DOE Bioenergy Technologies Office (BETO)
2017 Project Peer Review

Synthetic Design Microorganisms for Lignin Fuels and Chemicals

3/9/2017
Synthetic Biology
Biochemical Conversion

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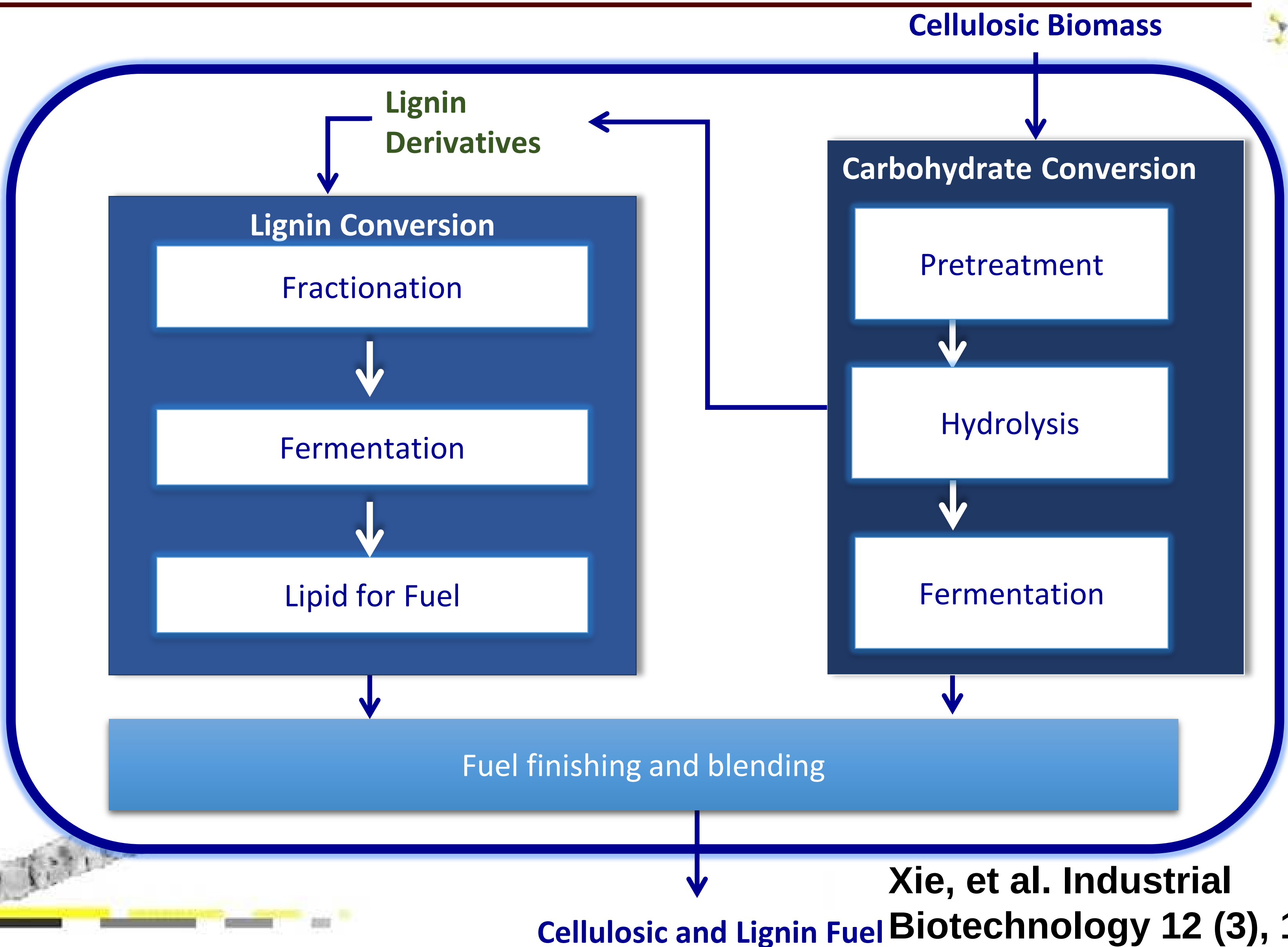
Project Goal: Design of Microorganisms for Lignin Fuel

- Address one of the most challenging issues in biofuel production: the utilization of lignin for fungible fuels.
- Create a process to convert real biorefinery lignin streams into lipids to enable biorefinery economics.
- Synthetic biology methods will be used to modify organisms, and process-relevant fractionation methods will be explored to produce lipids at comparable cost to biodiesel.

- BETO Missions:
  - Additional feedstock for biodiesel
  - Reduce carbon emission by complete biomass usage
  - Improve biorefinery economics and sustainability

Project Goal: From Lignin to Fuels and Chemicals



Xie, et al. Industrial Biotechnology 12 (3), 161-167

Quad Chart Overview

Timeline

- Project start date: 07/01/2013
- Project end date: 06/30/2017
- NCTE has been requested to: 06/30/2017
- Percent complete: 90%

Barriers

- Barriers addressed
  - Ct-A. Feedstock Variability
  - Ct-G. Efficient Intermediate Cleanup
  - Ct-J. Process Integration
  - Ct-L. Aqueous Phase Utilization

Budget

	FY 14-16 Costs	Total Planned Funding (FY 17-Project End Date)
DOE Funded	$1,798,044	$601,933
Project Cost Share (Comp.)*	$550,252	$0

Partners

- Partners
  - University of Tennessee, Knoxville/ Oak Ridge National Lab
  - Washington State University
  - University of British Columbia
- Other collaborators and Industrial Partners:
  - ADM provided the biorefinery slurry
  - ICM inc.

Project Overview

Project Title: Synthetic Design of Microorganisms for Lignin Fuel

Objectives:

(1) Synthetic design of secretion systems and functional modules in *Rhodococcus* to enable effective lignin depolymerization;

(2) Modification and integration of functional modules to improve carbon flux from aromatic compound catabolism to lipid production;

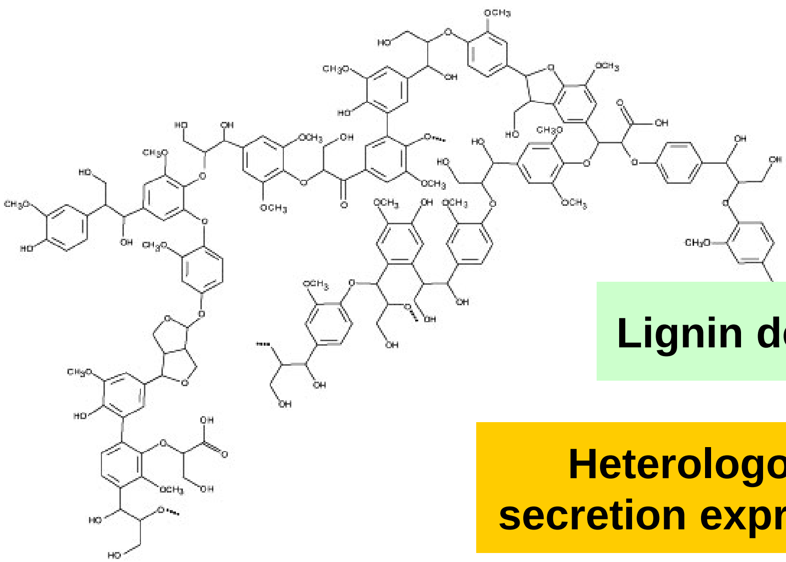
(3) Optimize the fractionation and fermentation of lignin to increase lipid yield using synthetic and wild type strains.

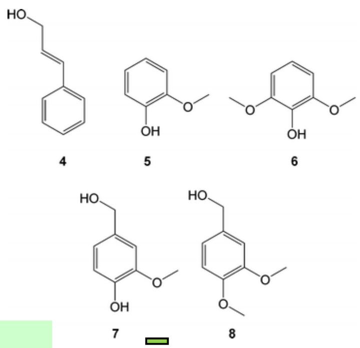
- Systems Biology-guided Biodesign
- Lignin Fractionation to Improve Processibility
- Fermentation Optimization for Yield

→ ~1,000 Times Increases of Lipid Titer

Xie, et al. Industrial Biotechnology 12 (3), 161-167

Technical Approach

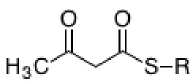




Lignin depolymerization

Heterologous secretion expression

Aromatic compound utilizition capacity



Systems biology-Guided Design

Lipid accumulation capacity

Lipid for Biodiesel

Critical Factors for Success:

(1) Titer: >10g/L Lipid

(2) Reaction Time: <96 hour

(3) Feedstock Load: >30g/L

Major Challenges:

(1) Lignin Depolymerization

(2) Carbon Flux Optimization for Higher Targeted Products

(3) Fermentation Optimization

Lin, et al., Green Chemistry 18 (20), 5536-5547

Management Approach

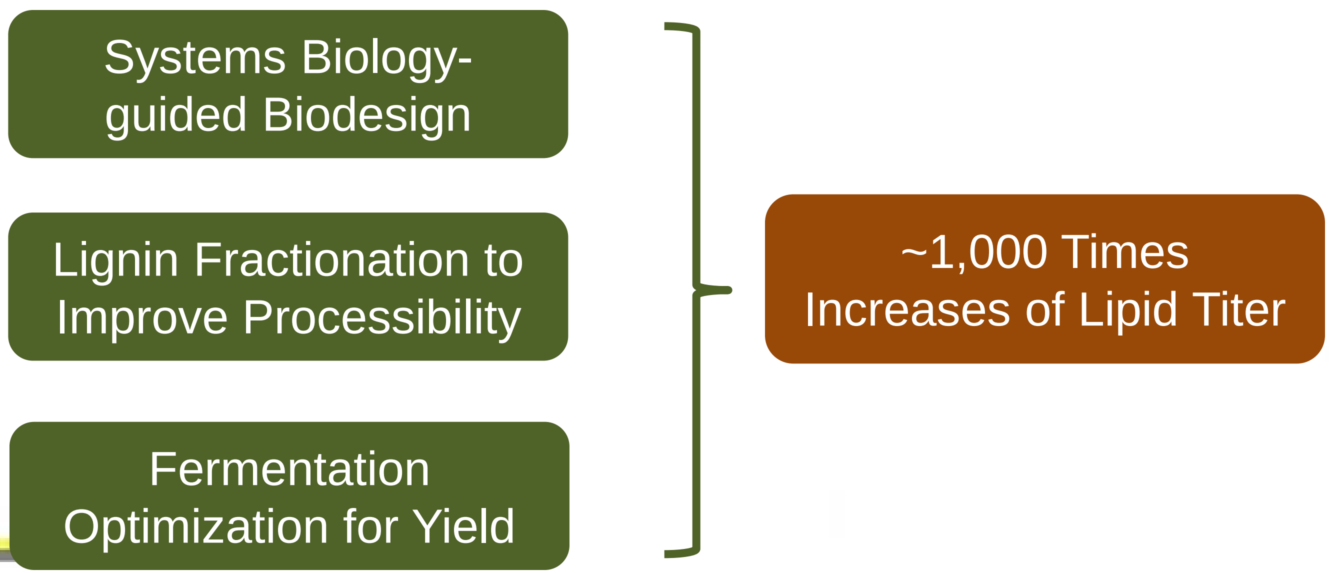
- Defined and measurable milestones were laid out for technology development and commercialization.
- Go/No-Go milestones were set at the end of the year.
- Monthly group teleconferences and semi-annual group meetings were implemented to evaluate the progresses against milestones. Regular teleconferences between the PI and the program management were implemented to evaluate progresses, mitigate risks, and address management issues.
- Engaging industrial partners for deliverables relevant to EERE MYPP. Meetings to engage industry.
- Timely publications to ensure data sharing with community.
- Technoeconomic evaluation are being carried out and industrial partners are engaged to promote commercialization.

Technical Accomplishments – All Major Milestones Met

Milestone Criteria	Actual Accomplishment
Secretion system (including peptide, promoter, rbs) that enables the production of secreted proteins at more than 2% of total secreted proteins.	Secretion system enables >90% of total secreted protein to be laccase, and laccase yield at 13.6g/L, record of bacterial heterologous secretion protein production.
More than one synthetic modules designed to have cell growth on aromatic compound or lignin 3 times more than the control.	Several functional modules enable >1000 times increase of cell growth on lignin substrate.
96 hour fermentation will reach 40-100g/L cell density, 20-40% lipid content (total lipid titer 10g/L).	11.2g/L of lipid titer was achieved after 96 hour of fermentation.

Technical Accomplishments In Details

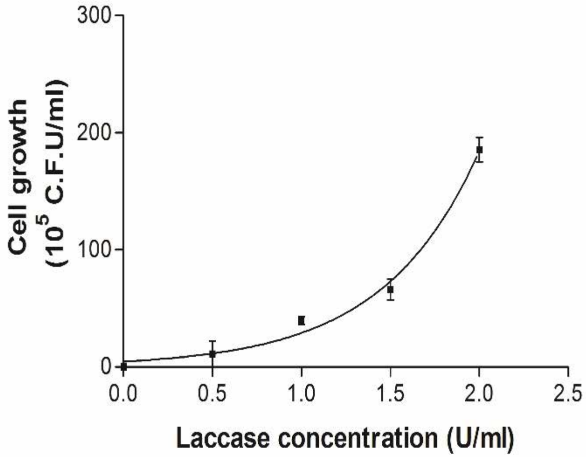
- Discovering and characterizing the enzyme-cell synergy for lignin degradation.
- Biological and chemical design to engineer efficient lignin depolymerization and lipid production functional modules.
- Development of multiple chemical and biological fractionation methods to enhance lipid production.
- Fermentation optimization to enhance lipid titer.

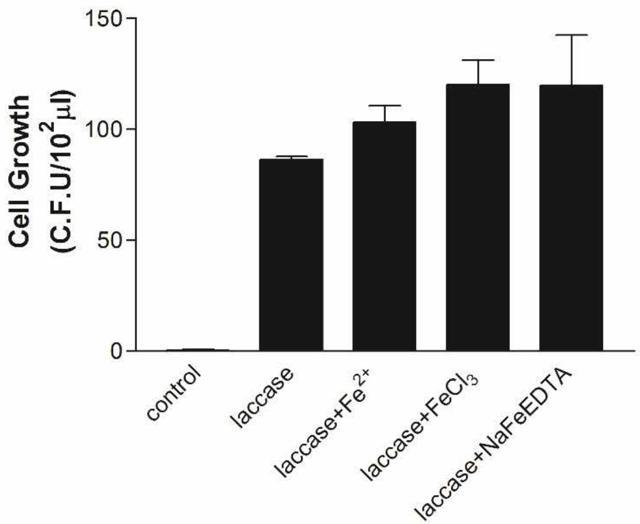


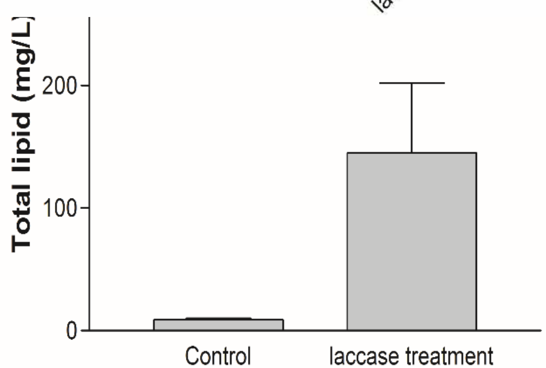
Technical Accomplishment
-- Laccase as an Effective Enzyme to Synergize with Cell for Depolymerization

Zhao, et al., Green Chemistry 18 (5), 1306-1312

Laccase and Cell Synergy



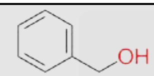
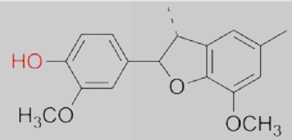
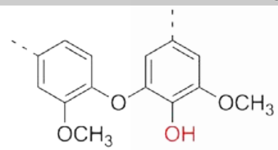
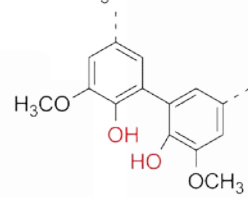
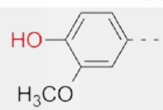
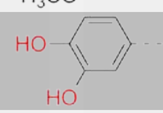
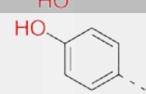
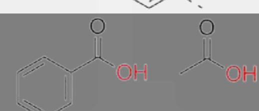




- Laccase-cell co-treatment can promote the cell growth and lipid yield on Kraft lignin significantly.
- Between laccase and Fenton reaction treatments, laccase is much more effective, which may be due to the availability of radicals.
- The synergy leads to 17 times of lipid productivity increase.

Zhao, et al., Green Chemistry 18 (5), 1306-1312

Chemical Validation of Lignin Degradation

Functional Group		Integration region (ppm)	Examples	hydroxyl contents/(mmol/g lignin)				
				I[a]	II[b]	III[c]	IV[d]	V[e]
Aliphatic OH		150.0-145.2		2.38	2.32	1.88	1.98	1.99
C_5 substituted condensed Phenolic OH	β-5	144.6-142.9		0.15	0.02	0.02	0.01	0.01
	4-O-5	142.9-141.6		0.01	0.02	0.01	0.02	0.01
	5-5	141.6-140.1		0.00	0.05	0.02	0.03	0.03
Guaiacyl phenolic OH		140.1-138.8		1.32	1.40	0.98	1.00	1.02
Catechol type OH		138.8-138.2		0.04	0.02	0.01	0.02	0.02
p-hydroxy-phenyl-OH		138.2-137.3		0.08	0.06	0.02	0.03	0.03
Carboxylic acid OH		136.6-133.6		0.50	0.15	0.16	0.29	0.06

Hydroxyl content of lignin determined by quantitative ^{31}P NMR after derivatization with TMDP

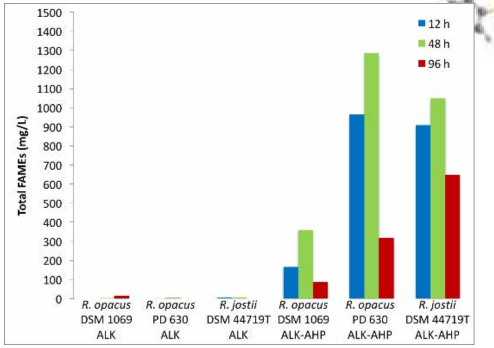
Technical Accomplishments – Development of Fractionation Methods to Improve Lignin Conversion

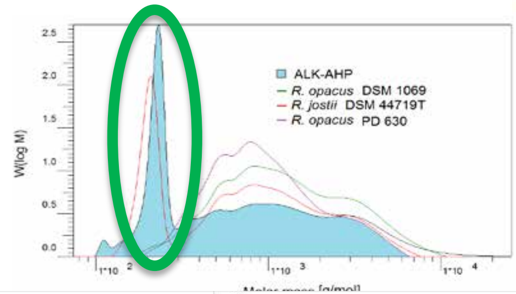
Xie, et al., ACS Sustain. Chem. Eng. 2017 (in press)
Le, et al., ACS Advances, 2017 (in press)
Sadeghifar, ACS Sustain. Chem. Eng. 2016 (in press)

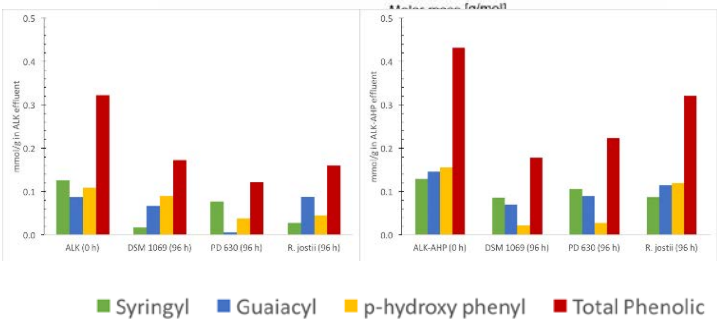
Viability of *Rhodococcus* on ALK and ALK-AHP Fractionated Effluents

- *Rhodococci* strains are able to proliferate on ALK and ALK-AHP effluents
  1) **Alkaline Pre-treatment (ALK)**
  80 °C, 1 h, solids 10% (w/v) and 8% NaOH/g dry biomass
  2) **Alkaline Pre-treatment + Alkaline Hydrogen Peroxide Pre-treatment (ALK-AHP)**
  60 °C, 0.5 h, 25 mg peroxide per g biomass

- ALK-AHP is the more ideal substrate for viability and oil production
  - **~1.5 g/L FAMEs were produced by the strains grown on ALK-AHP effluent**
    - **~10 mg/L FAMEs produced on ALK**
  - Aggressive catabolysis of low MW aromatics 100 – 300 g/mol
  - Preferred H-type lignin, followed by G and S-type
  - PD 630 and *R. jostii* exhibited oleaginous yields, 42.1% and 23.3%, respectively

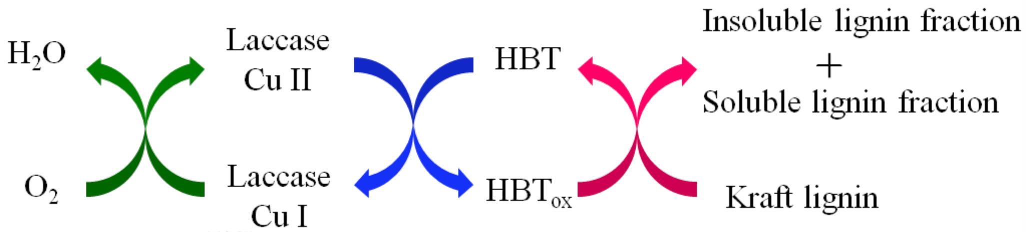


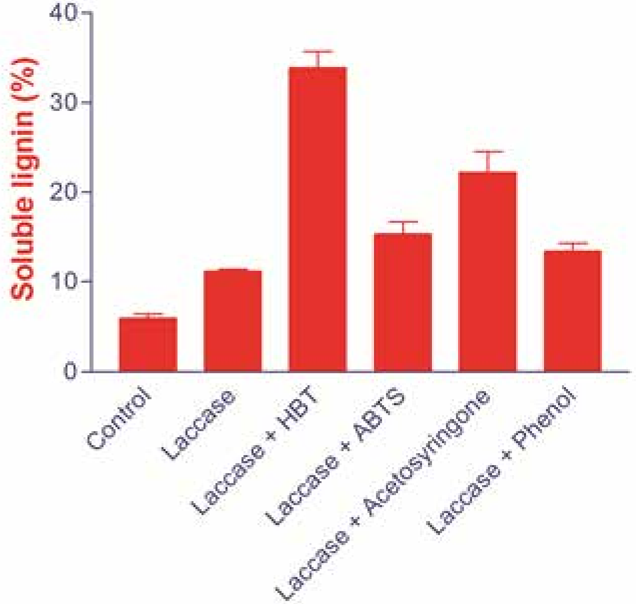


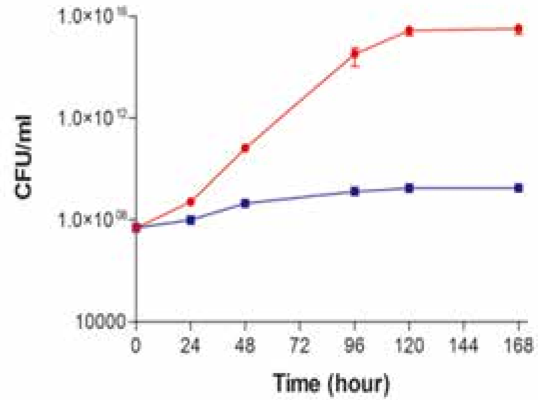


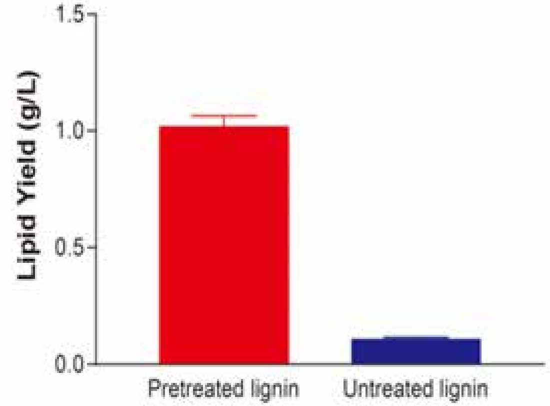
Le et al. RSC Advances In press (DOI: 10.1039/c6ra28033a)

Lignin Depolymerization and Solubilization by Laccase-Mediator system





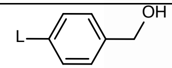
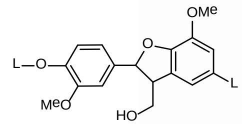
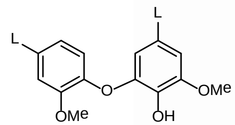
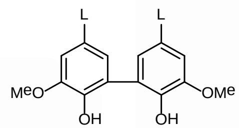
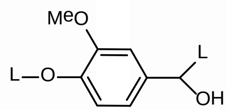
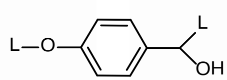
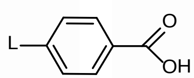




Laccase-mediator system has enhanced redox transfer in lignin depolymerization, released >35% of lignin, and enabled >1g/L titer in conversion.

Xie, et al., ACS Sustain. Chem. Eng. 2017 (in press)

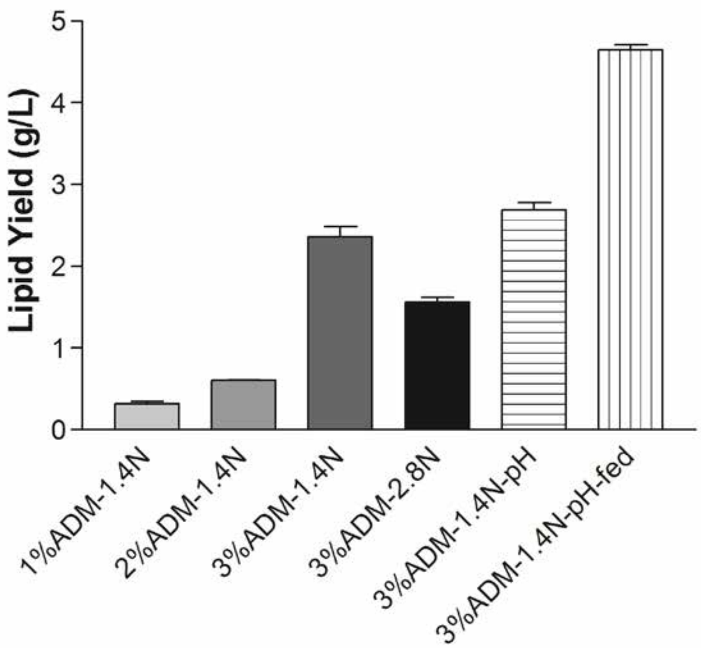
Lignin Depolymerization and Solubilization by Laccase-Mediator system

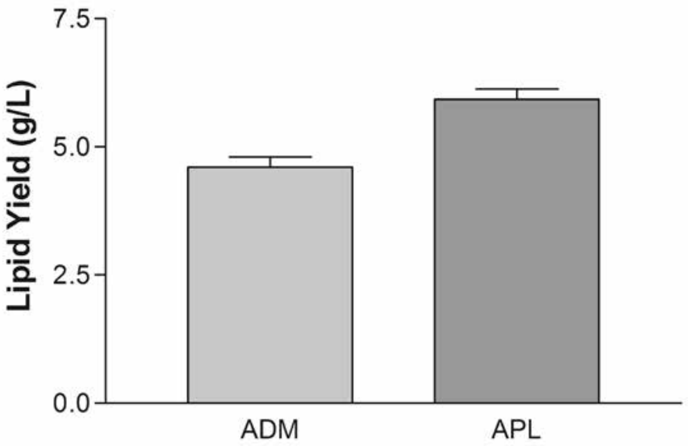
Chemical shift range (ppm)	Assignment	Structure sample	Consistency (mmol/g)		
			Untreated lignin	Lac	Lac+HBT
150.0 – 145.5	Aliphatic OH		2.57	2.33	1.90
144.70 – 142.92	β-5		0.53	0.41	0.29
142.92 – 141.70	4-O-5		0.37	0.35	0.26
141.70 – 140.20	5-5		0.59	0.51	0.40
140.20 – 138.81	Guaiacyl		1.90	1.37	1.10
138.18 – 137.30	p-hydroxylphenyl		0.22	0.21	0.12
136.60 – 133.60	Carboxylic acid OH		0.46	0.32	0.14
144.7---140.0	C5 substituted “condensed”	--	1.56	1.35	0.98

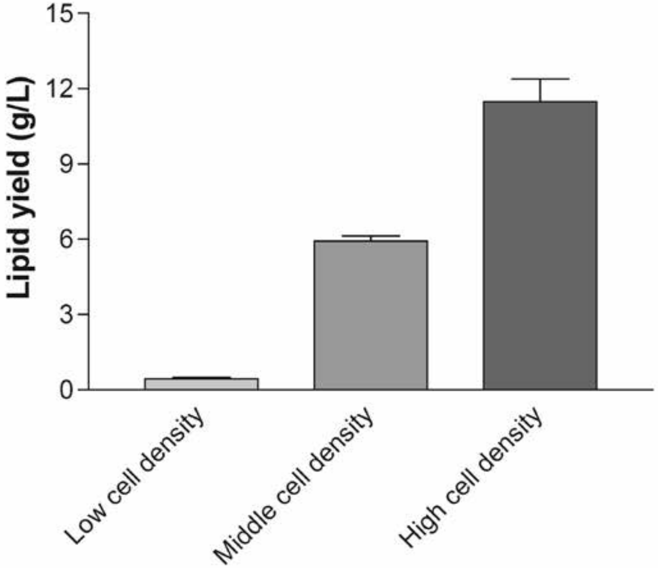
Xie, et al., ACS Sustain. Chem. Eng. 2017 (in press)

Technical Accomplishments – Fermentation Optimization

Fermentation Optimization for High Lipid Yield – Nitrogen Fermentation







The nitrogen concentration optimization and cell density optimization has led to lipid yield >10g/L for biorefinery waste conversion. The combination of strain engineering, lignin fractionation, and fermentation optimization allows us to accomplish our technical targets.

Technical Accomplishments

- **All major technical milestones were met. We are working on achieving final project target yield in a 2 Liter bioreactor.**
- **13.6g/L laccase yield in secretion expression system represents the world record on heterologous secretion protein expression in bacteria.**
- **>10g/L lipid titer on lignin represents more than 1000 times increase of previous published state-of-the-art on EOL lignin.**
- **Cell growth increased about 1000 times on Kraft lignin, which is also a significant improvement over the previous reports.**
- **Biorefinery waste solid load can be between 1 to 5%.**
- **15 publications published and more underway.**
- **Two comprehensive provisional patent profilios filed. The commercialization partners have been identified.**

Publication List

1. Qiang Li, Shangxian Xie, Wilson K. Serem, Mandar T. Naik, Li Liu, *Joshua S. Yuan**, Quality Carbon Fiber from Fractionated Lignin, *Green Chemistry*, In Press.
2. Shangxian Xie; Qining Sun, Yunqiao Pu, Furong Lin, Su Sun, Xin Wang, Arthur J. Ragauskas, *Joshua S. Yuan**, Advanced chemical design for efficient lignin bioconversion, *ACS Sustainable Chemistry & Engineering*, In press.
3. Hasan Sadeghifar, Tyrone Wells, Rosemary K. Le, Fatemeh Sadeghifar, *Joshua S. Yuan*, Arthur J. Ragauskas, Fractionation of organosolv lignin using acetone: water and properties of the obtained fractions, *ACS Sustainable Chemistry & Engineering*, In press.
4. Tyron Wells Jr., Rosemary K. Le, Parthapratim Das, Xianzhi Meng, Ryan Stocklosa, Adita Bhalla, David B. Hodge, *Joshua S. Yuan* and Arthu J. Raguaskas, Conversion of corn stover alkaline pre-treatment waste streams into biodiesel via rhodococci, *RSC Advances*, In press.
5. Shangxian Xie, Authur J. Ragauskas, *Joshua S. Yuan**, Lignin conversion: opportunities and challenges for the integrated biorefinery, *Industrial Biotechnology,* 2016, 12 (3), 161-167.
6. Cheng Zhao, Shangxian Xie, Yunqiao Pu, Rui Zhang, Fang Huang, Arthur J. Ragausaks, *Joshua S. Yuan**, Synergistic enzymatic and microbial conversion of lignin for lipid, *Green Chemistry*, 2016, 18, 1306-1312.
7. Su Sun[$], Shangxian Xie[$], Hu Chen, Xin Qin, Yanbing Cheng, Yan Shi, Susie Y. Dai, Xiaoyu Zhang, *Joshua S. Yuan**, Genomic and molecular mechanisms for efficient biodegradation of aromatic dye, *Journal of Hazardous Material*, 2016, 9, 302:286-295.
8. Shangxian Xie, Xing Qin, Yanbing Cheng, Weichuan Qiao, Su Sun, Scott Sattler, Zhanguo Xin, Susie Y. Dai, Katy Gao, Bin Yang, Xiaoyu Zhang, and *Joshua S. Yuan**, Simultaneous conversion of all cell wall components with oleaginous fungi without chemical pretreatment, *Green Chemistry*, 2015,17, 1657-1667.
9. Shangxian Xie, Ryan D. Syrenne, Su Sun, *Joshua S. Yuan**, Exploration of Natural Biomass Utilization Systems (NBUS) for advanced biofuel--from systems biology to synthetic design, *Current Opinion in Biotechnology*, 2014, 27:195-203.
10. Yucai He, Xiaolu Li, Peiyu Leu, John R. Cort, and Bin Yang*, "Lipid Production from Dilute Alkali Corn Stover Lignin by *Rhodococcus* Strains," ACS Sustainable Chemistry & Engineering, 2016. DOI: 10.1021/acssuschemeng.6b02627.
11. Yucai He, Xiaolu Li, Xiaoyun Xue, Marie S Swita, Andy Smith, and Bin Yang*, "Biological Conversion of The Aqueous Byproduct from Hydrothermal Liquifaction of Algal and Pine Wood with Rhodococci," *Bioresource Technology*, 2017, 224: 457–464.
12. Libing Zhang, Yunqiao Pu, John R. Cort, Art J. Ragauskas, a, and Bin Yang*, "Revealing the Molecular Structure Basis for the Recalcitrance of Hardwood and Softwood in Dilute Acid Flowthrough Pretreatment," ACS Sustainable Chemistry & Engineering, 2016. DOI: 10.1021/acssuschemeng.6b01491, Publication Date (Web): October 3, 2016
13. Yan Lishi, Pu Yunqiao, Bowden Mark, Ragauskas Arthur, and Bin Yang*, "Physiochemical Characterization of Flowthrough Pretreated Lignocellulosic Biomass," ACS Sustainable Chemistry & Engineering, 2016, 4 (1), pp 219–227, DOI: 10.1021/acssuschemeng.5b01021.
14. Libing Zhang, Lishi Yan, Zheming Wang, Dhrubojyoti lascar, Marie Swita, John R. Cort, and Bin Yang*, "Characterization of Lignin Derived from Water-only and Dilute Acid Flowthrough Pretreatment of Poplar Wood at Elevated Temperatures," Biotechnology for Biofuels, 2015, 8 (1):1-14 doi: 10.1186/s13068-015-0377-x.
15. Sawsan Amara, Nicolas Seghezzi, Hiroshi Otani, Carlos Diaz-Salazar, Jie Liu & Lindsay D. Eltis, Characterization of key triacylglycerol biosynthesis processes in rhodococci, Scientific Report, 6, 24985.

Relevance

- The research provided an effective approach to convert lignin to lipid as biofuel precursor, which well aligns with the MYPP goal to improve biorefinery efficiency and cost effectiveness. In particular, the biorefinery waste stream will improve biorefinery sustainability and reduce the cost of fuel toward $3/GGE, which addresses the need of BETO, the MYPP goals, and the challenges in biofuel industry.

- The technology paved the path for multistream integrated biorefinery to produce multiple products for biomanufacturing.

- The research significantly advanced the current state-of-the-art. The advanced scientific design can be translated for commercialization. The technologies can be integrated with different platforms to produce valuable compounds from lignin.

- The technology has been licensed and will be scaled up for commercialization as solutions for biorefinery.

Future Work

- **We are scaling up the lipid production in 2 liter reactors.**
- **We are finishing the technoeconomic analysis to evaluate the value adding to the biorefinery.**
- **We are working on the project final validation and report.**

Acknowledgement

Project Management:

Jay Fitzgerald   Joshua Messner

CoPIs:

Dr. Art Ragauskas   Dr. Bin Yang

Dr. Lindsay Eltis   Dr. Susie Y. Dai

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ADM

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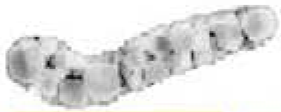

U.S. DEPARTMENT OF ENERGY | Energy Efficiency & Renewable Energy

Aspen Plus model of Lignin to Lipids