Multiomics analysis of mitochondrial versus cytosolic compartmentalization of the isobutanol pathway in *Saccharomyces cerevisiae*

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Project Goals: We aim to establish which subcellular compartment in *S. cerevisiae* the isobutanol pathway should be localized to in order to produce isobutanol at high yields.

Isobutanol, a branched-chain higher alcohol, is considered a promising alternative biofuel to ethanol. As the dominant bioethanol producer, *Saccharomyces cerevisiae* has a demonstrated high alcohol tolerance and thus is considered a suitable host for isobutanol production. Our overarching goal is to redirect *S. cerevisiae*'s native metabolism from ethanol to isobutanol production. Efforts in eliminating ethanol production have proven challenging because ethanol synthesis plays an essential role in recycling reducing equivalents necessary for glycolysis and cell growth[1]. In order for us to establish isobutanol as the predominant NADH-oxidizing pathway in yeast, we need to substantially increase *S. cerevisiae*'s ability to turnover this essential cofactor via isobutanol fermentation. A number of different metabolic engineering techniques have been implemented to balance this requirement and thus increase isobutanol flux. One strategy entails localizing the five enzymatic steps involved in isobutanol biosynthesis into a single compartment: either the mitochondria or the cytosol. However, there is debate as to what subcellular compartment is optimal for maintaining flux through the pathway. In previous reports, the mitochondrial localized isobutanol pathway outperformes the cytosolic version, but the cytosolic version is likely to be more robust under industrially relevant anaerobic conditions where the mitochondria enter a minimal energy-requirement mode. Thus, we seek to understand what barriers in the cytosolic strain can be addressed in order for us to develop a platfrom isobutanol producing *S. cerevisiae* strain.

In our work, we have equipped yeast with either the cytosolic or mitochondrial localized isobutanol pathway and performed a multiomics analysis to elucidate why the mitochondrial compartmentalization strategy favors isobutanol production. During the course of the fermentation, we collected samples for metabolomics, proteomics, and transcriptomics analyses at early-exponential, mid-exponential, and early-stationary phase. In agreement with previous reports, the metabolite data showed that the strain harboring the mitochondrial-localized isobutanol pathway outperformed the cytosolic version by 5.5-fold and produced 2.32 mM isobutanol after the 48-hr fermentation. An increase in the upstream isobutanol metabolite levels in the strain with the cytosolic localized pathway suggests that the difference in titer is due to a

bottleneck at the third enzyme in the pathway, dihydroxy-acid dehydratase (DHAD). DHAD requires a [4Fe-4S] cluster which is synthesized by both mitochondrial ISC (iron-sulfur cluster) and cytosolic CIA (cytosolic iron-sulfur cluster assembly) proteins. Thus, we hypothesized that the DHAD activity was impaired when expressed in the cytosol due to this complex, cross-compartmental assembly. Furthermore, the protein and transcript data of the strain showed increased ISC/CIA activity and altered sulfur metabolism; functional enrichment analysis showed proteins involved with cysteine biosynthesis/homocysteine degradation, siroheme biosynthesis, and sulfate assimilation were positively enriched compared to the strain with the mitochondrial localized isobutanol pathway. Taken together, we hypothesize that in the strain with the cytosolically localized isobutanol pathway, DHAD activity is impaired because the CIA machinery insufficiently makes, targets, and/or loads the Fe-S cluster into the cytosolically localized DHAD resulting in non-functional protein. Since the cytosolic strain is likely to be more robust in industrial conditions, our current objective is to address this observed DHAD bottleneck. The knowledge gained from this multiomics study will inform the design of the next isobutanol producing strain.

Publications

1. Gambacorta, F. V., Dietrich, J. J., Yan, Q. & Pfleger, B. F. Rewiring yeast metabolism to synthesize products beyond ethanol. *Current Opinion in Chemical Biology* **59**, 182–192 (2020).

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