**Developing the yeast *Kluyveromyces marxianus* as a thermotolerant bioproduction host**

**Ian Wheeldon,**[1*] (wheeldon@ucr.edu), Xuye Lang[1], Mengwan Li[1], Pamela Besada-Lombana[2], Danielle Bever-Sneary[2], Nancy Da Silva[2]

[1]University of California, Riverside; and [2]University of California, Irvine

**Project Goals: This systems and synthetic biology project seeks to understand and engineer the native stress tolerance phenotypes of the yeast *Kluyveromyces marxianus* with the goal of developing a new synthetic biology chassis for fuel and chemical production.**

The non-conventional yeast *Kluyveromyces marxianus* is one of the fastest growing eukaryotes, is thermotolerant to temperatures upward of 50°C, and has the capacity to assimilate a wide range of $C_5$ and $C_6$ sugars. These traits make *K. marxianus* an attractive host for the industrial production of biochemicals. However, in comparison to the common yeast synthetic biology chassis, *S. cerevisiae*, there is a clear lack of genome editing tools and standardized genetic parts for biosynthetic pathway construction. In this work, we expand the synthetic biology toolbox by identifying and characterizing a set of 25 different promoters and apply these new genetic parts to engineer the overproduction of a native metabolite, 2-phenylethanol, and a heterologous product, triacetate lactone (TAL). We first developed a one-step markerless multigene integration system that can effectively integrate three unique expression cassettes in a single round of strain engineering. We used this new technique to rapidly create a 27-member strain library that varied the expression of Shikimate pathway genes ARO4, ARO7, and PHA2. This refactoring experiment identified an engineered strain with a five-fold increase in 2-phenylethanol production and demonstrated new capabilities in the rapid engineering of *K. marxianus*. We have also developed a new high-efficiency CRISPR system for our toolbox. Using this system, new pathway knowledge, and computer predictions (using the OptKnock algorithm/*K. marxianus* genome-scale model), we can rapidly engineer metabolic pathways for increased synthesis of TAL from various carbon sources. Initial gene knockouts or heterologous gene integrations have resulted in up to four-fold increases in TAL production from xylose or glycerol. Taken together, the genetic engineering tools and metabolic engineering presented here demonstrate significant advancement in *K. marxianus* as a viable host of biochemical production.

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