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Engineering and optimization of lignin catabolic pathways in *Rhodosporidium toruloides*

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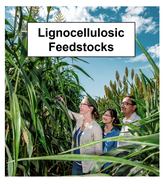
Abstract

Rhodosporidium toruloides is an ideal chassis for valorizing lignocellulosic biomass, with substantial malonyl-CoA pools and the ability to co-utilize multiple carbon sources[1]. Here, we engineer the yeast to better utilize the lignin portion of biomass by manipulating its native *p*-Coumarate consumption pathway. We explore production of six *p*-Coumarate-derived compounds and obtain significant titers for many (6.7 g/L PCA, 4.6 g/L 4HBA, and 0.4 g/L PDC). Finally, we employ Tolerance Adaptive Laboratory Evolution (TALE) to enable robust growth of *R. toruloides* in 20 g/L *p*-Coumarate.

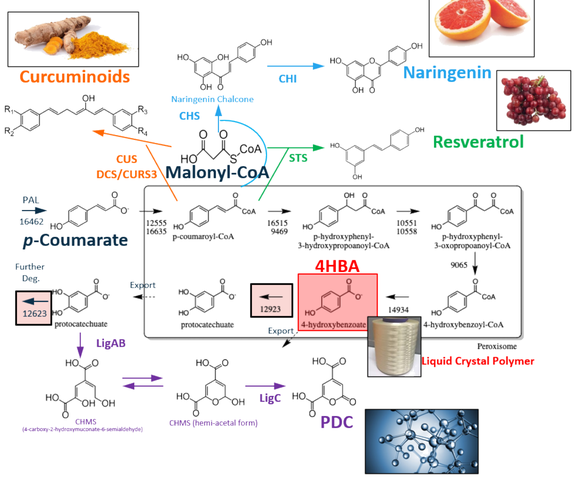
Lignin Valorization in *R. toruloides*



Biomass Deconstruction

- Cellulose → C6's (glucose) 10-80%
- Hemi-Cellulose → C5's (xylose) 10-50%
- Lignin → *p*-Coumarate 5-35%

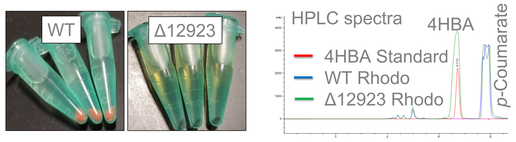
To utilize all Carbon in biomass, we must utilize lignin



Native *p*-Coumarate degradation in *R. toruloides* (in black) and engineered pathways (in color). Completed gene deletions are highlighted in red.

Deleting Key Steps in Pathway

Employing CRISPR-Cas9[2] to delete 12623, 12923 enables conversion of *p*-Coumarate into 6.7 ± 2.2 g/L PCA or 4.6 ± 0.1 4HBA respectively in minimal media

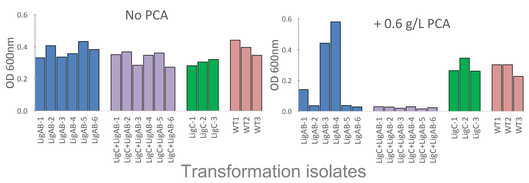


Successful KOs give brown phenotypes.

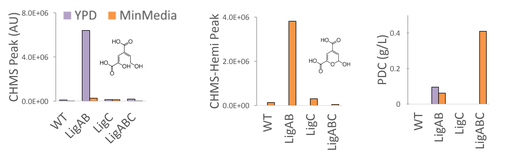
Growth of Δ12923 in Ensiled Sorghum Hydrolysate produces 1.3 ± 0.1 g/L 4HBA

Production of PDC

LigABC pathway for producing PDC from PCA integrated in Rhodo, grown with PCA

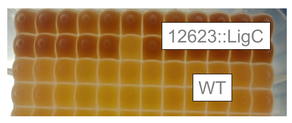


PCA hindered growth only of strains harboring LigAB. Best explained by LigAB conversion of PCA to CHMS, an aldehyde (& potentially toxic) PDC precursor

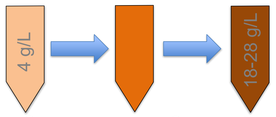


Observed peaks for PDC, CHMS on GC-MS. Follow-ups failed, due to bad storage

Integrated LigC @ 12623 to prevent PCA consumption. Stacking with LigAB



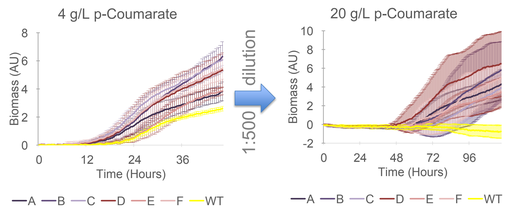
p-Coumarate Adaptation



Slowly increase *p*-Coumarate concentration as sole carbon source

Six independent cultures (A-F) adapted over ~10^{12} cumulative cell divisions

Adapted strains grow much better than WT, easily tolerating 20 g/L *p*-Coumarate



Growth is robust in *p*-Coumarate; Strain "F" even grows directly in 20g/L

Resveratrol Degradation Products

Integration of Resveratrol STS pathway resulted in dark brown color. Two unique peaks observed in HPLC spectra

Rhodo's cousin, *U. maydis*, has a known Resveratrol cleavage enzyme Rco1[3], with homology to two *Rhodo* genes

Rhodo could be producing, & subsequently cleaving, resveratrol

Enhancing Substrate Availability

In general, *p*-Coumaroyl-CoA based pathways failed, possibly due to localization in the peroxisome

Moving fist step in *p-Coumarate* out of peroxisome by deleting PTS's

12555: …SVYEEKPR**AKL***
16635: …LYTKAEGKAR **AKL***

Conclusions & Future Work

- High titers of PCA (6.7 g/L), 4HBA (4.6 g/L) were recovered from *R. toruloides.*
- Strong signs of CHMS production, and of potential PDC production (0.4 g/L detected).
- *R. toruloides* adapted to high *p*-Coumarate levels, growing directly in 20g/L. Incorporate product pathways into these evolved strains
- Resveratrol degradation products appear from STS expressing *R. toruloides*. Peroxisome localization may hinder product formation. Delete *rco1* and PTS's to fix this.

References

1. Yaegashi, "*Rhodosporidium toruloides*: a new platform organism for conversion of lignocellulose into terpene biofuels and bioproducts". Biotech. Biofuels, 2017
2. Otoupal, "Multiplexed CRISPR-Cas9-based genome editing of *Rhodosporidium toruloides*". mSphere, 2019
3. Brefort, "Cleavage of resveratrol in fungi: Characterization of the enzyme Rco1 from *Ustilago maydis*". Fungal Genetics and Biology, 2011

Acknowledgments

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JBEI 5-Year Goal: Develop fungal strains that deconstruct biomass and generate **10g/L** of targeted bioproducts