Exploring Oleaginous Yeast *Rhodosporidium toruloides* as a Platform Organism for Production of Chemicals and Fuels

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Oleaginous yeast *Rhodosporidium toruloides* is viewed as a potential platform organism for production of biofuels and bioproducts such as biodiesel, lubricants, polyols, jet fuels, and alcohols from renewable lignocellulosic biomass[1,2]. *R. toruloides* naturally accumulates lipids from multiple sugars when some other essential nutrients such as nitrogen is limiting[3]. Recently, we have engineered *R. toruloides* for increased lipid production during growth on glucose[4,5]. In addition to lipid-based chemicals, *R. toruloides* also produces a number of sugar alcohols at high titers. For example, during growth on xylose in nitrogen-rich medium, *R. toruloides* produced D-arabitol[6]. However, present efforts to engineer the organism are hampered by the limited availability of genetic engineering tools. Targeted knock-in and knock-out are available using strains which have had the non-homologous end-joining (NHEJ) gene *KU70* removed, allowing homologous recombination (HR) to dominate, albeit at the expense of the cells' DNA repair capabilities and requiring tedious and low-throughput construction of long homology arms to target the modification.

As part of this project, we seek to both improve the fundamental understanding of *R. toruloides'* metabolism by elucidating the mechanisms of substrate utilization, metabolite identification, and identification of the key genes governing the lipogenesis process, as well as to develop more advanced genome editing tools to facilitate metabolic engineering efforts.

We performed transcriptome and metabolite analysis of *R. toruloides* IFO0880 during growth on plant-based sugars, acetic acid and lipids. The results from transcriptomics and metabolomics indicate global metabolic shifts resulting from growth on different substrates. We mapped differential gene expression and metabolite levels on the metabolic pathways for *R. toruloides*, which revealed the activation of different pathways by different substrates. We also identified and functionally characterized a few putative sugar transporters from *R. toruloides* in *Saccharomyces cerevisiae*. These results provide more clarity regarding substrate utilization in *R. toruloides* and their associated pathway.

We also report the first development of a functional CRISPR/Cas9 system in *R. toruloides* for modular, high-efficiency targeted gene knockout[7]. Different Cas9 and gRNA expression systems were evaluated, and reporter genes in the β-carotene biosynthetic pathway (phytoene synthase, phytoene desaturase) were knocked out with up to 98% deletion efficiency using a novel hybrid 5S rRNA-tRNA promoter for gRNA expression. Multiplexed deletion of phytoene synthase and β-isopropylmalate dehydrogenase (*LEU2*) was demonstrated with 78% efficiency.

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