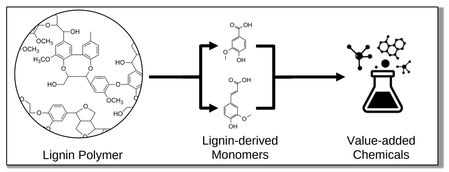
Engineering *Pseudonomas putida* for Improved Utilization of Syringyl Aromatics

Qixing Wei[1], Joshua Mueller[1], Jiantao Guo[2], Wei Niu[1]*

1. Department of Chemical and Biomolecular Engineering, University of Nebraska – Lincoln, Lincoln, NE 68588
2. Department of Chemistry, University of Nebraska – Lincoln, Lincoln, NE 68588 *Corresponding Author, wniu2@unl.edu

Bio-based Chemicals from Lignin

Lignin, a complex organic polymer found in plants, is essential in the formation of cell walls. Its hydrophobic nature enables plants to conduct water. Lignin typically cross-links with polysaccharides, conferring mechanical robustness to the plant cell wall. These characteristics, combined with resistance to degradation, enhance plant survival. Meanwhile, lignin is recalcitrant to depolymerization, which limits its utilization for other purposes. Currently, lignin stream from biomass processing is mainly used as a source of fuel.[1] Further valorization of lignin for bio-based chemical production maximizes economic values that can be extracted.[1,2]



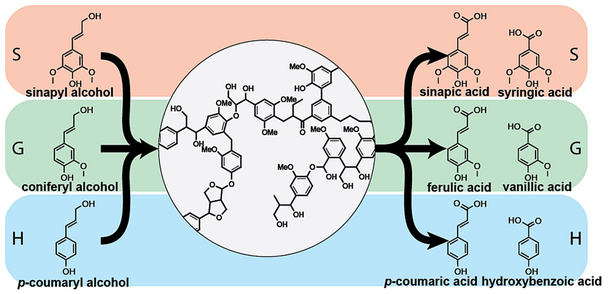


Figure 1. Schematics for the polymerization of monolignols (left) into polymeric lignin (center) followed by its depolymerization into model monomeric aromatic products (right), including hydroxy (H), guaiacyl (G), and syringyl (S) lignin subunits.

Pseudomonas putida KT2440

Pseudomonas putida KT2440 is naturally capable of metabolizing H and G lignin depolymerization products through the *β*-ketoadipate pathway. Meanwhile, KT2440 has limited capability of utilizing S lignin-derived compounds, e.g., syringic acid. The strain encodes a gallic acid metabolic pathway. We have proposed and engineered the conversion of syringic acid into gallic acid in KT2440 by taking advantage of the promiscuous activity of its native vanillate demethylase (VanAB).[3]

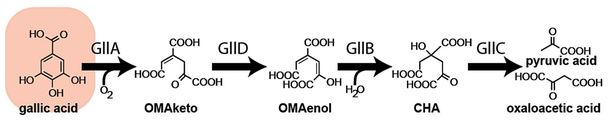


Figure 2. Metabolism of gallic acid.

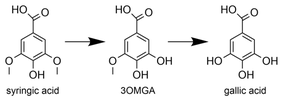


Figure 3. Proposed conversion of syringic acid to gallic acid.

Engineering Syringate Metabolism in KT2440

We have observed that vanillate demethylase (encoded by *vanAB*) can catalyze the demethylation of both meta methoxy groups in syringate. When the *vanAB* gene was overexpressed in *P. putida* KT2440, syringate can be consumed as a carbon source.[3]

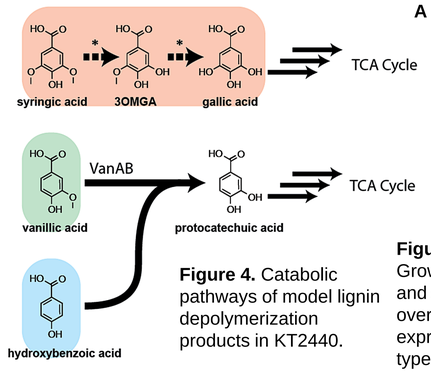


Figure 4. Catabolic pathways of model lignin depolymerization products in KT2440.

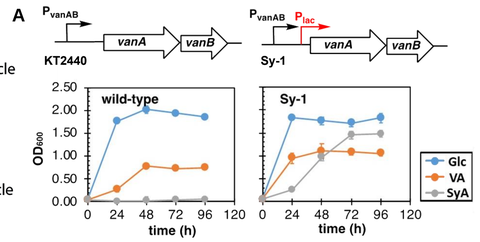


Figure 5. Characterization of wild-type and Sy-1 strains. **A.** Growth characterization on glucose (Glc), vanillate (VA), and syringate (SyA). **B.** Concentration change of syringate over time in culture media. **C.** qPCR analysis of vanA expression normalized to expression level of vanA in wild-type *P. putida* grown in glucose.

Adaptive Lab Evolution of Sy-1 Strain

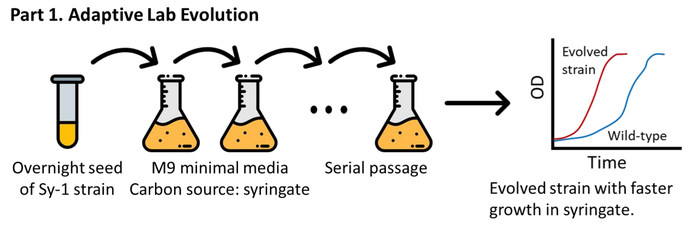


Figure 6. Schematic diagram of ALE procedure. ALE experiments were initiated by starting seed culture in M9 media containing syringate as the sole carbon source at an initial OD of 0.1. Cell growth was monitored daily until the OD was greater than 1.[3]

Adaptive Lab Evolution (ALE) is used to study and engineer the molecular evolution and adaptive changes in microbial populations undergoing long-term selection under specific growth conditions.

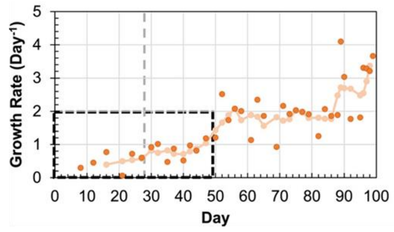


Figure 7. Average growth rates of adaptive laboratory evolution cultures (Sy-1) for 100 days.

Characterization of ALE Strains

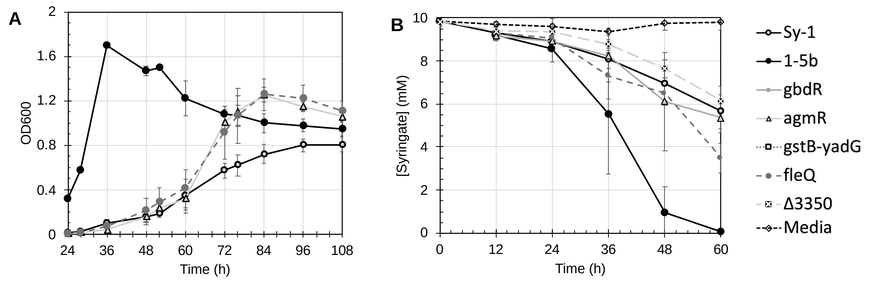


Figure 8. Characterization of Sy-1 mutant on the utilization of syringic acid. **A.** Comparing the growth of modified Sy-1 strain with the growth of Sy-1 and the evolved 1-5b strain. **B.** Consumption of syringate over time by all strains.

Following ALE, the best isolated mutant 1-5b showed improved growth in syringate media. Mutations in *amgR*, *fleQ*, *gbdR* and *gstB-yadG* gene regions were identified in 1-5b strain after genome sequencing and comparison to the Sy-1 strain.[3]

The above mutations were reverse engineered into Sy-1 individually to explore the effect of each mutations on the growth improvement.[3]

All evolved strains showed improved growth characteristics comparing to the parental Sy-1 strain. Benefited with all four mutations, **1-5b** showed **better growth characteristics** than Sy-1 strain with any single mutation.

Characterization of Sy-1 and 1-5b

Growth of Sy-1 and 1-5b stains in media containing various carbon sources, including glucose, ferulic acid, and *p*-coumaric acid, was monitored. Extended growth phase and higher stationary phase ODs were observed for 1-5b in all carbon sources.

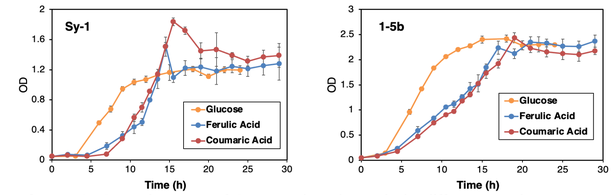


Figure 9. Growth curve of Sy-1 and 1-5b strain in different carbon sources. Each data point represents triplicate measurements.

FleQ is a transcription regulator of biofilm matrix components. The mutation in the *fleQ* gene of 1-5b led to the loss of cell mobility, which may contribute to more efficient carbon utilization.

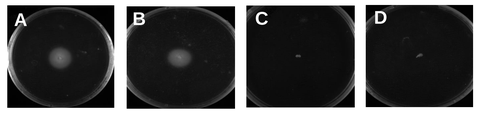


Figure 10. Mobility assay on 0.3% agar LB plate. Cells with flagellum diffuses and forms large growth zone. **A.** KT2440; **B.** Sy-1; **C.** KT2440-fleQSNP; **D.** 1-5b.

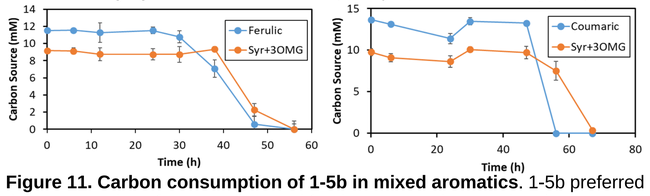


Figure 11. Carbon consumption of 1-5b in mixed aromatics. 1-5b preferred the consumption of *p*-coumarate and ferulate over syringate when it was cultured in media containing all the three carbon sources.

Conclusions and Future Work

- We successfully engineered *P. putida* KT2440 for robust growth using syringate as the sole carbon source through a combined approach of rational strain design and adaptive lab evolution.
- Genome sequencing and genetic analysis revealed mutations that contribute to the enhanced fitness. Mutation in the FleQ protein likely led to higher cell density.
- Future efforts will focus on engineering simultaneous utilization of all model compounds that can be derived from lignin degradation.

References

1. *Science* **2014**, 344, 6185.
2. *Metabolic Engineering*, **2020**, *59,* 156.
3. *Biotechnology and Bioengineering* **2022**, 2541-2550.

Acknowledgement

NEBRASKA CENTER FOR ENERGY SCIENCES RESEARCH

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