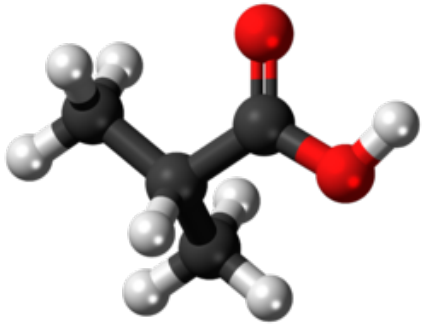
DOE Bioenergy Technologies Office (BETO)
2021 Project Peer Review

Implementing a Design, Build, Test, Learn *P. kudriavzevii* Engineeering Cycle for Production of an Organic Acid Product



March 10, 2021
Agile BioFoundry Consortium
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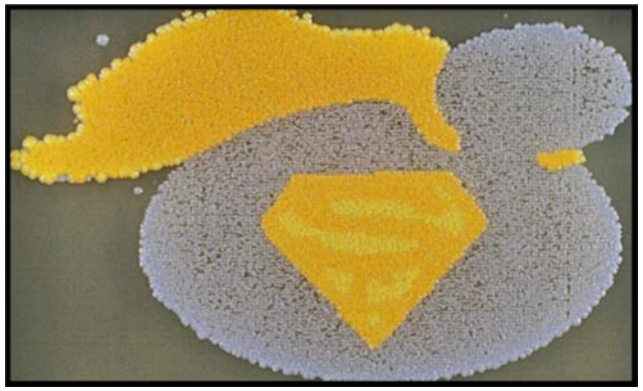
Lygos Inc.

Project Overview (1)

- Lygos uses *Pichia kudriavzevii* (Pk), an acid-tolerant 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* robust nature provides an advantage over more traditional biomanufacturing strains like *E. coli* and *S. cerevisiae*, working with Pk presents unique challenges.
- Lygos' experience working with this non-conventional yeast is extensive; however, there remained room to improve the engineering cycle, providing an opportunity to partner with the Agile BioFoundry (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 (IBA), which can be converted to the large market material methyl methacrylate (aka. Plexiglass).

Super Yeast



Project Overview (2)

Project Mission

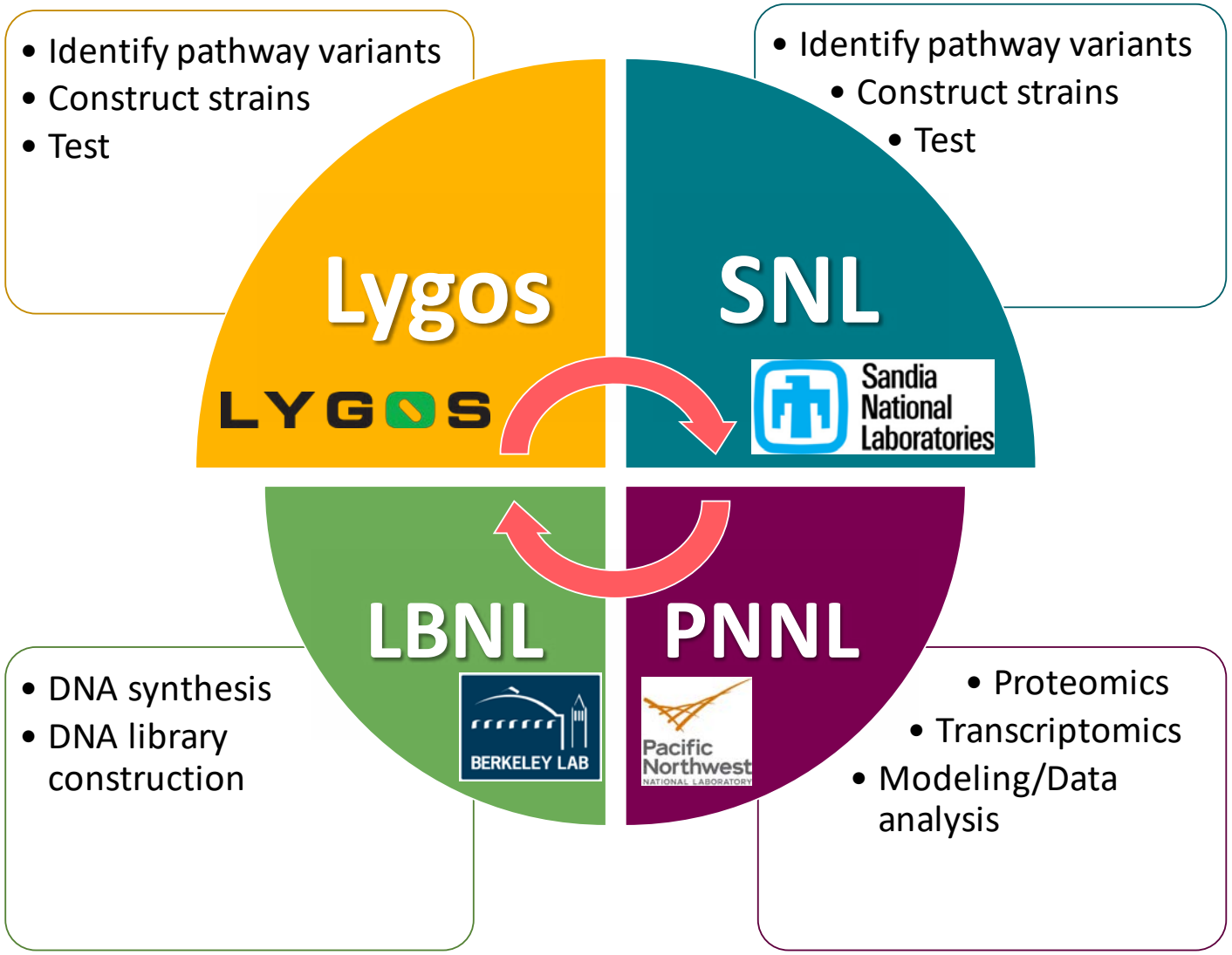
1. To apply the ABF's Design, Build, Test, Learn (DBTL) tools and technologies to establish and optimize production of isobutyric acid (IBA) in *P. kudriavzevii*.

2. To onboard *P. kudriavzevii* as a production host within the ABF.

This project aimed to replace an environmentally hazardous, and harmful process used today to manufacture IBA (***) with a safe, cost-effective and renewable route using microbial technologies to the same product in order to achieve a more sustainable future.

(***) Isobutyric acid is traditionally produced via high pressure hydrocarboxylation from unsustainable petroleum feedstocks. This process involves strong acids, carbon monoxide and hydrogen fluoride, which may cause environmental damage.

1 – Management



- To accelerate on-boarding of the Pk host to the ABF, a post-doctoral scientist from SNL worked on-site at Lygos for the duration of this project.

- Monthly project meetings were held with all project stakeholders:
  - To support clear communication of progress and open discussion about challenges encountered
  - To ensure proper alignment of priorities and resources

2 – Approach (1)

- Lygos
  - Provided a proprietary engineered *P. kudriavzevii* strain that accumulates high levels of pyruvate (precursor to the IBA pathway) while minimizing biomass production.
  - Generated the baseline IBA strain and provided lab infrastructure for strain construction, testing and bioreactor cultivations.

- SNL
  - Conducted strain engineering and bench-scale testing.
  - Worked with Lygos to conduct several DBTL cycles.

- PNNL
  - Conducted multi-omic analysis of strains to assess strain performance and identify potential bottlenecks.

- LBNL
  - Provided construct synthesis through ABF's Design Implementation Verification Automation (DIVA) platform.

2 – Approach (2)

- The IBA biosynthetic pathway is comprised of multiple non-native enzymes.
  - Three of the biocatalytic steps required enzyme homolog exploration to increase IBA production efficiency to industrially relevant levels.

- ABF capabilities were leveraged to improve the effectiveness of the DBTL cycle to improve IBA production in *P. kudriavzevii*.

- **Challenge 1:**
  - Efficient testing of the pathway design-space.
    - Achieving optimal pathway activity typically requires testing multiple homologs of each enzyme, different enzyme expression levels, and different protein codon optimizations, etc.

- **Challenge 2:**
  - Lygos typically uses fermentation product and byproduct profiles to build basic models of glucose flux.
    - Collecting metabolite-, transcript-, and protein-level data could improve the model quality and enable more efficient strain engineering.
    - A key challenge is developing and implementing the workflows to collect, analyze, and act on the data gained from these three methods.

3 – Impact

Scientific/technical

- On-boarded a new host to the ABF that is well suited for use with lignocellulosic feedstocks and capable of tolerating high concentrations of many different organic acid byproducts.
- Provided an opportunity to “kick the tires” of the ABF’s DBTL technologies in collaboration with its industrial partner Lygos.

Publications, presentations, and IP

- Lygos had previous IP on the IBA pathway.
- Lygos and the ABF are currently in the process of publishing press releases around this project.
- Potential scientific publication → **“Engineering an industrially relevant non-model yeast to produce isobutyric acid”.**

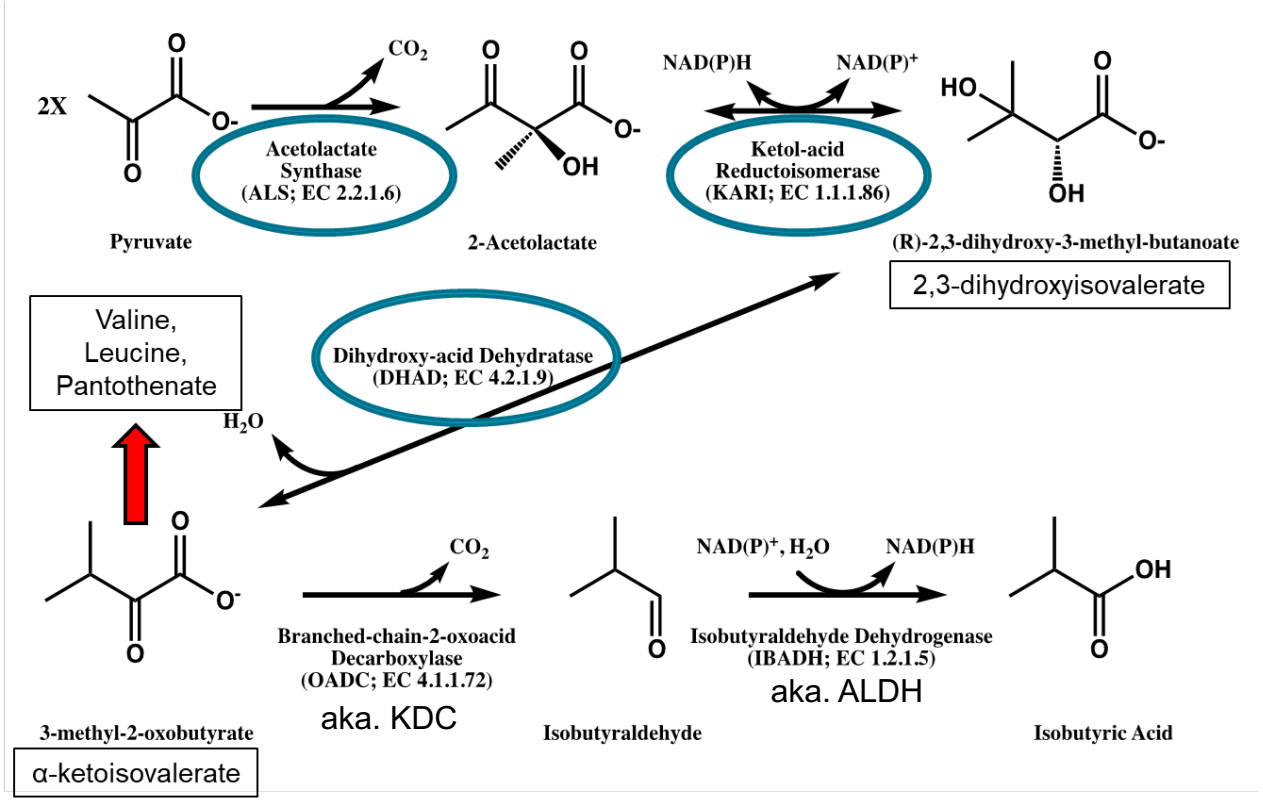
Commercial

- Lygos is continuing R&D and commercialization activities given the successful completion of this ABF project.
- Lygos is interested in converting IBA to methyl methacrylate (aka. Plexiglass) which is currently produced in a quantity of 2 MM tons per year.
- Lygos is discussing with two large chemical companies to scale this technology to make bio-based plexiglass.

4 – Progress and Outcomes: Project Goals

Engineer *P. kudriavzevii* to produce isobutyric acid (IBA)

- Lygos developed the baseline strain
- Use the ABF DBTL capabilities to optimize the pathway
- Goal was to produce high levels of IBA from DMR hydrolysate

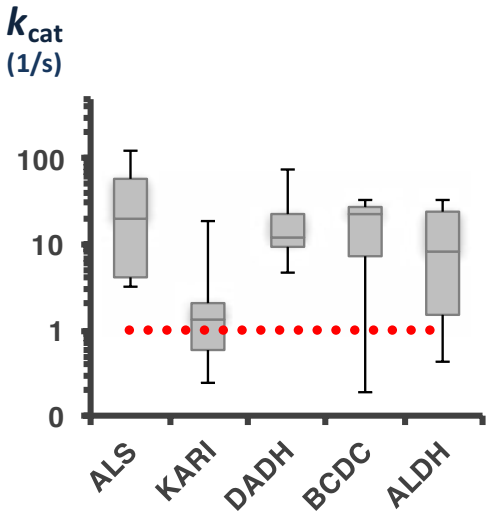


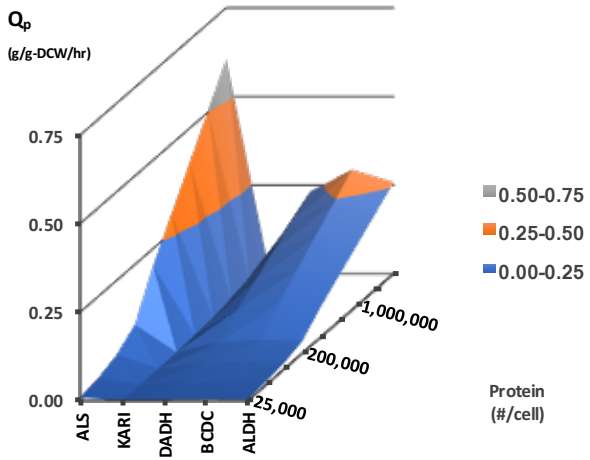
4 – Progress and Outcomes: Project Milestones

All tasks have been completed

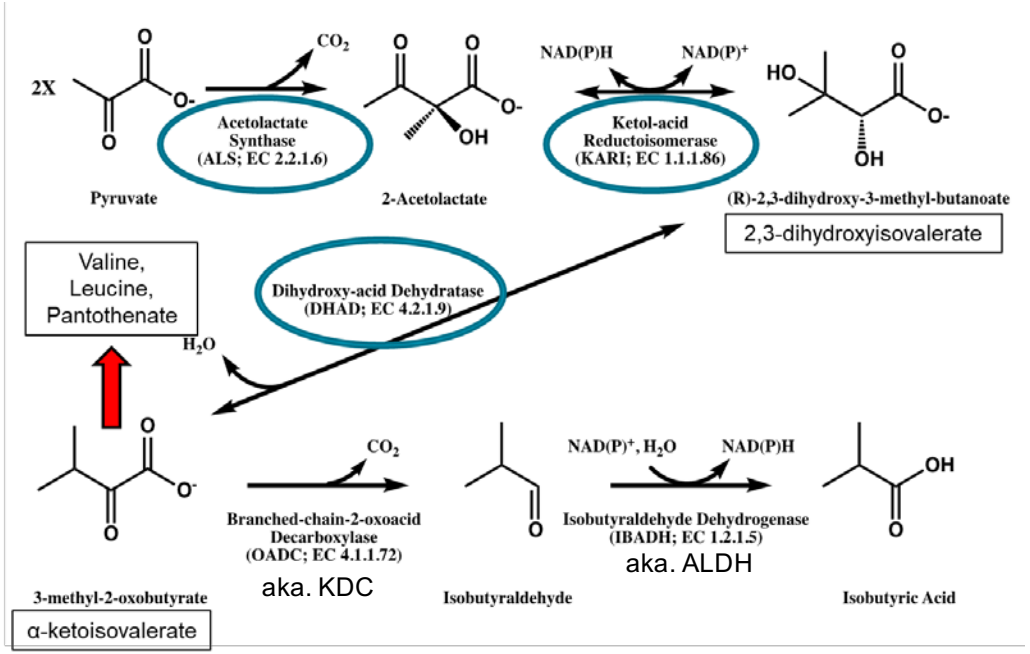
Task No.	Task Description	Duration (Starting & Ending Project Month)	Responsible Parties
1	Identify OA1 pathway enzyme variants, use ICE/DIVA to Design constructs.	01-09	NTESS/LBNL/Lygos
2	Transfer P. kudriavzevii protocols to Build team. Initiate baseline proteomic and metabolomics testing of the base P. kudriavzevii strain.	07-09	NTESS/PNNL/Lygos
3	Test OA1 constructs in P. kudriavzevii and achieve ≥1 g/L titer. Identify correlations between variants and OA1 titers and select strains for multi-omic analysis.	10-12	NTESS/PNNL/Lygos
4	Refactor the OA1 pathway, initiating the 2nd DBTL cycle. Complete multi-omic Tests and use data to inform 2nd DBTL cycle Designs.	13-18	NTESS/LBNL/PNNL/ Lygos
5	OA1 pathway refactoring, strain construction, and fermentation optimization.	19-24	NTESS/LBNL/Lygos
6	Prepare Final Report.	24-24	NTESS/LBNL/PNNL/ Lygos

4 – Progress and Outcomes: Initial Pathway Analysis





Enzyme	k_{cat} - 75th Percentile (1/s)	Qp (g/g-DCW/hr) @ 500K copies/cell	Qp (g/g-DCW/hr) @ 1MM copies/cell
ALS	57.05	0.24	0.48
KARI	2.06	0.01	0.02
DADH	21.83	0.09	0.18
BCDC	27.68	0.12	0.23
ALDH	24.35	0.10	0.20

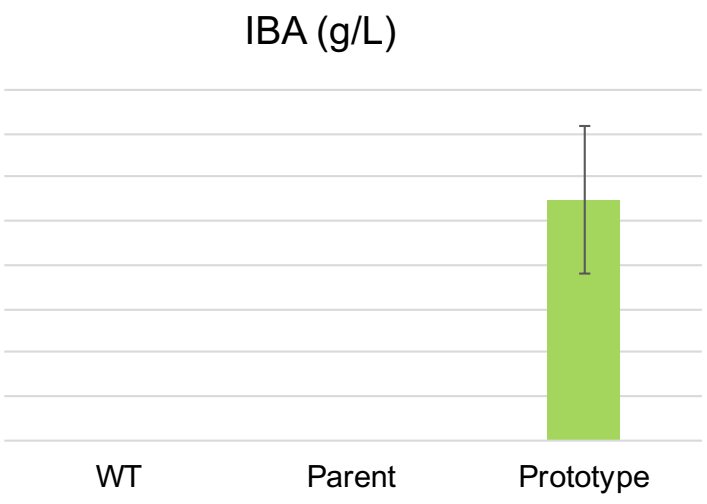


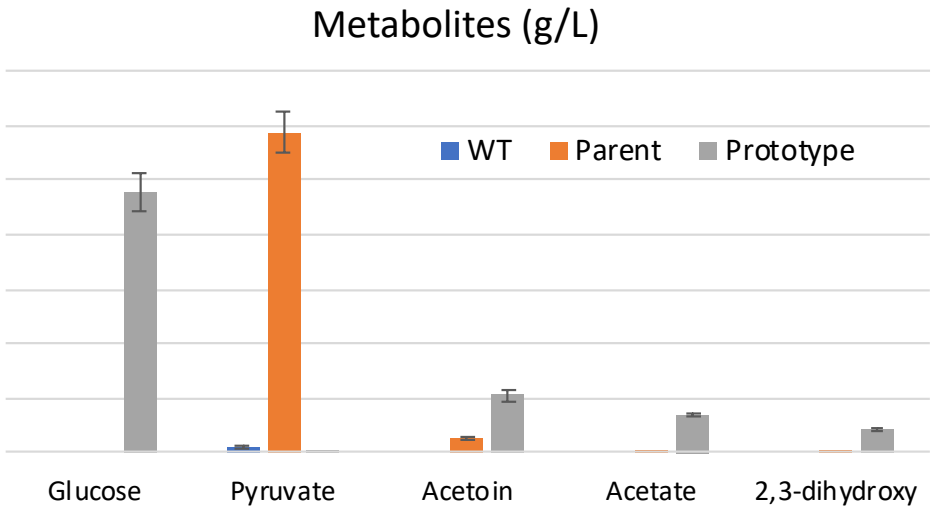
- Initial analysis (redox, kinetic, thermodynamic, cofactor, etc) yielded:
  - KARI kinetically slow to achieve high production
  - Change KARI preference from NADPH to NADH to improve redox
  - DHAD needs Fe-S as a cofactor. In yeast, cytosolic Fe-S has been shown to be limiting for high heterologous enzyme activity
  - IBADHs are generally quite promiscuous

4 – Progress and Outcomes: Prototype Strain

- Prior to the start of this project, Lygos had generated a platform strain that could accumulate high levels of the intermediate molecule pyruvate.

- Lygos generated the IBA prototoype strain by introducing the 5-enzyme IBA pathway to the above-mentioned platform strain.

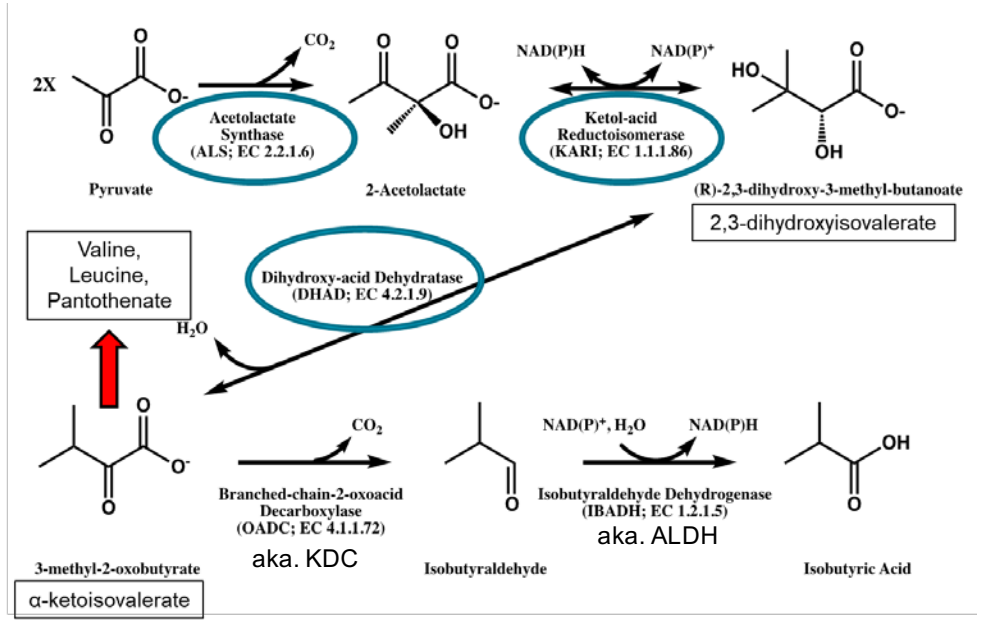




4 – Progress and Outcomes: Results (1)

Task 1: Identify IBA pathway variants

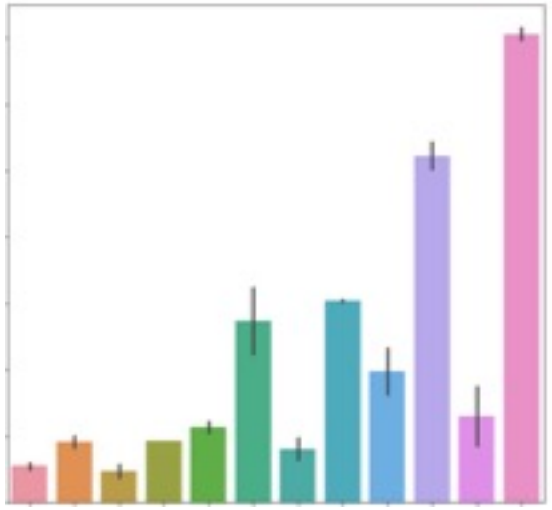
- 15 KARI homologs were identified by Lygos before the CRADA started.
- 40 DHAD homologs and 10 IBADH homologs were identified during this CRADA using EFI-EST (https://efi.igb.illinois.edu/efi-est/).
  - Vectors were designed and constructed by the DIVA team.
- Many additional ALS and KDC variants were evaluated after the multi-omic analysis was completed.



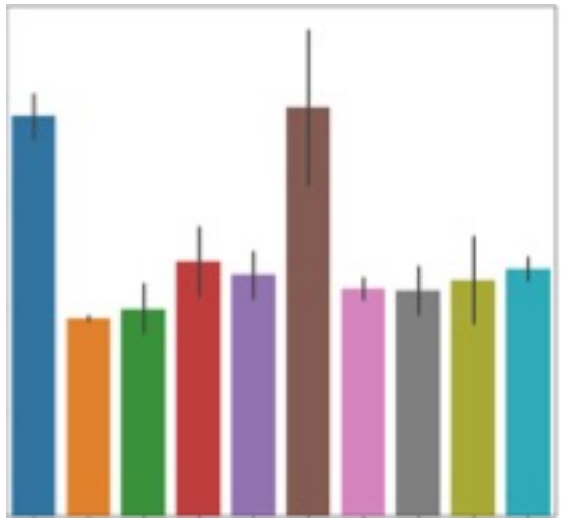
4 – Progress and Outcomes – Results (2)

Task 2: Conduct multi-omic analysis on the baseline prototype strain

- Initial multi-omic analysis suggested that 2-ketoisovalerate accumulated, so 2-ketoisovalerate decarboxylase (KDC) activity may have been limiting.
- Thiamine supplementation increased IBA titers.
  - Thiamine is a cofactor required by two enzymes on the IBA pathway, acetolactate synthase (ALS) and 2-ketoisovalerate decarboxylase (KDC).
- Additional copies of ALS improved IBA production.



IBA titer with different cofactor supplementations

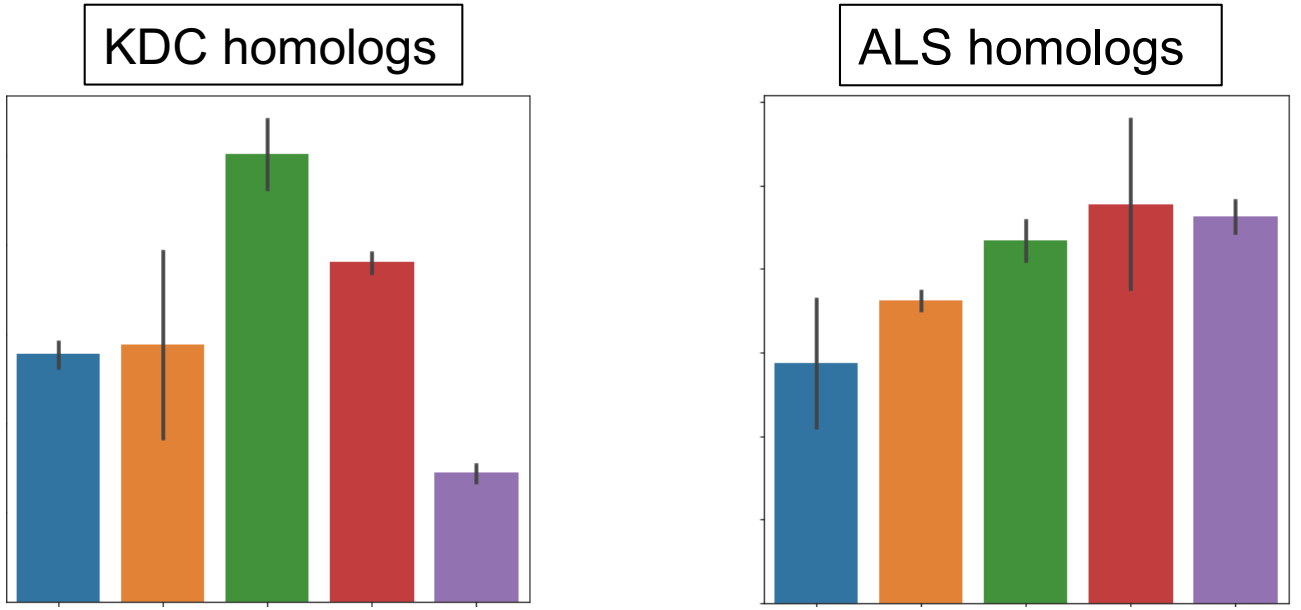


IBA titer from strains expressing different IBA enzyme homologs

4 – Progress and Outcomes – Results (3)

Task 3/4: Test enzyme variants and refactor pathway

- Initial screening of DHAD and IBADH did not improve IBA titers.
- KDC and ALS homologs were also screened, new KDC variants significantly improved IBA production.
- ABF's proteomics and metabolomics capabilities were key to identifying and resolving bottlenecks in the process.
  - The omics data had a huge impact on understanding the microbial system that otherwise wouldn't have arisen without this collaboration.



IBA titer when evaluating new KDC or ALS homologs

4 – Progress and Outcomes – Results (4)

Task 3/4: Test enzyme variants and refactor pathway

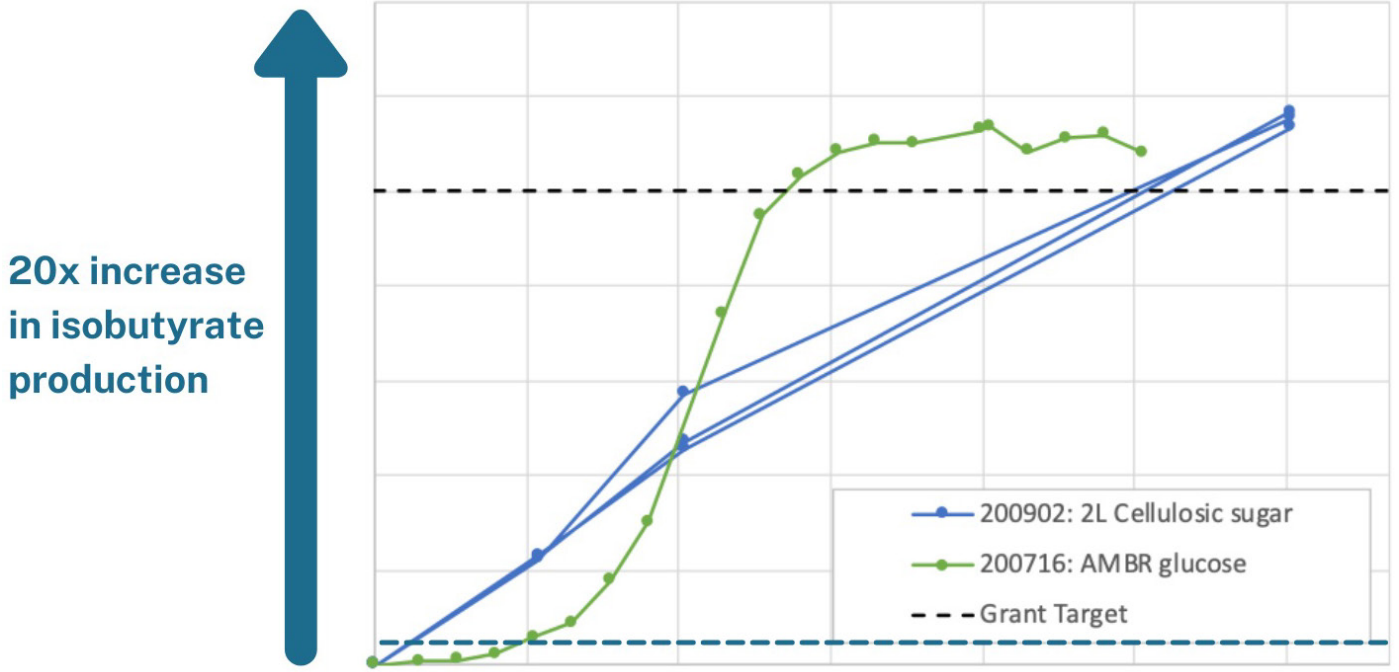
- Additional approaches that boosted strain performance
  - Knocking down the competing branched-chain amino acid pathway.
  - Expression of the IBA pathway in both the cytosol and mitochondria.
  - Optimized ALS expression through promoter tuning.

- Beneficial strain modifications and process conditions were combined resulting in significantly improved performance in small-scale shake flask cultivations.

4 – Progress and Outcomes – Results (5)

Task 5: Fermentation optimization

- A variety of media formulations and fermentation conditions were tested.
- Fermentations were initially performed in AMBR 250-mL fermenters using regular glucose feedstock and then performance was benchmarked against the NREL DMR corn stover hydrolysates in 2-L fermentations to assess the potential of this strain to convert lignocellulosic feedstocks.
- IBA titers exceeded the final project milestone for this project.



4 – Progress and Outcomes – Results (6)

Task 6: Prepare final report

- The final report has been finished and submitted.

Onboarding of *Pichia kudriavzevii* to the ABF

- Lygos has transferred strains and protocols for Pk onboarding to the ABF
  - 23 plasmid strains containing key genetic parts.
  - 21 *P. kudriavzevii* platform and IBA strains.
  - Protocols for performing *P. kudriavzevii* transformations, genetic engineering and shake plate/flask screening.

Summary

- Lygos and the ABF successfully collaborated to complete several DBTL cycles and were able to rewire the host organism to produce high levels of isobutyric acid.

- Leveraging the ABF's capital investments and deep expertise (e.g. advanced systems biology) removed serious bottlenecks in the project, leading to a 20-fold increase in the production of isobutyric acid.

- Onboarded *P. kudriavzevii* as a production host to the ABF and confirmed it as being a robust microbe well suited for industrial use.

- Lygos is planning to continue R&D and commercialization activities towards biomanufacturing of the high market valued bioproduct isobutyric acid.

Quad Chart Overview

Timeline

- 04-05-2018
- 01-20-2021

	FY20 Costed	Total Award
DOE Funding	$304,706	$1,000,000 (all spent)
Project Cost Share	$113,362	$428,572 (all spent)

Project Goal

To apply ABF DBTL tools and technologies to establish and optimize production of a new, valuable, organic acid product in *P. kudriavzevii*.

End of Project Milestone

To generate a strain and bioprocess capable of efficiently utilizing cellulosic hydrolysate and producing sufficiently high titers of isobutyric acid in 2-L fermentations to warrant further investment in this product program.

Project Partners

- SNL, PNNL, LBNL
- Lygos Inc.

Funding Mechanism

This was a Directed Funding Opportunity (DFO) for industry and academic partners to utilize ABF capabilities through a CRADA mechanism.