Implementing a design, build, test, learn *P. kudriavzevii* engineering cycle for production of an organic acid product

PIs: John Gladden (SNL), Jeffrey Dietrich (Lygos), Taek Soon Lee (LBNL), Jon Magnuson (PNNL)

Project Type

- Cooperative Research and Development Agreement (CRADA) between the Agile BioFoundry and Lygos for the implementation of a DBTL cycle to engineer *Pichia kudriavzevii* for the production of isobutyric acid.
- CRADA No. FP00006776

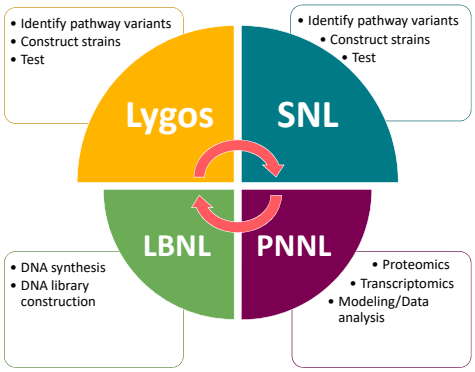
Timeline

- **Project Start Date:** 04/01/2018
- **Project End Date:** 03/31/2020
- **Percent Completed:** 45%

	Total Funding Pre-FY17	FY 17 Fundin g	FY 18 Funding	Total Planned Funding (FY 19-Project End Date)
DOE Funded	$0	$0	$250,000*	$750,000*
Project Cost Share	$0	$0	$94,365	$334,207

*DOE funding split between SNL (50%), LBNL (25%) and PNNL (25%)

Management Approach



- Lygos and SNL determined the necessary pathway enzymes and homolog candidates for each of the biocatalytic steps and are charged with constructing and testing these engineered *P. kudriavzevii* strains.
- LBNL provides DNA synthesis/construction to Lygos and SNL to facilitate high-throughput strain engineering.
- PNNL receives samples from Lygos and SNL and performs proteomics and metabolomics analyses.

Technical Approach

- Lygos provided a proprietary engineered *P. kudriavzevii* strain that accumulates high levels of pyruvate while minimizing biomass production.
- The biosynthetic pathway is comprised of multiple non-native enzymes that convert pyruvate into isobutyric acid (IBA).
- At least two of the biocatalytic steps require enzyme homolog exploration and/or engineering to increase IBA production efficiency to industrially relevant levels.
- A second optimization area focuses on IBA export from the cell.
- The third optimization area targets the reduction of undesired metabolic byproducts.
- ABF capabilities will be leveraged to reduce the DBTL cycle length to improve IBA production in *P. kudriavzevii*.
- **Challenge 1:** Efficient testing of the pathway design-space. Determine and build combinatorial libraries of enzyme homologs, promoters, codon-optimization, etc.
- **Challenge 2:** Developing analytical tools that better guide strain engineering. Collect and analyze transcriptomics, proteomics and metabolomics data from *P. kudriavzevii* strains.

Technical Progress

- Lygos demonstrated the production of IBA in a *P. kudriavzevii* prototype strain prior to the start of this CRADA.
- Lygos and ABF have identified the IBA pathway limiting steps and new pathway variants have been designed. Potential pathway bottlenecks were identified by analyzing thermodynamics, calculated equilibrium fluxes, reversibility, and published enzymatic kinetic constants. Fifty new pathway variants were established and the DNA parts were added to ABF ICE registry. DIVA was used to design the plasmid construction protocols to generate these genetic constructs.
- Fifteen new variants of a specific enzyme in the IBA pathway were integrated in the *P. kudriavzevii* genome and the resulting strains were tested for IBA production (Fig. 1) and their *in vitro* enzymatic activity was measured.
- Lygos and ABF have started to collect proteomics and metabolomics data from prototype and control strains. Various media for multi-omics analysis were tested and samples at different time points were taken.
- PNNL has established the baseline for metabolomics and proteomics and initial multi-omic data is currently being analyzed.
- Gene knock-outs are underway in order to reduce potential by-product formation.
- All planned milestones for Budget Period 1, ending Mar 31st, 2019 have been achieved.

Relevance and Impact

- The first goal of this project is to apply the Agile BioFoundry DBTL tools and technologies to establish and optimize production of IBA in *P. kudriavzevii.* The second goal is to onboard *P. kudriavzevii* as a production host within the ABF.
- IBA is a commercial product that is difficult and costly to make petrochemically. As an added benefit, IBA is also an intermediate useful for the production of other important chemicals.
- Lygos' manufacturing process for BioMalonic acid confirmed that *P. kudriavzevii* is a robust microbe well suited for industrial use. While Lygos has developed protocols and tools for building and testing *P. kudriavzevii* strains, there remains room to improve the DBTL cycle by partnering with the ABF to further advance this microorganism.

Future Plans

- DNA parts are being synthetized by LBNL which will be integrated into relevant *P. kudriavzevii* strains, and will be tested for IBA production.
- Combinatorial libraries of different enzyme homologs and promoter strengths will be designed and built.
- Multi-omics data will be used to infer potential genes to overexpress, delete or downregulate.

Figure 1

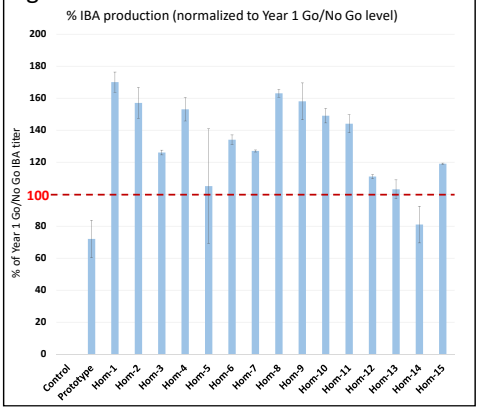


Figure 2