Accelerating Pathway Engineering of Non-Model Organisms Through Novel Cell-Free to In Vivo Workflows

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Project Goals: Non-model organisms have unique traits and offer significant advantages and benefits for biomanufacturing. One example is gas fermenting acetogens capable of converting low cost waste feedstocks to fuels and chemicals, deployed today at commercial scale for conversion of steel mill emissions to ethanol. Yet, engineering these non-model organisms is challenging due to lower transformation and recombination efficiencies, longer cycle times and a more limited set of genetic tools compared to model organisms *E. coli* or yeast.

Cell-free systems can guide and accelerate non-model organism strain development. We are establishing a new interdisciplinary venture, the clostridia Foundry for Biosystems Design (cBioFAB) that combines advancements in cell-free and *Clostridium* engineering metabolic engineering to develop industrial-robust production strains for conversion of lignocellulosic biomass to next-generation biofuels and bioproducts such as acetone, butanol, 3-hydroxybutyrate (3-HB), 1,3-butanediol (1,3-BDO) or monoethylene glycol (MEG).

Climate crisis and rapid population growth are posing some of the most urgent challenges to mankind. The accelerating rate of extraction and combustion of fossil resources for fuel, energy and chemicals over the past 100 years has resulted in carbon dioxide (CO_2) accumulation in the atmosphere to levels unprecedented since the Pliocene Epoch (5.3 - 2.6 million years ago). Although the effect that elevated atmospheric CO_2 will have on the Earth's climate has been predicted by scientists for several decades, it was only in 2016 through the Paris Agreement that nations formally laid plans to abate atmospheric CO_2 release. In each case, these plans necessitate that "above ground" carbon resources increasingly displace fossil resources as feedstocks for fuel and chemical production.

Gas fermentation offers a solution using carbon-fixing chemolithoautotrophic microorganisms. After a decade of scale up, the technology has recently been commercialized by LanzaTech with the first 48k MTA plant turning emissions from the steel industry into fuel ethanol operating successfully and additional units under construction. The process has been demonstrated to accept a broad range of feedstocks including waste gases from various industrial sources (e.g., processing

plants or refineries) or syngas generated from any biomass resource (e.g., agricultural waste, unsorted and non-recyclable municipal solid waste, or organic industrial waste) (1).

In order to maximize the value that can be added to the array of gas resources that the process can use as an input, LanzaTech has pioneered the development of a genetic toolbox for acetogenic clostridia, considered genetically inaccessible not even a decade ago (1). While automated high-throughput engineering is possible for these anaerobic organisms today, cell-free systems offer a path to further reducing cycle times and maximizing throughput, accelerating pathway engineering by more than an order of magnitude beyond what is feasible today.

We have demonstrated the application of cell-free systems to guide various aspects of strain engineering, including selection of best pathway variants and optimal expression levels (2) or prioritizing gene knock-outs for competing reactions. Furthermore, we developed a new framework that allows to seamlessly go from cell-free to cell designs and feed into ensemble and machine learning models.

First, we established an *in vitro* Prototyping and Rapid Optimization of Biosynthetic Enzymes (iPROBE) platform where cell lysates are enriched with biosynthetic enzymes by cell-free protein synthesis and then metabolic pathways are assembled in a mix-and-match fashion to assess pathway performance. Through this approach, we demonstrated optimization of two multistep pathways, leading to 20-fold improvement in cellular production (2). Often times, introduction of a new pathway into a cellular host leads to formation of unwanted byproducts through native reactions. Identification and iterative knock-outs of the responsible enzyme(s) can be cumbersome and time-consuming. We have demonstrated that the iPROBE platform can rapidly identify candidate enzymes to guide cellular engineering.

To enable a seamless transition from iPROBE to cellular engineering, we have established a GoldenGate based vector system that work in context of cell-free protein synthesis and allow identified gene variants to be directly assembled into cellular expression constructs in a single step without re-synthesis or complex cloning and we showed automation of the whole workflow.

Using this platform, we have optimized pathways for acetone, butanol and 3-HB and have demonstrated completely new biosynthesis routes to 1,3-BDO and MEG.

References

1. Liew et al. (2019) Gas Fermentation – A Flexible Platform for Commercial Scale Production of Low Carbon Fuels and Chemicals from Waste and Renewable Feedstocks. *Front Microbiol* 2016, **7**:694.
2. Karim et al. (2019) *In vitro* prototyping and rapid optimization of biosynthetic enzymes for cellular design. *BioRxiv* doi: https://doi.org/10.1101/685768

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