and to improve organization and clarity. We will also be adding links to task-specific websites associated with strain and DNA information, HOD, and "learn" tools. Some links exists presently, like one to the EDD, but we will be working to make this link as well as others more prominent. Specific information regarding timing and pricing are not included on the website because the ABF is not a service provider, per se, and companies are encouraged to reach out to the ABF to discuss their specific needs. In addition, our social media presence on Twitter and LinkedIn continues to grow and increase awareness of the ABF's mission, capabilities, and achievements. We also maintain and are continually building a mailing list that we use to announce important updates and opportunities. We adhere to best practices in our mailing list management; this includes only adding recipients who have given us permission to contact them. We continue to explore and pursue new strategies to help us reach the biomanufacturing community. This emphasis on marketing will continue to expand to communicate with wider audiences. Currently, with relatively limited resources, we must prioritize our primary audiences. The IEO team appreciates the need to evaluate the impact and success of IEO efforts, which is inherently challenging given that the outcomes are not easily quantified. To this end, in our quarterly interactions with the IAB, we have begun to utilize a dashboard that highlights expanded metrics of publications, publication impact, tools developed, intellectual property, CRADAs, SPPs, Work for Others, and alumni involvement in biomanufacturing. Due to the brevity of Peer Review presentations, this was not shown. Now that we are collecting these metrics, they could be made publicly available on the ABF website in the future. The appropriateness of these metrics will be continually evaluated as we learn more about industry needs and amend as needed.

Intro to Directed Funding Opportunities

ABF

WBS:	ABF5
Presenter(s):	James Gardner



COMMENTS

- An approximately 7- to 8-month turnaround from submission to start of DFO—that's pretty good for a government contract. Keep working on streamlining as much as possible. To industry, time is major money. Investors want rapid return on investments, and especially small startups, who are likely to want to use ABF services, do not have a lot of time to spend before they need to show progress. As discussed during the reviews, 20% cost share is very hard to demonstrate and contribute for academic labs, especially because they are government funded! ABF/BETO is working on making it easier for academic labs to deal with this requirement, which is good. Overall, the DFO approach is working well (for industry) and has had successful outcomes. The early funded projects were in line with BETO/ABF goals at the time, although these have since evolved. Perhaps goals will change based on this review process; keep making sure DFO projects are in line with the current ABF goals and priorities. A "4" for approach is for the difficulties academic labs have in dealing with the cost-share requirements.

- The large number and the diverse nature of industrial applicants from the DFO has reflected the relevance of ABF's capability to current biomanufacturing. Most of the DFO awardees used ABF's omic tools and onboarded strains. It is not clear how these DFO partners can benefit from ABF's core technology development, such as from beachhead development. It is also not clear how ABF has

benefited from DFO projects. There should be some clear demonstration on how each DFO project helps to shorten ABF's DBTL cycle, and help to speed up new DFO projects. ABF should consider using this as a metric to manage and evaluate the progress of DFO projects.

- The ABF has established a carefully organized and well-managed system to seed adoption of ABF capabilities by industry via DFOs. These engagements help prove relevance while generating interest from industry. The cost-share requirement helps secure industry commitment, but it also largely excludes academia. (We later heard about two academic partners having to jump through difficult hoops to be able to participate.) As the structures for executing DFOs mature, it will become increasingly important to evaluate them in terms of appropriateness of the use of DOE funds. The funding strategy should balance three different priority areas: supporting industry, increasing uptake and visibility of ABF capabilities, and developing ABF capabilities in a manner that will be of relevance to others. From the presented projects, the first of these seems to have had the clearest priority. The second priority needs some refinement: Although it is clear that the ABF is helping these partners, it is much less clear what specific DBTL capabilities were deployed, at what scales, and, more importantly, how they may transfer to a new interaction. (Consider, for example, an unrelated company seeking to learn from the interaction.) For the third priority, it was not clear whether it is considered important that these interactions help the ABF build reusable infrastructure that will aid its other partnerships. There should be clear communication of how ABF DBTL infrastructure is boosted by DFOs, beyond simple research collaborations and publications. If ABF DBTL improvement is not a priority of a particular engagement, then it should probably be treated like a contract manufacturing organization arrangement, as ABF is not gaining as much as the company from the DOE funds.

- The goal of DFOs is to develop partnerships between the ABF and industry and nonprofits to (1) accelerate the commercialization of bioproducts and (2) advance the ABF's capabilities. The management of the DFO is well-structured, with a number of systems in place to monitor progress on the different projects, and a well-defined strategy for proposal review and project adjudication. Each project has a technical contact at ABF, which provides clear lines of communication between the partners and ABF. With respect to approach, there is a decrease in the number of applications for 2021 (4x to 2.4x oversubscription). Further, compared to other funding opportunities, DFO has a low oversubscription rate. Broader advertising of DFO or lowering the cost-share requirement would expand the number of projects that come to the ABF for evaluation. In particular, the 20% cost-share requirement may exclude some potential nonprofit partners that may bring new capabilities to ABF. Thus, a tiered cost-share system may be worth exploring. The impact of the DFO is high for both the companies involved, in terms of advancing products to market, and the ABF, in terms of advancing its in-house capabilities. The DFO should strive for a balance between projects that are clearly beneficial to companies, but are run of the mill for the ABF, and projects where the ABF has a significant potential to expand or refine its capabilities. Potentially, the total project award could be tied to the potential of the ABF to expand its capabilities. At this point, with some exceptions, most companies collaborating with ABF are startups or small companies. It may be worth expanding collaborations with companies of different sizes, supply chain location, and industry segments. The progress of the DFO has been substantial, both in terms of advancing companies toward product scale-up and identifying novel opportunities for ABF to tackle.

- The management of this program seemed robust: there was not a ton of attention paid to this in the presentation, but there seemed to be an adequate focus on communication, and there was an emphasis on improving this with each iteration. The approach seemed sound: this appears to be a highly cost-effective way for the ABF consortium to make technical progress. However, the cost-share barrier seemed very

significant for academic and industry participants, and some of the rules on what count as allowable costs were unnecessarily restrictive. The potential impact of the topic areas, including culture heterogeneity, AI-enhanced manufacturing, and new host onboarding are all high. There seemed to be substantial progress across many target areas and hosts for 15 projects, but it was hard to fully evaluate this given the level of detail presented.

- The management structure appears sound. In particular, the clear delineation of responsibilities for key participants and stakeholders, use of program management tracking software, and identification of risks and mitigations is an important foundation for management. The approach used for soliciting, reviewing, choosing, and launching the DFO awards is well conceived. It is commendable and notable that this approach has evolved over time in response to feedback and observations, which is always good to see (rather than an inflexible or dogmatic attachment to the initial approach). One concern is the >three months for the CRADA, NDA, MTA, and safety evaluations; it would be good to try to shorten that, so that the launch of R&D can occur sooner after the awardees are selected. Another thing to consider is whether it makes sense to target a certain ballpark of overapplication, defined as the amount of money or value that the DFO applicants request compared to that awarded ($5–$5.7 M). For example, National Institutes of Health (NIH) Research Project Grant (R01) success rates are around 20%, and while this is too low, it does reflect that NIH grants likely favor the cream of the cream of the crop and can also favor grants that advance their overall strategic vision. If ABF were to solicit and receive more DFO applications from more organizations, would the quality and impact of the work be higher because the committee would need to be more selective? The impact, progress, and outcomes of the DFO awards are a bit hard to judge, because some of the awards are still in the early stages of work and other awardees presented anonymized data or descriptions that were hard to assess rigorously. One thing that the ABF staff should do (if they are not already) is to ask incisive questions of the key staff for each DFO award, and at the end of the award period, judge the impact of the work. In many cases in this relatively nascent industry, we have a hypothesis about the type of approach that will prove fruitful in generating key insights that lead to improvements in TRY to drive the development of manufacturing-ready technology. But these are only hypotheses, and some will be right and others wrong (or not suited to particular problems), even if the proposed work is executed flawlessly with high rigor, rich data sets, and deep analyses. For example, strain performance may fall over time in fermentation, and I may hypothesize that if I measure changes in mRNA abundance or metabolite levels then I will understand the mechanism responsible for the performance drop, and thus be able to solve it. I might then make such measurements, but perhaps they might prove unhelpful in understanding the underlying mechanism and thus not have a positive impact in increasing performance. This is a valuable negative result that should be tracked and used to learn what types of experiments are most valuable in particular circumstances, and perhaps in the future the ABF staff will decide not to pursue such approaches and instead find ones that are more impactful.

- The overall process for DFOs seems to be well refined and working from a programmatic perspective. The critical issue in my opinion is that projects selected need to be rigorously evaluated based on their alignment with the best interest of the ABF mission in mind. It is not that these projects bear a conflict of interest per se; instead, the projects selected could have stronger strategic priorities. Projects should inherently take on more risk to create a higher likelihood that the investment will lead to a step-change of capabilities, instead of incremental improvements. None of the projects evaluated reflect that opportunity for ABF to be impactful. Almost all of the FOA projects presented would be a better fit under the rubric of DFOs because they will be more impactful. Too many of the projects seemed to be more of a fee-for-service model instead of an enabling capability for academia and industry to create that step-change. As

stated on slide 19, the DFO provides a unique opportunity for ABF to get insights into the emergent challenges within industry; however, the process could exploit that opportunity much more than it is currently doing to select more high-risk projects. ABF's DFO investment is working in a unique space that allows it to be attractive and impactful for industry. Reviewers should be challenged to review ABF's mission more closely and select projects that create enabling/disruptive capabilities. The projects reviewed do not solve tough challenges. It was striking that one of the current performers under review, TeselaGen, very clearly stated that the protracted CRADA process was a major hurdle that dissuaded any future collaboration with ABF. It highlights that the CRADA negotiation process still requires improvement, beyond the tremendous accomplishments already achieved.

PI RESPONSE TO REVIEWER COMMENTS

- We agree with the reviewer's assessment that the inherent cost-share requirements of DFOs pose a challenge for academic collaborators. In the four years since its establishment, the ABF has developed collaborations with the University of Georgia, UCSD, University of Washington, UC Berkeley, University of Delaware, and Washington University in St. Louis. However, many academic groups do not have significant nonfederal cost share to offer DFO-based project proposals, which puts them at a distinct disadvantage and prevents the ABF from more frequently engaging in strategically meaningful academic collaborations, especially by way of the DFO. As a partial response to this dilemma, the ABF is working to establish an interagency collaboration with the National Science Foundation, to set up a funding opportunity that would allow for applicants to utilize their funds as part of their cost-share requirement, opening the door for greater academic engagement.

- We agree with the reviewer's assessment that the goals of DFO projects, from the ABF perspective, should balance industry support with growth of ABF knowledge and capabilities, so that collaborators advance their technology and ABF applies learnings and new capabilities in ways that benefit future projects and the biomanufacturing community as a whole. This requires careful consideration for the rights of outside parties engaging in CRADA agreements and the resulting ways in which the ABF may negotiate the use of intellectual property and proprietary information arising from such projects. In keeping with the reviewer's observation, the support-of-industry part of the equation arguably received greater emphasis in the ABF's initial DFOs, while the ABF built its visibility in the community, the third facet the reviewer describes above. But these collaborations have indeed offered value to the ABF, beyond visibility. As just one example, the ABF has benefited from the onboarding of new hosts, dramatically expanding its library of microbial hosts, which are now much more poised for other genetic engineering. Perhaps more to the reviewers' broader point, the focus of the presentation was more squarely on the unique value that the ABF has immediately offered its DFO collaborators, and less so on the corollary of those relationships. While the ABF evaluates its prospective DFO relationships for their internal strategic value too, that aspect could have been better communicated during the "Intro to DFOs" discussion.

- The ABF will take the reviewer's recommendation to more fully communicate all aspects of these relationships in future presentations. We agree with the reviewer's assessment regarding the large number and diverse nature of the industrial applicants as a reflection of the ABF's relevance. The ABF's capital-intensive operations and rare expertise needed to make use of such capabilities have been a driver in ABF's ability to serve the community as fully and immediately as it has in such a short period of time. This is true not only for omics tools and onboarded strains, but for every leg of the DBTL cycle, along with various process integration capabilities (e.g., scale-up and TEA/LCA). Meanwhile, as the reviewer

highlights, the ABF has strategically developed other core technologies, beachheads in this case, in order to dramatically cut DBTLs for target molecules that depend on flux through the cognate pathways.

- The ABF looks forward to testing the beachhead approach through future collaborations. We will continually and carefully evaluate this strategy, and its execution, but we predict it will prove to be a valuable offering to the biomanufacturing community. The prior reviewer made a similar and fair point, regarding unclear articulation of the benefit of DFOs for the ABF. Excerpting from above, "While the ABF evaluates its prospective DFO relationships for their internal strategic value too, that aspect could have been better communicated during the 'Intro to DFOs' discussion. The ABF will take the reviewer's recommendation to more fully communicate all aspects of these relationships in future presentations." Additionally, in reference to this reviewer's suggestion of a metric, the ABF will take this under advisement as an additional means of evaluating its DFO investments, and we thank the reviewer for the suggestion. The ABF is attuned to the time-sensitive nature of business and how that can conflict with the process of government contracting. We work with our contracting officers, tech transfer experts, IP attorneys, and other stakeholders from the ABF consortium national laboratories so that the contracting process may move as swiftly as possible. Advancements such as the non-negotiable CRADA and a recently established blanket NDA are part of an overall strategy of shaving time off the process.

- A reviewer above echoed the sentiments for the inherent difficulties for academic labs to meet cost share. Please refer to our first response about the collaborations with academic institutions.

- The academic community is a key driver in the development of new bioproduct technologies; the ABF is keenly aware of the importance of these collaborations and is working with agencies and internal stakeholders to grow the ABF's academic relationships. Demonstrating cost share, however, is arguably not difficult and an area where there is considerable guidance from DOE and the code of federal regulations: see (1) recent DOE FOA, and (2) DOE's Guide to Financial Assistance. The ABF strives to provide a straightforward process via a simple Google form, for a cost-share declaration that aligns with DOE guidelines and requirements but does so in a process-economical fashion. We thank the reviewer for the comments and appreciate the assessment of the ABF's approach to managing the DFO process. The ABF is committed to continually improving this process and avoiding adherence to approaches that are less effective or fail to change with evolving circumstances. One vision would be to have the agreement process collapse to the time required to read and sign each agreement. To that end, the ABF has recently crafted a blanket NDA, pre-signed by the seven national labs that constitute the ABF consortium. The team is continuing to consider other ways to shorten this shared process and streamline related but independent internal processes (e.g., material transfer agreements). Comparing the ABF DFO to the NIH's funding machinery is fraught with incongruities, though the point is still well taken. For the three DFO funding cycles (FY 2017, FY 2020, and FY 2021), the ABF has enjoyed an average oversubscription of about three-fold, so with some minor adjustments in the early stages of the timeline, a five-fold oversubscription (i.e., a 20% funding line) is certainly feasible, and we would welcome that scenario. Our ardent outreach has thus far resulted in a level of response that arguably reflects the newness of the organization, the relatively narrow scope of the funding opportunity, and of course, the self-limiting cost-share requirements.

- To that last point, the FY 2021 DFO received 22 initial abstracts. Those whittled down to 17 applications, largely as a function of drop-off by academic groups who couldn't define a path to covering the cost share. Had all applied, the ABF may indeed have hit a crème de la crème 20% funding line, such as what the reviewer describes. As noted in the presentation, the ABF is identifying paths to augment its ability to collaborate with academic groups, to deepen and enrich the pool of collaborations overall. The ABF appreciates the reviewer's remarks on the value of negative data, and as projects conclude, will work to systematically catalogue such information, for precisely the purpose the reviewer describes. The ABF appreciates the reviewer's comments on the successful facets of the ABF's approach to its DFO, and we acknowledge that success is a moving target that requires continual assessment and recalibration. Earlier responses to reviewer comments allude to the inherent barriers posed by cost-share requirements, 20% being a common DOE figure. As was presented, the ABF is seeking paths to reduce that barrier, for instance through collaborative funding opportunities with the National Science Foundation. However, DOE allowable costs are arguably not restrictive, and as noted previously, this is an area where there is considerable guidance from DOE and the code of federal regulations.

- We thank the reviewer for the comments regarding DFO proposal and project management. It is unclear as to which "other funding opportunities" the reviewer is referencing, in comparing and critiquing the DFO oversubscription rate. However, as our responses to previous comments describe, the 20% cost share is an inherent barrier that does certainly dampen the number of academic applicants (see previous comments). The suggestion of a tiered cost-share system is intriguing, and the ABF will broach this idea with BETO. Regarding collaborator benefit versus ABF benefit, the ABF strives to develop mutually beneficial collaborations with all of its DFO-based projects. Admittedly, and as stated in earlier responses to reviewer comments, the presentation should have been more explicit in addressing the benefit that the ABF itself aims to reap from each project. The reviewer accurately observed that most, but certainly not all, DFO collaborators are small companies or startups. The continued success of the DFO may itself generate a larger and more diverse pool of applicants, as knowledge of and demand for the DFO grows year-to-year.

- The ABF's IEO team will review the DFO outreach strategy and consider paths and strategic costs and benefits of expanding collaborations to include companies of different sizes, supply chain location, industry segments, and other considerations. The ABF thanks the reviewer for the comments and agrees that it is important for the ABF strike a balance between (1) supporting collaborators in a manner that is otherwise unavailable in the private sector and (2) gaining learning and technologies from each project that are applicable to future projects, for the broader benefit of the industry. The reviewer suggests that the ABF cultivate more high-risk/high-reward projects that create enabling/disruptive capabilities and that, in the reviewer's opinion, the projects reviewed do not solve tough challenges. Would-be collaborators come to the ABF for enabling technologies and expertise that are unavailable in the private sector. Further, there is often an incremental quality to the DBTL cycle and its iterative processes, a core operational feature of the ABF, which thus far is serving the ABF mission well.

- Obtaining step-function improvements where possible, say through the pursuit of nonrational AI-based hypotheses, is just one area where the ABF offers cutting-edge capabilities. Similarly, the onboarding of noncanonical hosts is enabling genetic engineering for new realms in biomanufacturing; industry demand itself is helping define the direction of this effort. As stewards of public dollars and purveyors of industry-defined projects, it is important for the ABF to balance project risk with reward. However, to acknowledge the reviewer's suggestion, the ABF will internally review its strategic approach to risk within the DFO application review and selection process, so that the ABF may best serve the

biomanufacturing community at large. As previous responses mentioned, the CRADA contracting process is part and parcel of working with the federally funded research and development centers. This is an area where the ABF continually strives to coordinate and streamline, across the ABF's seven national laboratories. Although the contracting process is more time-consuming than we want, multiple groups have repeatedly applied to the ABF DFO, pointing to the net positive value it offers for many. However, as it has done for every DFO, the ABF will execute its future DFO planning with an eye toward shorter paths to contracting completion.

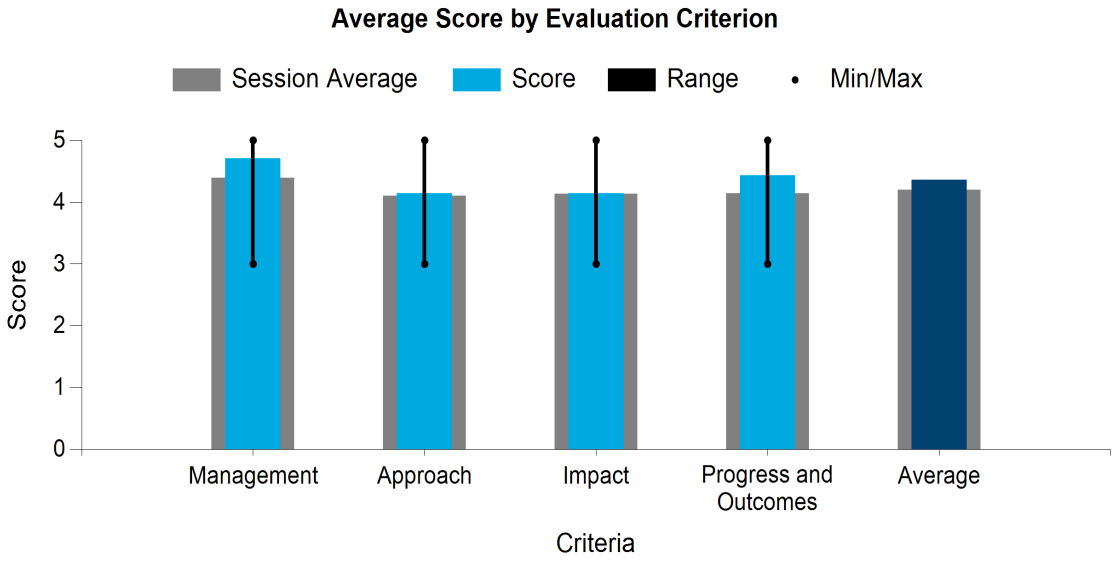
ABF Industry Engagement Lab Call—Lygos

Lygos

PROJECT DESCRIPTION

WBS:	ABF6
Presenter(s):	Andrew Conley
Project Start Date:	04/01/2018
Planned Project End Date:	01/08/2021
Total DOE Funding:	$1,428,472

The ABF is a flexible platform that can adjust to the needs of its partners, thus enabling them to rapidly develop and optimize the production of a wide range of bioproducts into the market. To demonstrate this capability, three ABF labs (National Technology and Engineering Solutions of Sandia, LLC; LBNL; and PNNL) partnered with the biomanufacturer Lygos to rapidly develop a microbial host for the production of high-value organic acid. Lygos uses *Pichia kudriavzevii*, an acid-tolerant BSL-1 yeast, to produce organic acids, chemicals that are generally expensive to manufacture petrochemically but that can be produced at high yields and for low cost biologically. While *P. kudriavzevii's* general robustness provides an advantage over more traditional biomanufacturing strains like *E. coli* and *S. cerevisiae*, working with *P. kudriavzevii* presents unique challenges. Lygos' experience working with this nonconventional yeast is extensive. However, there remained room to improve the engineering cycle, providing an opportunity to partner with the ABF to further advance this microorganism. The high-value organic acid chosen to demonstrate the beneficial collaboration between Lygos and the ABF was isobutyric acid, a useful platform chemical that can be converted to methyl methacrylate (aka Plexiglas, a large market material). Over the course of this project, the ABF's DBTL tools and technologies were used to diagnose and optimize many pathway bottlenecks in a high-throughput manner, resulting in a rewired host organism with improved production of the isobutryic acid bioproduct. At the end of this project, the ABF and Lygos identified a strain and fermentation conditions that produced ~20-fold more isobutyric acid using lignocellulosic feedstocks compared to the original prototype strain. These results showcase the successful cooperation between the ABF and Lygos to produce isobutyric acid in *P. kudriavzevii*.



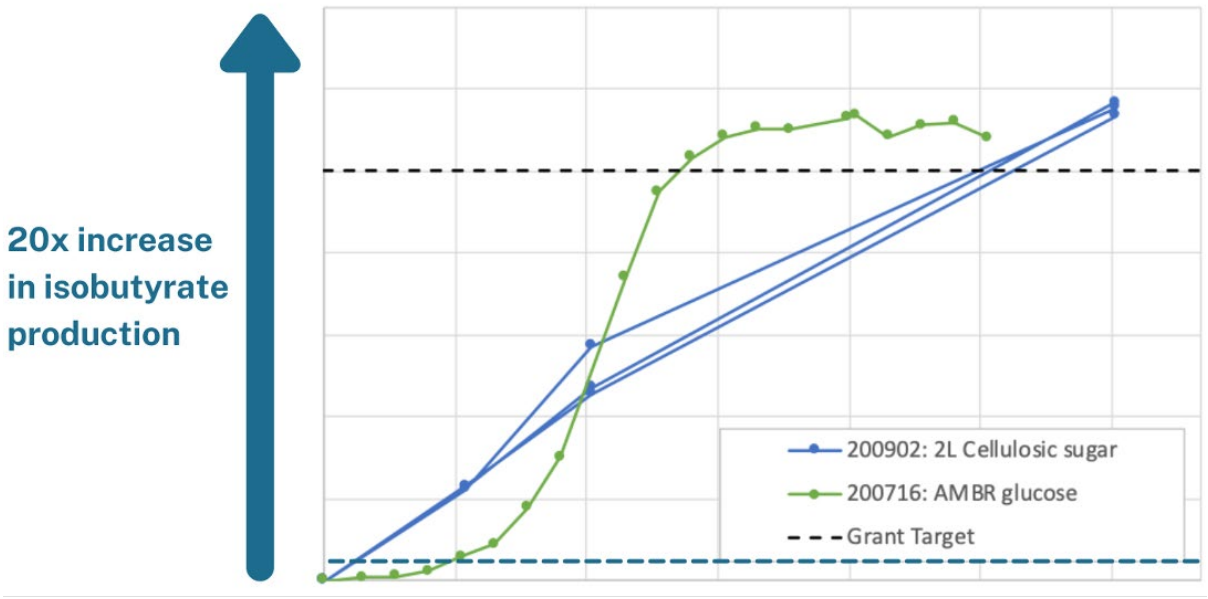


Photo courtesy of Lygos

COMMENTS

- As stated in the slide, this was an early DFO project that "Provided an opportunity to kick the tires of the ABF's DBTL technologies in collaboration with its industrial partner Lygos." Overall, it was highly successful at this goal and provided gain to both ABF and Lygos. As this project is finished, and ABF has refined their priorities and vision going forward, it is not that relevant to review in detail, as there is nothing to change. Overall, this project was successful, well managed, and met milestones. As discussed in other reviews, ABF/BETO needs to reevaluate the necessity to require industry partners to use hydrolysate feedstocks. This is not relevant or useful to industry. Industry use of hydrolysate feedstocks is at least a decade away. Requiring this work diverts resources that can be used to make progress on relevant industrial processes. Also, making a strain to use hydrolysate is very likely to move a strain into a certain metabolic state that will negatively impact TRY on traditional sugars. As a compromise, let industry use standard sugars, but require them to test hydrolysate to benchmark performance to understand the impact on pathway and TRY metrics. If the industrial microbe is theoretically able to use biomass sugars, then ABF can further develop that microbe on their own funding for better usage of hydrolysate (but not part of industry partnered DFO). Future DFO's should have an emphasis on (1) beachhead/host development, (2) how many DBTL cycles were completed, (3) showing progress on DBTL cycle time reduction, and (4) clear metrics on host technology tier level progress or tier increases.

- Strengths: All proposed tasks have been completed. Final product titer reached the grant target. The project clearly demonstrated the use of ABF's capability to complete the DBTL, which otherwise might be difficult for Lygos to complete by itself. This project onboarded a yeast strain to ABF's list, although the amount of genetic tools is not at the level with other ABF strains.

- Weaknesses/areas for improvement: The DBTL cycle of this project is longer than other projects. As a result, only a few DBTL cycles were completed. Besides onboarding a strain, it is not clear what other capability this project has added to ABF. The benefit of this project to ABF is not high considering its budget size.

- The goal of this project is to apply ABF DBTL cycles to engineer *P. kudriavzevii* for the production of isobutyric acid, a precursor to methyl methacrylate. The project has clear lines of communication between Lygos and ABF participants. The approach involves strain engineering at Lygos and strain design and testing (scale-up, omics) at the ABF. The impact to Lygos is high, as the ABF provides capabilities (omics, scale-up) that are not present at a small company, and in this sense, the project fits within the ABF's mission to reduce the time to bioprocess scale-up. However, the impact of the project to the ABF is medium. The project is too small-scale to significantly push the ABF's capabilities (e.g., only 15 enzymes in the homology screen provide limited data for the learn component of the DBTL cycle). Further, while Lygos is providing expertise in working with *P. kudriavzevii,* ABF is onboarding this organism without clear delineation of whether the organism fits within ABF's strategic plan for organism onboarding and development (physiology, metabolism, phylogeny). Progress on this project is high, greatly outperforming the originally set metrics for isobutyric acid production.

- The management of this program was strong based on the sensible distribution of responsibilities across participants, the communication between participants, and the residency of scientists at different participant labs (i.e., a Sandia person was put at Lygos). The approach was smart: The selection of a *P. kudriavzevii* strain that accumulates a precursor while minimizing biomass, the initial focus on three enzymes and expansion to five based on progress, and the full utilization of the expertise of each participant were all compelling. The impact is high: *P. kudriavzevii* strains are of high potential commercial relevance, especially for replacing petrochemical-derived products. The progress was great, with several DBTL cycles completed and a 20-fold increase in isobutyric acid production in under two years. This was an excellent example of ABF advancing a commercial objective that may not have otherwise been possible given the capital and equipment investment required to complete this work.

- The management structure described seems well suited to the need. The approach is solid, but not particularly innovative; the greatest innovation in the project from the perspective of the ABF (it seems to this reviewer) is the onboarding of a new host species (*P. kudriavzevii*), which is beneficial for the ABF to achieve its numerical targets for host strains, and *P. kudriavzevii's* special, potentially beneficial attributes are nicely complementary to the other strains in the ABF stable. However, applying these generalizable omics approaches to a new species and target product is welcome and provides a further successful test of the hypothesis that such omics can help identify bottlenecks and guide efforts to resolve the bottlenecks. The impact is quite favorable in the sense that Lygos has been talking with outside partners who appear to be interested in helping with scale-up and commercialization of isobutyric acid, and the results from the ABF work were described as sufficiently promising for Lygos to continue the technology optimization after the conclusion of the grant, including >20-fold increase in production. Another favorable outcome is that it was stated that the ABF proteomics and metabolomics identified bottlenecks in the pathway, and subsequently they were able to resolve those bottlenecks. However, with respect to impact, two things are not clear. First, it is not clear how close the technology is, at the end of the funding period, to what is needed for commercialization. Second, while replacing environmentally damaging and hazardous waste-producing petrochemical processes with a bio-based process is welcome, the other justification on the basis of sustainability was not well elucidated; it was said that the current process uses "unsustainable petroleum feedstocks," but it wasn't stated how long the petroleum feedstocks may last into the future, and what the environmental footprint may be of the bio-based process. The project seems to have completed all of its objectives on the original (revised) timeline.

- The project is a good exercise for ABF to test, learn, and refine its current pathway development platform and collaboration across ABF. The project is only adding limited value to the ABF capabilities. If the ABF wants to reach its goal of achieving 50% reductions in time to bioprocess scale-up, this project is not the project that helps move the needle in the correct direction to meet that goal. There are no measurable improvements to the ABF DBTL cycle, and the onboarded chassis is not necessarily a better strain than others to handle organic acids. Looking beyond yeast strains will make ABF's HObT program even more valuable.

- Through this DFO, Lygos was able to leverage the ABF's capacities for multi-omics analysis and DNA assembly to improve the economic viability of isobutyric acid production. Significantly, based on the successes of this project, Lygos intends to continue to pursue commercialization of this product. In this respect, this project is a clear win for the DFO program. In terms of the ABF activities articulated in the presentation, it appears that ABF assembled a few dozen vectors, performed multi-omics analysis on one baseline production strain, and contributed expertise for metabolic engineering and fermentation optimization. It is assumed that other ABF contributions not described account for the remainder of the considerable DOE funding for this project. From a HOD perspective, it is fantastic that ABF staff were able to receive training on a new host at Lygos. However, to ensure continuity and uptake, it would be preferable to have the capability for *Pichia* engineering move in-house as a specific milestone for the project.

PI RESPONSE TO REVIEWER COMMENTS

- The project team is appreciative of all of the constructive feedback received from the reviewers and will consider this feedback in future ABF industry projects. As an update to this project, the on-boarding of *Pichia* strain engineering and fermentation has now been moved in-house at the ABF.

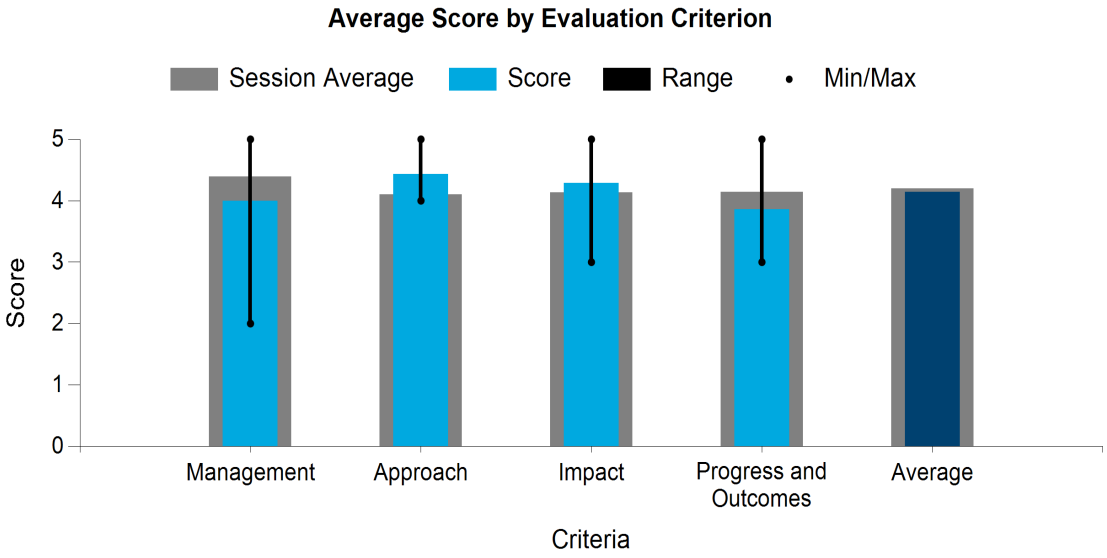
ABF Industry Engagement Lab Call—Kiverdi

Kiverdi

PROJECT DESCRIPTION

WBS:	ABF7
Presenter(s):	John Reed; Dan Robertson
Project Start Date:	07/24/2017
Planned Project End Date:	09/30/2021
Total DOE Funding:	$1,285,700

The objectives of this project are to improve transformation in *Cupriavidus necator*, develop CRISPR/Cas for *C. necator* genome editing, systematically screen RBSs for *C. necator*, develop and initially optimize a heterologous 1-dodecanol pathway in *C. necator*, and perform fermentation of *C. necator* under chemolithoautotrophic conditions. The project will support the ABF's goal to translate new IP and manufacturing technologies to U.S. industry to ensure market transformation, add a new host organism to ABF portfolio, and contribute to the identification of underperforming ABF unit operations.



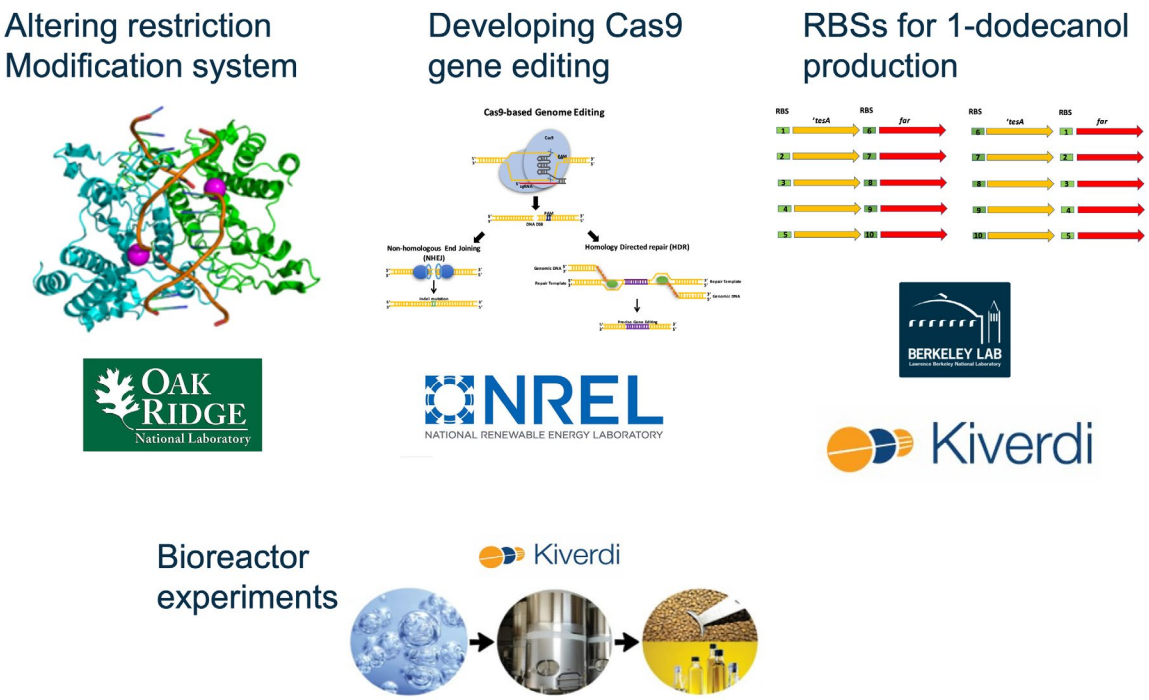


Photo courtesy of Kiverdi

COMMENTS

- This is a good project that increases the ABF's capabilities; it looks to enhance the ABF's strain repertoire, adding a good microbial chassis. Project adds value to the DBTL cycle of ABF by testing various tools and approaches by the ABF team. The struggle is for ABF not to continue to be a fee-for-service organization while only providing incremental improvements of their own technology development process relevant to the mission of ABF.

- Strengths: Good progress was made on genetic tool development. The project is on track to complete the proposed milestones. This project efficiently used ABF's capability in onboarding new strains.

- Weaknesses/areas for improvement: So far, Kiverdi's contribution to this project is limited. The first DBTL cycle has not been completed. This probably reflects a problem with the structure of project: The Kiverdi team has to wait for the genetic tools to be developed to start their project. Given this problem, it is unlikely for this project to perform multiple DBTL cycles to obtain high TRY metrics. It may just end and produce some C12 alcohol in their organism. The overall impact of this project is probably limited.

- The goal of this project with Kiverdi is to develop *Cupriavidus necator* as a host for the upgrading of knallgas to chemicals. This is primarily a synthetic biology tools development project. The tasks performed by each team member are clearly defined. It is unclear what Kiverdi's contribution to the project is, apart from microbial fermentation, as the ABF is conducting the host onboarding, tools development, and strain engineering. Progress on this project has been good from the ABF side, but the tasks led by Kiverdi have not been started yet. The impact of the project is medium. The impact for Kiverdi is to more rapidly engineer *C. necator* to produce dodecanol from knallgas. The impact for the

ABF is less clear. While Kiverdi is providing expertise in working with *C. necator*, ABF is onboarding this organism without clear delineation of whether the organism fits within ABF's strategic plan for organism onboarding and development (physiology, metabolism, phylogeny). Nor is it clear whether other companies, in addition to Kiverdi, are likely to use the tools developed for this organism or whether *C. necator* is the best organism to onboard and develop for knallgas biotransformation. Answers to these questions will address the impact of this project to the general bioindustrial field.

- The management of the program was marginal; it seems that COVID significantly disrupted timelines, as expected, but that this compounded other issues with changing scope. Estimates on the number of DBTL cycles possible in the remainder of the program seemed greatly off. The planned approach seemed good, in that it addressed several foundation gaps simultaneously, including transformation efficiency and CRISPR systems. The potential impact is high: Direct conversion of CO_2 would be a transformative step for making our economy circular. Without seeing specific results, it was hard to assess progress, but it seemed like they met several of their goals, though on an extended timeline.

- The management plan seems generally good, although perhaps it would be better to have more frequent check-ins than the quarterly updates described in slide 7, because there can be many changes and risks that need to be mitigated that arise within a quarter. Also, from the management perspective, it was unfortunate that staffing was not in place for all of the tasks, which led to a delay in Task 2 (slide 13). The approach is innovative and nicely complementary to other activities in ABF, with *C. necator* providing an exciting opportunity to make products from knallgas. It is a win-win situation for the ABF to onboard a potentially useful new strain and for Kiverdi to accelerate development of the genetic tractability of this species. The potential impact is very high, both from a greenhouse gas perspective and from the perspective of Kiverdi having a strategic partnership in place to commercialize the fatty alcohols with a world leader in surfactants (slide 9). Given the results shown, there are many exciting achievements demonstrated; however, the project seems to have a long way to go to complete the remainder of its tasks, and thus is at risk of being delayed or incomplete. For example, there were a few tasks that were 10%–50% complete as of 3/8/21, but were targeted for completion on 3/31/21. In addition, Tasks 5 and 6 are very exciting and ambitious, but given the percent finished to date, may be at risk of not being successfully completed by the end of June or September, respectively.

- This project is a good demonstration of how the ABF's technologies and funding mechanisms can support small businesses who lack access to critical technologies. In particular, this collaboration has produced new genetic manipulation tools for *C. necator*, an interesting C1 chassis, and can already claim several victories via screening of 10 integrases, 18 promoters, 14 ribosomal binding sites, and a T7-polymerase-based inducible system. The project is experiencing a few delays due to the pandemic, but these are understandable. Overall, these new genetic tools will certainly increase the viability of using *C. necator* as a production host. From the Kiverdi perspective, a key outstanding question is whether these improvements will lead to improvements of commercial relevance. To this end, it appears that a large number of expression variants of two pathway genes have been prepared and are awaiting testing. From the ABF perspective, the progress was good, but hard to distinguish from standard low-throughput metabolic engineering that could be accessed via a regular academic collaboration. Based on the power of ABF's DIVA system, one could imagine ABF testing hundreds of integrases, promoters, and RBSs, as appropriate.

- This was an early DFO project that provided a very good opportunity to develop one of the new host strains on ABF's list as well as help Kiverdi advance their technologies to produce a relevant molecule

for the bioeconomy. Although not completed, it is on track to achieve close to, if not all, of the milestones. Overall, it has been highly successful and is a gain to both ABF and Kiverdi. It was nice to see a project use a non-sugar feedstock and really develop that technology. More and more, it is looking like converting biomass to gasses as feedstocks is going to be more economically viable than enzymatic hydrolysate feedstock. These types of projects should be sought after for future funding. In the future, DFOs and their reviews should have an emphasis on (1) beachhead/host development, (2) how many DBTL cycles were completed, (3) showing progress on DBTL cycle time reduction, and (4) clear metrics on host technology tier level progress or tier increases. This project did show host-onboarding progress, progress on DBTL cycle time reduction, and clear metrics on host technology tier level progress or tier increases.

PI RESPONSE TO REVIEWER COMMENTS

- We would like to thank the reviewers for their constructive comments. We regret the delays caused by COVID lockdown of labs, but with the approved extension of the project to 9/30/21, we plan to achieve the amended project goals.

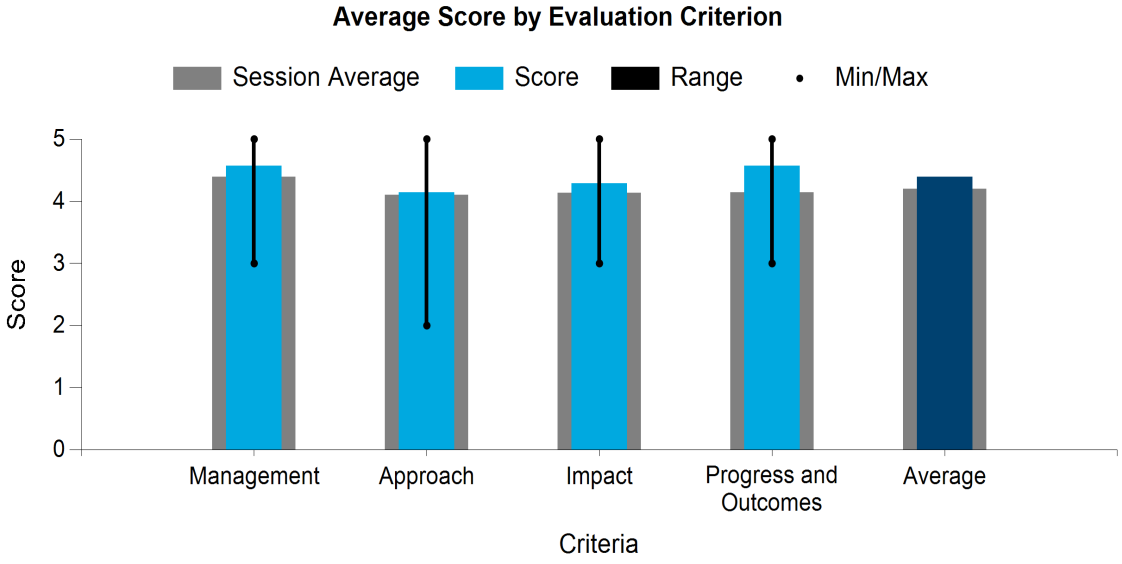
ABF Industry Engagement Lab Call—Agilent

Agilent

PROJECT DESCRIPTION

WBS:	ABF8
Presenter(s):	Alex Apffel
Project Start Date:	9/30/2019
Planned Project End Date:	10/29/2021
Total DOE Funding:	$689,835

Elucidating multiple types of information about engineering biosynthetic pathways and the host biological processes is necessary to generate groundbreaking insights critical to driving bio-based chemical optimization processes. Significant advances in synthetic biology, genome editing, and DNA synthesis capabilities have propelled the ability to design and construct novel strains for biomanufacturing research. Generating thousands of unique strains for a given target molecule covering multiple routes, tuned protein expression, and targeted genome modification is now routine. Yet, analytical tools to match this throughput have lagged far behind. There is great need for high-throughput analytical workflows that reduce time and resource needs to enable synthetic biology research at the ABF. Agilent Technologies' developments in high-throughput liquid chromatography-mass spectrometry methods coupled with advanced software to predict which methods will be successful offer valuable components for these types of workflows. And, interfacing these technologies with ABF mass spectrometers, software (i.e., EDD), and workflows for strain design and construction would have significant impact on foundry operations, strain development processes, and mathematical modeling efforts.



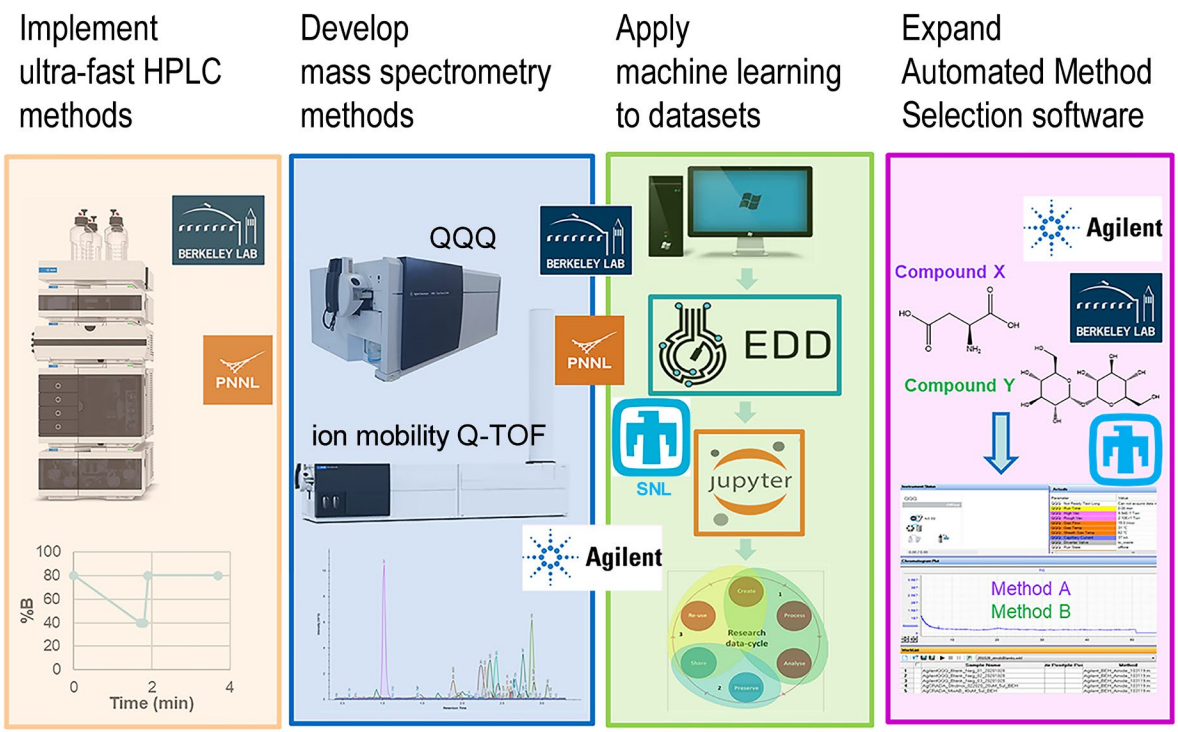


Photo courtesy of Agilent

COMMENTS

- I found this program difficult to assess based on the level of detail provided during the presentation, which felt to me to be filled with broad, vague statements. For management, it seemed that the participants were each able to contribute to meet goals and that the planning of the program was successful. For approach, the technical details seemed fine, but it was not evident to me that efforts to maximize the collaboration were made; it seemed like the labs gave Agilent data and some assistance on improving their machine learning, but that there was not a lot of back and forth. I think the impact level is moderate: Automated method selection capabilities would enhance some programs, but I think the level that this would influence industry is lower than the other DFO projects. The goals seemed to have all been completed and on time.

- Strengths: The high-throughput metabolite analytical resource is needed widely by the biomanufacturing community, and thus will provide value in metabolic engineering. This project will advance ABF's metabolite characterization capability and improve machine learning prediction. The project has been making the planned progress. Weakness/area for improvement: None.

- The goal of this project is to combine Agilent's ultra-performance liquid chromatography/mass spectrometry (UPLC/MS) capabilities with the ABF's machine learning expertise to develop algorithms to predict UPLC/MS methods to test the microbial production of various chemicals. The management of this project has clear lines of communication between Agilent and ABF. The overall impact of this project is to reduce the UPLC/MS method development time, thus accelerating the testing step in the DBTL cycle. The impact of this project to Agilent is high, as the predictive UPLC/MS method tools can

be integrated with its commercial software. Agilent can then make these tools available to their customers in the wider bioindustrial community. As part of this project, ABF is honing its machine learning capabilities. The approach to the project is well reasoned. Ideally, ABF could quantify the reduction in the DBTL cycle time enabled by this UPLC/MS method prediction software. The progress of this project has been good, with the system able to predict methods not only for model compounds but also for real samples. Of note, this is the only collaboration between ABF and a large-sized company, and lessons learned from this interaction could potentially increase ABF collaborations with other large-sized companies.

- The management structure seems sound overall, with biweekly meetings and distributed work. The approach described will advance the state of the art, relative to BETO goals, and will be generally useful if the method predictor can be demonstrated to function well. While this is an important advancement, it is not clear how innovative this will be and how significant its potential is. Also, with regard to approach and impact, it was not clear how the team is planning to investigate the accuracy, precision, or reproducibility of the actual measurements (for example, did a time course of samples from the same fermentation give sensible trends?). Moreover, it was not clear to what extent this proposed new technology will address a stated goal of increasing sample measurement throughput and decreasing development time. The progress and outcomes are mixed. While the team made good progress as it transferred the methods, made 116 measurements, addressed some gaps in data processing and disambiguation of charge states, and created an automated method selection tool, they are somewhat (at least 1.5 weeks) behind schedule on testing whether the most innovative goal was met. What is the extent to which their initial automated method selection tool can accurately predict the best method to use for a given novel metabolite? It will be very interesting to see how good the initial tool is, and the how much the team can improve it before the final milestone deadline at the end of June.

- The need for ultra-high-throughput screening is a must-have in the world of biotech/synthetic bio. Metabolomics is an especially problematic field to tackle, and yet it is so valuable for debottlenecking strains and making rapid strain advances. This is a fantastic collaboration example for the ABF. As stated, this work grew out of the initial collaboration with Amyris and Agilent in an earlier Defense Advanced Research Projects Agency (DARPA)-funded project. One of the main barriers to implement the work developed under that partnership was the software and user interface. One of the big gains in this DFO appears to be the use of ABF's EDD. As stated: EDD is a publicly available online tool designed as a repository of experimental data and metadata. EDD can uptake experimental data, provide visualization of these data, and produce downloadable data in several standard output formats. The latter outcome is of huge value to the synthetic bio industry. As Agilent stated, what was so positive about this partnership was bringing real-world problems and data sets for them to test and develop their systems. Again, a great example of partnerships that ABF should be looking toward in future DFOs. There was a question if Agilent could do 10,000 samples a day. The answer was no, and even 1,000 a day was hard but doable. It's not clear what project/company could even use/need 10,000 samples a day on a consistent basis. I'd agree with that. 1,000 a day seems like a very good reasonable goal for now. Machine learning and data analysis will need to seriously increase by orders of magnitude in order to be able to process and make sense of 10,000 data points a day. Even 1,000 data points a day, right now, is too much to handle with current learn technology. ABF should continue to invest in learn to process the data generated in test for omics. In the next review, I would like to see more information on DBTL cycle time and progress on cycle time reduction.

- This project differs from the others in that it utilizes ABF's expertise to enable an instrument vendor to develop and validate a new test technique. Here, Agilent seeks to merge multiple mass spectroscopy platforms with machine learning to enable profiling of samples at scales of thousands a day. The approach is also nicely bridged into ABF's EDD platform. Although fast test with some intrinsic desirability, it was not entirely clear how these developments will translate. First, we are still awaiting key results to validate utility of the key machine learning component, which aims to automate target-dependent method selection. Second, because the approach depends on multiple high-end instruments, the number of entities outside of the ABF that would be able to utilize it is likely small. Last, it was not made clear how this approach can be made to match up with ABF's throughput on DNA assembly or strain construction, which is currently far from generating thousands of samples a day.

- This project is a good example of how ABF should leverage partnerships to expand current capabilities and leverage unique insights into industry technology development to better DBTL processes. The benefits for this project are clearly defined and lay out a good strategy for how the work will deliver greater analytical capabilities to ABF, which benefits industry partners as a whole. The project is focused on the overall mission of ABF to reduce processing time and increase productivity. The collaboration shows how the industry partnership is creating a workflow in coordination with ABF laboratories, which also provides a clear path for how this work will improve product development. Overall, it is a very good collaboration, with clear benefits to ABF and industry at large.

PI RESPONSE TO REVIEWER COMMENTS

- Response to reviewer 1: Three clarifications: (1) The current workflows implemented at PNNL and LBNL are not approaching the throughput capacity potential of the approach. At this point, throughput is not limited by the UPLC-MS technology, but by the demands of the strain production.
(2) The current workflows have been designed and demonstrated on three separate MS platforms (triple quadrupole, quadrupole time-of-flight (QTOF), and ion mobility-QTOF). However, they are not all required; any one alone will produce actionable data. (3) Machine learning models have been demonstrated based on existing data that have a 70%–80% accuracy rate. As additional data are gathered and new, improved machine learning models are developed and refined, this will only improve.

- Response to reviewer 2: We appreciate the supportive comments.

- Response to reviewer 3: The current workflows implemented at PNNL and LBNL are not approaching the throughput capacity potential of the approach. In a related project, a throughput of 1,000 samples/day has been demonstrated. At this point, throughput is not limited by the LC-MS technology, but by the demands of the strain production.

- Response to reviewer 4: Method selection based on preliminary machine learning models has been demonstrated using existing data that yield a 70%–80% accuracy rate. As additional data are gathered and new, and improved machine learning models are developed and refined for our final milestone, this will only improve. The ABF test mass spectrometry-based omics workflows utilize technical and biological replicates for quality control measures and statistics. Likewise, the accuracy, precision, and reproducibly of measurements from these new metabolomic workflows are evaluated with technical replicates (repeated measurements of the same sample) and biological replicates (parallel measurements of distinct samples) across time course experiments for different host organisms.

- Response to reviewer 5: Unfortunately, it appears the close and ongoing collaboration between Agilent and PNNL/LBNL/Sandia was not sufficiently communicated. To clarify, semiweekly meetings were held during the initial development stages. Analytical methods were transferred from Agilent to the ABF test/learn partners at PNNL, LBNL, and Sandia. As implementation and testing progressed, frequent interaction took place to refine and troubleshoot methodologies. Initial machine learning models were implemented at Agilent using PNNL omics data, and current work is underway with machine learning models being developed and refined at Sandia, so the collaboration has been very active and in both directions.

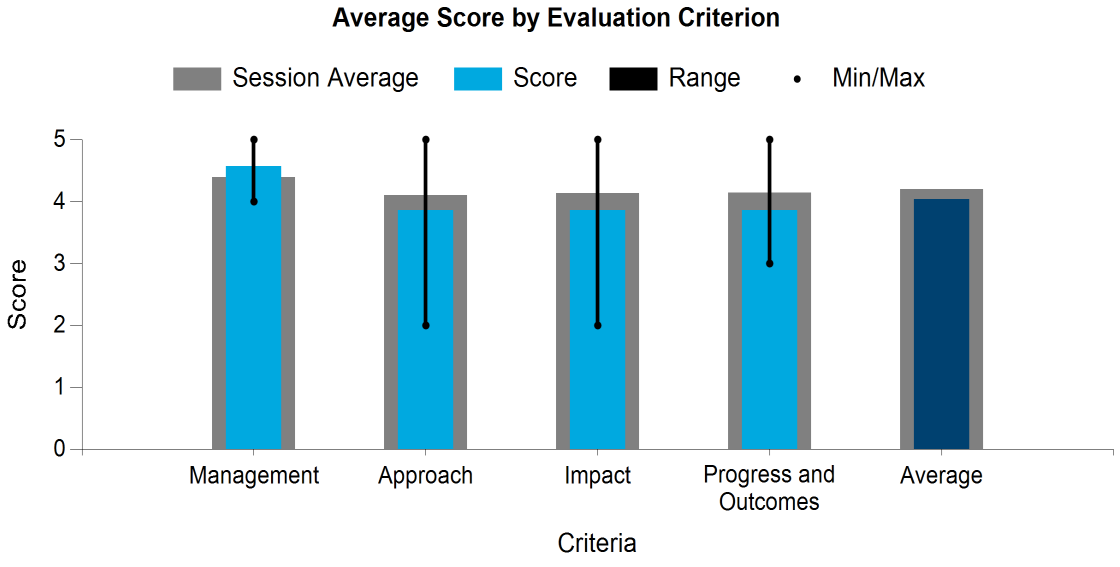
ABF Industry Engagement Lab Call—TeselaGen

TeselaGen

PROJECT DESCRIPTION

WBS:	ABF9
Presenter(s):	Mike Fero
Project Start Date:	06/29/2018
Planned Project End Date:	07/10/2021
Total DOE Funding:	$1,142,858

Companies are facing enormous challenges accelerating their synthetic biology efforts. Computer-aided design and manufacturing are taking an ever-increasing role in the forward engineering of biology. Efforts to close the design-build-test loop while incorporating a machine learning-based "learn" step require better data management and control. Experience in both academic and industrial biofoundries has shown a real need for a much better approach to fighting "data-dispersal," where valuable data are generated on a plethora of laboratory devices without much attention to bringing the data together into an organized cohesive whole. TeselaGen Biotechnology is a synthetic biology software company that has developed a powerful, AI-driven, cloud-based, computer-aided design and build platform for accelerating synthetic biology. TeselaGen's platform is well suited for working with a diverse set of bacterial, fungal, and plant genomes. We plan to partner with the ABF, including staff at LBNL, Sandia, and PNNL to develop new bioinformatic capabilities that will enhance various tools developed at the national labs (e.g., BOOST, BLiSS, EDD). These developments will advance all aspects of the DBTL biomanufacturing cycle. We plan to integrate these new enhancements into TeselaGen's platform and evaluate emerging TeselaGen functionality against ABF workflows in the interest of significantly increasing ABF operational efficiency.



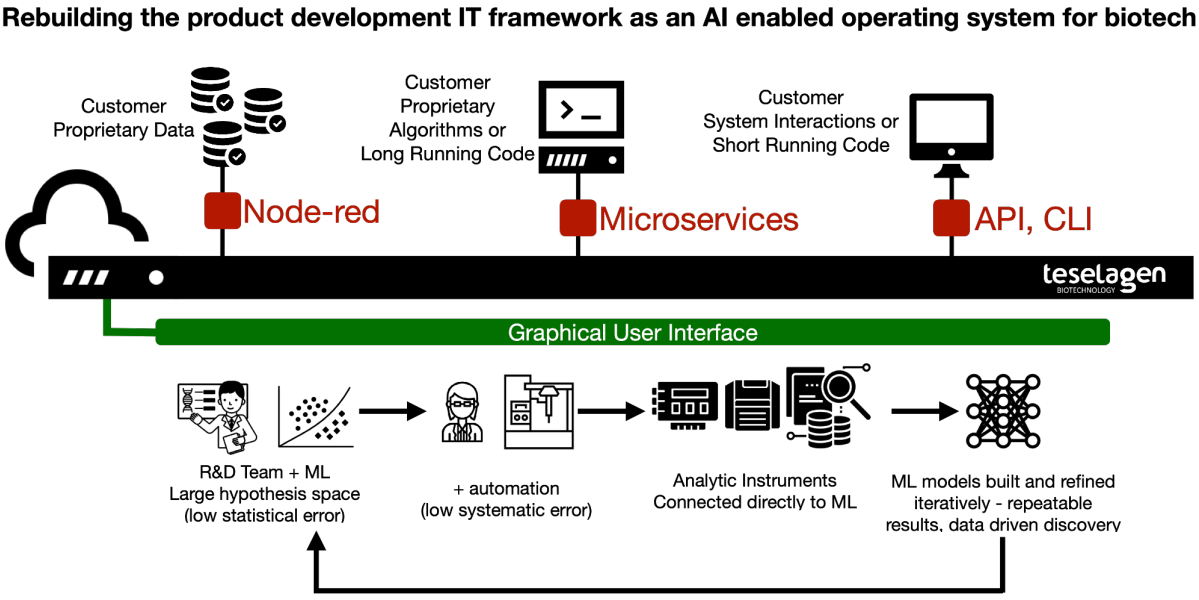


Photo courtesy of TeselaGen

COMMENTS

- For management of this project, the team seems to be well integrated and meeting frequently. In addition, having interviews with commercial users as a subtask of Task 3 will help ensure that the technology developed addresses unmet needs of the industry. The example given on slide 18 of a decision to recreate the EDD test functionality from scratch in TeselaGen, rather than make the existing EDD compatible with TeselaGen, seems to be an example of a well-functioning cross-stakeholder management team. The approach seems sound and has the potential benefit of enabling outside commercial entities to have access to the innovative computer programs and algorithms developed in the national labs via a more user-friendly and well-supported interface (in TeselaGen's software). One thing that was totally unclear from this presentation and others is how the raw data are subjected to processing and quality control before being uploaded into the EDD or its analog within TeselaGen; one would hope that this data processing could also be automated, but that was not mentioned in the talks from ABF, Agilent, or TeselaGen (as far as I can recall). Agilent said something about various Skyline apps, but according to my colleagues this labor-intensive data processing is still done largely manually at most organizations and is error-prone and creates risks. The impact seems high for several reasons. First, the potential for an integrated, user-friendly, well-supported platform to provide access to the DOE computational tools could be helpful for many companies and nonprofit researchers in this space. Second, the company partner (TeselaGen) has some contracts already for their software, demonstrating the commercialization potential, and is also soliciting feedback from their customers on what they need and want. Third, the September 2020 publication illustrates how these approaches have been used to improve strains. While the project team is on track with delivering all their milestones plus an additional one, the outcome is disappointing, in that the integration between DOE tools and TeselaGen is more limited than originally envisioned. In the end, it seems that none of the tools (EDD, BLiSS, BOOST) will be integrated into TeselaGen and actually offered to their customers. EDD functionality was recreated in TeselaGen, which is fine. However, BOOST will not be part of TeselaGen due to separate licensing and commercialization activity, and BLISS is "difficult to integrate." Given the difficulties with integrating BLiSS and BOOST, what proportion of the work in this proposal provided lasting

value? Were there side benefits, for example, did the new features in the DOE software help them with other activities they are pursuing, independent of TeselaGen?

- I see the benefit to TeselaGen by getting access to lots of real-world data to test and train their systems on, as well as connection to companies and endpoint users outside of academia. TeselaGen commented that the ABF was very practical, goal-oriented, and therefore a great partner. I see the benefit to ABF to "demonstrate ABF functionality integrated into TeselaGen's platform with industrial partners" as well as "evaluating emerging TeselaGen functionality support for ABF workflows." What was not clear from the presentation is how ABF/BETO sees this work continuing in the future and further deployment within the ABF. As outlined in slide 17, there are major differences between a private company and publicly funded ABF. It was not clear in the presentation how these differences were resolved or plan to be resolved. On slide 18 it says, "It was taking longer to make TeselaGen's TEST module compatible with EDD than to simply recreate EDD functionality de novo. TEST is written in pure JavaScript from front to back, using NodeJS as a backend. EDD is written in Python. With the guidance of the EDD team, it was decided to simply rebuild EDD functionality in TeselaGen's TEST module in pure JavaScript for speed and tight integration with advanced web technologies." How does this advance EDD and public release of tools, if advances made in the DBTL cycle are within TeselaGen's software? Strongly agree with the conclusion that BLiSS is important from a public safety point of view, and DOE should support BLiSS and make it freely available to trusted parties through a web service. This will need to be done in EDD and be made fully public. "BOOST" has useful code but is protected by a license agreement. We will be happy to implement when we have pull from customer. In the meantime, the developer started a company to commercialize BOOST, which may make integrations easier." Who owns the license (TeselaGen or ABF)? Is this a problem for TeselaGen or ABF? Again, this speaks to the seemingly unresolved issues of this particular private/public collaboration. It is hard to see what is going to motivate TeselaGen to deploy this work if they will not profit from it. In the review discussion, TeselaGen even said that it felt like they were doing work for the ABF and not the other way around.

- It seems like the management of this program was adequately handled between TeselaGen and LBNL; the presentation described a pretty significant shift of approach (away from making a generic framework) at a decision point, but it seemed that the reasoning behind this change was sound. On approach, I think there are substantial risks that upgrading BOOST and BLiSS to better integrate with a single company's products will make those tools less useful for the rest of the industry, and was surprised to see this approach accepted by ABF. On impact, while better software tools for biotech are certainly needed, and the impact on commercialization of TeselaGen's product was evident, I did not find the efforts to make sure this is of generalized commercial relevance were adequate. The specifics of the progress were not entirely clear to me from the presentation, but it seemed that the goals were accomplished on the set timeline.

- Strengths: The team has provided a unique strategy to integrate ABF's design and learning capabilities that can largely enhance the team's efficiency. The project has a clear management plan. TeselaGen has attracted several industrial customers, demonstrating its impact to biomanufacturing industry.

- Weakness/area for improvement: There are a lack of quantitative outcomes to evaluate the progress and impact of this project.

- The goal of this project with TeselaGen is to integrate a series of ABF software and data repositories into the commercial TeselaGen software platform. The ultimate goal is to use ABF data sets to drive machine

learning approaches to strain engineering. The management of this project is well outlined, with frequent interactions between TeselaGen and ABF. Previously unforeseen roadblocks were quickly identified, and the project adjusted. The impact of this project is high to the general bioindustrial community, as TeselaGen will distribute the software in a more user-friendly platform. The impact of the project on TeselaGen is also high, as it will allow its customers to use advanced machine learning algorithms for protein engineering. The impact on the ABF is high as their software and data sets will be used by scientists outside the ABF. The progress of this project is good, taking into account the pivot required early on in the project due to incompatibility of operating systems.

- This DFO is interesting in that it had the effect of kicking the tires on ABF software to evaluate its accessibility and utility to a company. The focus was on integrating BLiSS, EDD, and BOOST with TeselaGen's synthetic evolution platform. The return on investment of this project was difficult for this reviewer to evaluate. There was little description of how the goals (slide 11) were specifically met and what was learned, with the exception of the explanation of mid-course corrections on Tasks 2 and 3. The fact that it was deemed preferable to re-implement EDD was a troubling indicator of its general utility, which should be addressed on the ABF side. In slide 26, most tasks are noted as completed, but task changes are described in the following slides, and tasks are listed as incomplete in slide 30. This reviewer would like to have heard more about the advanced visualizations, BOOST enhancement, and deployment with and feedback from industry partners. The impact section should have focused on the impact of this specific effort.

- This project aligns well with the ABF mission: to create improvements in in-house technical capabilities, get a better understanding of how to improve the DBTL cycle, and incorporate industry standards into ABF processes. The project tasks are nicely aligned with the intent of ABF's mission as well, to provide a coordinated test and learn experience to augment existing process management and increase data management and analytical capabilities. Overall, this is a complementary effort to the ABF's overall effort. What is difficult to understand are the actual metrics. While TeselaGen indicates that the metrics are driven by their bottom line, it is not clear how ABF is actually improving its capabilities. Publications are not a metric of performance. It is not clear what the benefits are in terms of successful outcomes of the project.

PI RESPONSE TO REVIEWER COMMENTS

- The reviewers' questions and comments are fair and reasoned. We attempted to be open about our engagement, which we enjoyed and found to be productive. We appreciate the reviewers' equally frank comments. One comment was that we were vague about quantifying the results of the engagement or inconsistent in our reporting. We attempted to quantify in terms of milestones and goals met, and we feel that we were successful against all of our goals, with the possible exception of adding additional visualization to EDD/TEST. This was a debate because very good tools exist outside of EDD/TEST for data visualization, so we focused on integration with those tools instead of recreating them yet again. Our TEST integration with Jupyter Notebook and the visualization available there is excellent, but we now realize that some simple immediate feedback visualizations could be useful and indeed are under development. We should note that in response to the comment about a lack of focus on automated data processing ... good catch! This is a difficult subject but one that TeselaGen has spent the better part of the last 24 months working on. These data pipeline issues are being worked on in a number of settings; there are expensive general commercial solutions such as Databricks that are aimed at large-scale data mining and machine learning, and highly focused solutions like TeselaGen for biotechnology applications where the language of biology rather than that of commerce must be spoken fluently. Our

solution has been to roll out an integration server in addition to the usual application programming interface (API)/command line interface (CLI). This advanced integration server has made integrating with any data source/sink with an API a much more straightforward and supportable task. We have demonstrated its capabilities by integrating TeselaGen with JBEI ICE and many other software tools and databases. We would be happy to demonstrate the power of this approach to those interested. Some of the critical comments perhaps reflect the reality of any private-public collaboration, particularly with software, where companies are faced with existential decisions about priorities and resource allocation. There is a good deal of confusion both in commercial software companies and in academia about what should be an open-source tool or resource and what is more appropriately developed privately. In general, we have found that the creation of discrete tools like j5, BLiSS, BOOST, and ART if well modularized and wrapped in an API can be successfully licensed or just made open source. More general infrastructure platforms like EDD are more difficult because they themselves are a composite of many different open- and closed-source tools and may not be as efficiently maintained or deployed as commercially built software. We are quite happy to use tools like BLiSS and BOOST within our platform for that reason. We may have left an erroneous impression that we did not intend to do so. We think BLiSS should be well supported and made available at cost. BOOST is also useful, but the market is small. If customers have the need, we are happy to implement BOOST tools into the TeselaGen platform. EDD was just a tough decision, but I think the correct one for TeselaGen as a software company.

- The role of EDD is different from those of BLiSS and BOOST. It is essentially a cloud app with a web and API interface to a structured database used to support machine learning exercises. As such, it is much closer to a commercial software product, and the design choices the EDD team made were not compatible with the choices that TeselaGen made. That does not mean that EDD is not appropriate as an in-house solution for an academic setting, but it is not really something that a commercial company can scale and support unless they have made exactly the same architecture and framework choices across dozens of possibilities. Our feeling is that EDD is a legitimate choice in an academic setting, whereas the TeselaGen TEST module might be more appropriate for industrial applications. In general, we greatly value the interaction with cutting-edge academic and national lab investigators and developers. Every engagement does not have to be flawless; I would not trust one that was!