**Rapid Prototyping for Development of A Novel Gas-to-1,3-Butanediol Bioprocess**

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https://www.energy.gov/science/ber/articles/cell-free-technology-accelerates-industrial-biotechnology

**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.

**Abstract:** We recently described optimization and scale-up of gas-fermenting *Clostridium autoethanogenum* strains for continuous at scale production of acetone or isopropanol from syngas at rates of up to ~3 g/L/h and ~90% selectivity and >160% greenhouse gas (GHG) savings over current production routes[1]. This was achieved through an interdisciplinary approach of combinatorial pathway analysis, cell-free prototyping, multi-omics analysis, genome-scale kinetic modeling and process development as a blueprint for accelerated development of new bioproducts. Here we validate and refine this blueprint for production of 1,3-butanediol (1,3-BDO), a precursor for butadiene used in nylon and rubber production with a $20 billion USD/yr market.

While optimizing the acetone and isopropanol pathway, we also observed production of 3-hydroxybutyrate (3-HB) as by-product through native reductase and thioesterase activity interacting with the heterologous pathway [1]. The genome of *C. autoethanogenum* further encodes two aldehyde::ferredoxin oxidoreductase (AOR) enzymes[2] which could further reduce 3-HB to 3-hydroxybutanal, which subsequently could be reduced to 1,3-butanediol (1,3-BDO) via native alcohol dehydrogenase activity.

In a first step to build a route to 1,3-BDO, we aimed at enhancing 3-HB production by prototyping heterologous thiolase (ThlA) and acetoacetyl-CoA reductase enzymes (Hbd/PhaB) using a cell-free system (iPROBE). An optimal set of ThlA-Hbd were able to improve 3-HB *in vivo* production by 8-fold to 13 g/L from syngas and also allowed us to detect low levels of 1,3-

BDO of up to 0.5 g/L[3]. To further optimize 3-HB formation, we next aimed at replacing the native thioesterase reaction with promiscuous phosphate butyryltransferase (Ptb) and butyrate kinase (Buk) enzymes that would allow for ATP generation via substrate level phosphorylation. Genome scale modelling confirmed improved growth coupling and yields. We performed cluster analysis to identify potential Ptb and Buk variants from UniProt database. In total, 30 Ptb and 10 Buk variants were selected and synthesized into a modular cell-free to cell vector system we recently developed, which allows for cell-free prototyping and also serve as donor vectors for Golden Gate combinatorial assembly for *in vivo* workflow in *C. autoethanogenum*[4]. Cell-free prototyping in *Escherichia coli* revealed that Buk enzyme is not the bottleneck so we focused our effort on screening Ptb variants. Four Ptb variants were found to improve *in vitro* 3-HB biosynthesis by up to 6-fold. Based on this result, we performed combinatorial analysis using down-selected Ptb, Buk, ThlA and Hbd/PhaB in *C. autoethanogenum* to identify strains with optimal flux towards 3-HB and 1,3-BDO while growing on gas. Fermentation optimization in continuously stirred tank reactor was performed, demonstrating increased 1,3-BDO production titers using gas as feedstock.

**References**


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**Funding Statement:** We acknowledge the Biosystems Design program of the U.S. Department of Energy (DOE), Office of Science (SC), Office of Biological and Environmental Research (BER) under Award Number DE-SC0018249. DNA synthesis for the gene libraries was supported by the Joint Genome Institute (JGI) Community Science Program (CSP) under Award No. CSP-503280.