DOE Bioenergy Technologies Office (BETO)
2015 Project Peer Review

Synthetic Design Microorganisms for Lignin Fuels and Chemicals

3/26/2015
Synthetic Biology

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This presentation does contain proprietary information

Project Goal: Design of Microorganisms for Lignin Fuel

- The proposed research aims to address one of the most challenging issues in biofuel production: the utilization of lignin for fungible fuels.
- Project Outcome: A viable biological platform for conversion of lignin into advanced biofuels.
- Technology Area Goals: Advanced synthetic biology design to enable biofuels from biorefinery waste
- Project started July, 2013 as part of the synthetic biology program.
- BETO Missions:
  - Additional feedstock for biodiesel
  - Reduce carbon emission by complete biomass usage
  - Improve biorefinery economics and sustainability

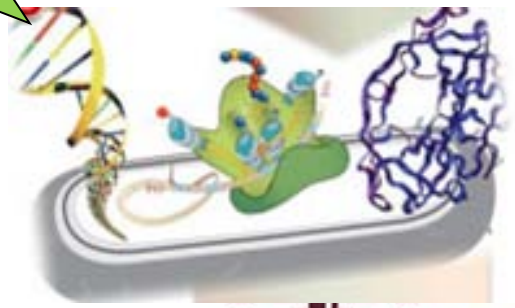
Project Goal: From Lignin to Fuel

30% replacement of the current US petroleum consumption with biofuels by 2030, and "will require more than approximately **one billion** dry tons of biomass feedstock annually"

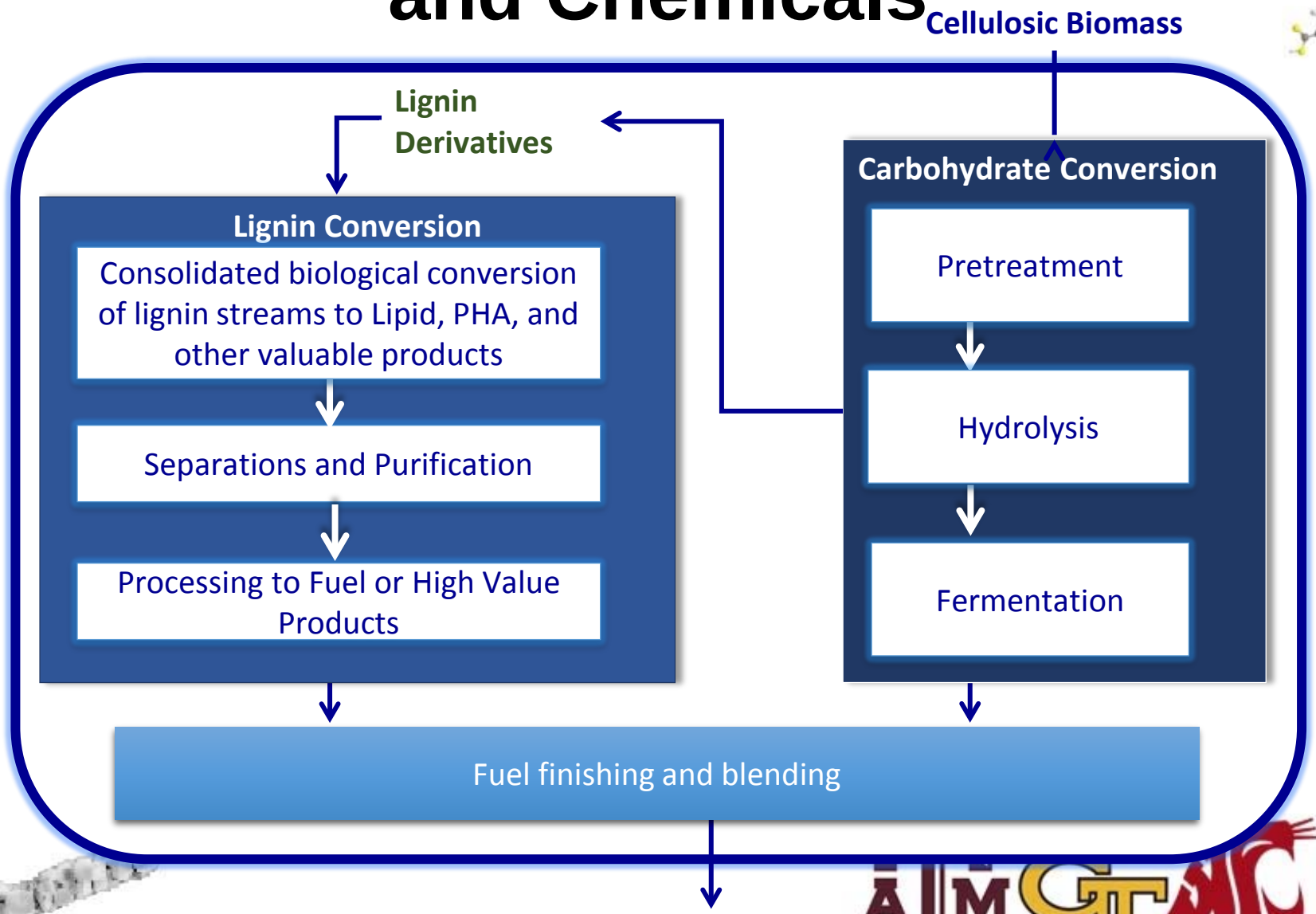
--The US Biomass R&D Technical Advisory Committee

150-300 million tons of lignin annually

- **Polymer of aromatic compound**
- **Higher energy content than cellulose 9000-11000 Btu/lb vs. 7300-7500 Btu/lb**
- **Difficult to decompose and toxic to environment**



Project Goal: From Lignin to Fuels and Chemicals



Quad Chart Overview

Timeline

- Project start date: 07/01/2013
- Project end date: 06/30/2015
- NCTE has been requested to: 06/30/2016
- Percent complete: 60%

Barriers

- Barriers addressed
  - Bt-H. Cleanup/Separation
  - Bt-J. Biochemical Conversion Process Integration

Budget

	FY 14 Costs	Total Planned Funding (FY 15-Project End Date
DOE Funded	676,582.49	$1,723,692.02
Project Cost Share (Comp.)*	166,020 (19.7%)	530,000 (23.5%)

Partners

- Partners
  - University of Tennessee, Knoxville
  - Washington State University
  - University of British Columbia
- Other Collaborations: ADM provided the biorefinery slurry
- Commercial Partner: Cleamol LLC: licensee for technology

Project Overview

Project Title:

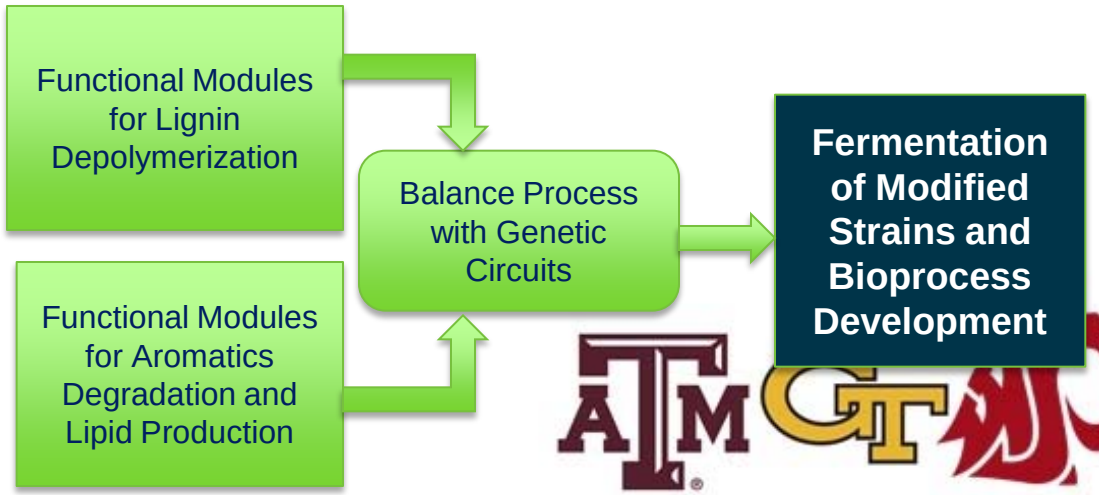
Synthetic Design of Microorganisms for Lignin Fuel

Objectives:

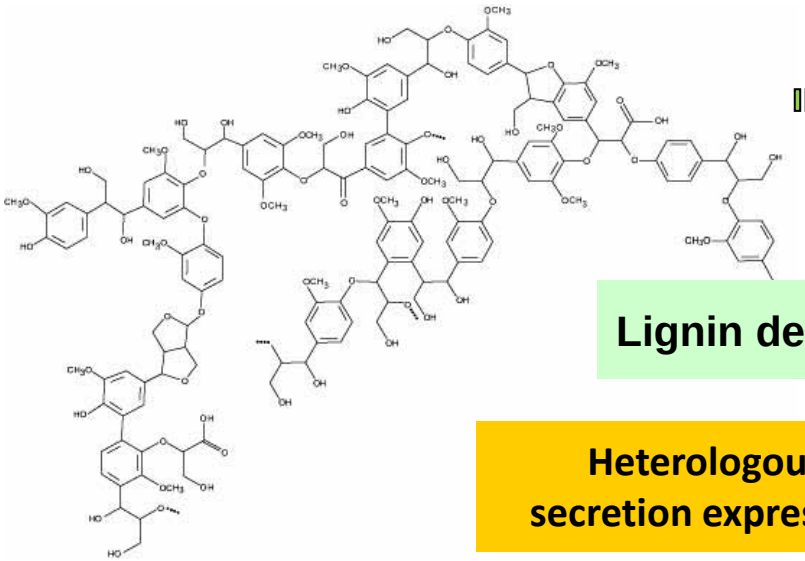
(1) Synthetic design of secretion systems and functional modules in RHA1 to enable effective lignin depolymerization;

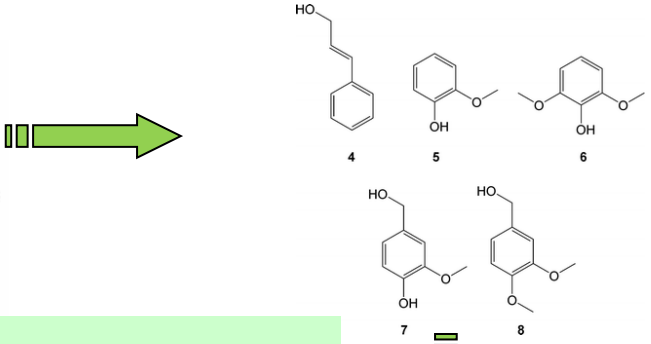
(2) Modification and integration of functional modules to improve carbon flux from aromatic compound catabolism to lipid production, as well as the design of genetic circuits to balance lignin depolymerization and derivative conversion for higher conversion efficiency;

(3) Optimize the fermentation of lignin to lipids using synthetic and wild type strains.



Technical Approach

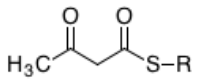




Lignin depolymerization

Heterologous secretion expression

Aromatic compound utilizition capacity



Systems biology-Guided Design

Lipid accumulation capacity

A Valuable Product

Critical Factors for Success:

(1) Titer: >4g/L Lipid

(2) Reaction Time: <72 hour

(3) Feedstock Load: >20g/L

Major Challenges:

(1) Lignin Depolymerization

(2) Consolidated Lignin Fermentation

(3) Carbon Flux Optimization for Higher Targeted Products

Management Approach

- Defined and measurable milestones were laid out for scientific development. Technical milestones were established for most of the quarters.
- Clear and measurable benchmarks and targets were laid out for technology commercialization. Preliminary technoeconomic evaluation was carried out to set the targets.
- Go/No-Go milestones were set at the end of budget period.
- Monthly group teleconferences were implemented to evaluate the progresses against milestones. Semi-annual group meetings were implemented to coordinate the efforts and strategize the future directions.
- Monthly teleconferences between the PI and the program management were implemented to evaluate progresses, mitigate risks, and addresses management issues.
- Engaging industrial partners for deliverables relevant to EERE MYPP.
- Rapid IP protection and commercialization by licensee.

Technical Progresses

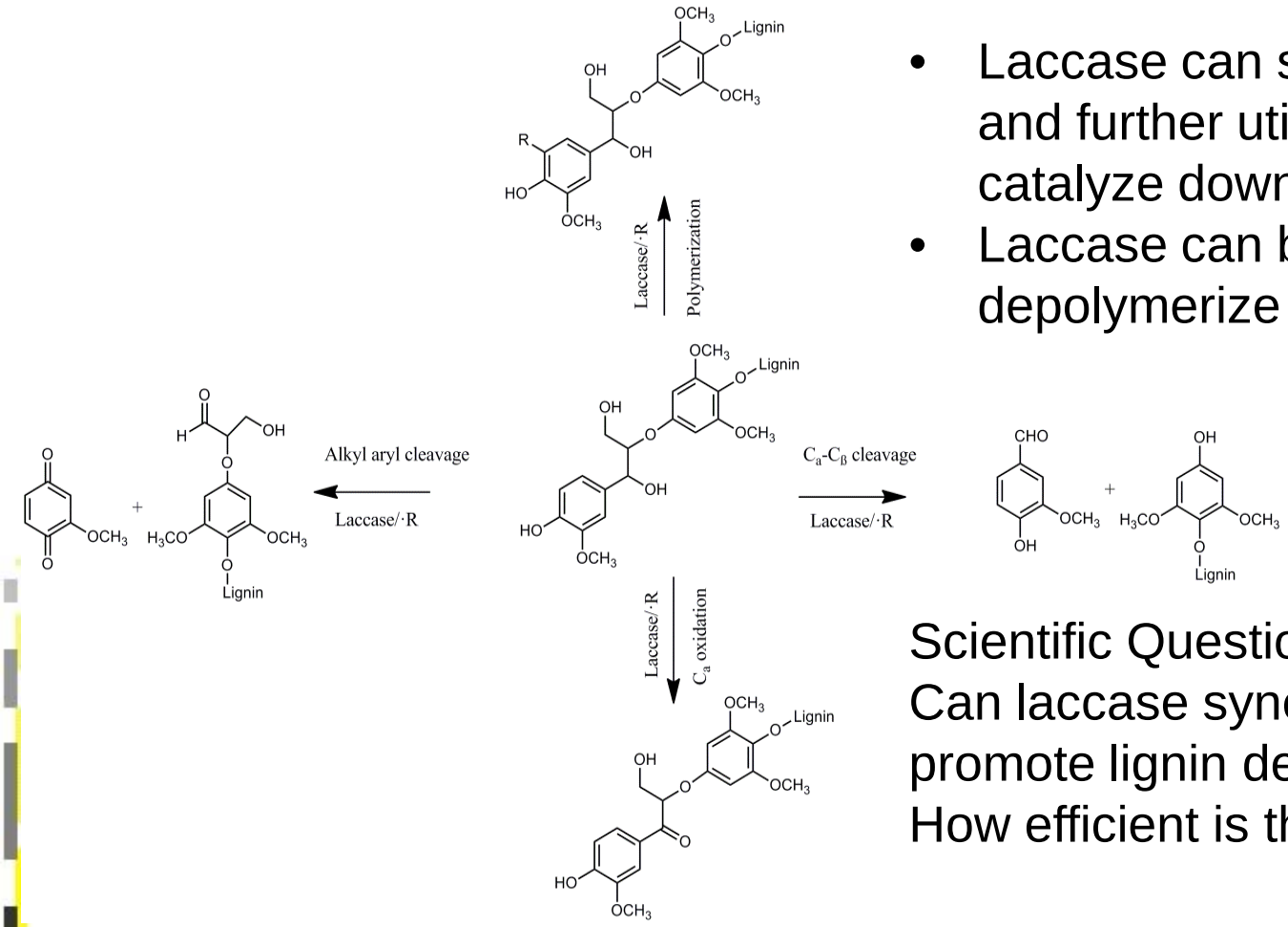
ID	Milestone	Progresses	Quarter
A.1. ML.1	A secretive expression system to secrete protein at 2% of total secreted protein	The milestone has been met, as shown by the results that has been validated by both gel picture and the enzyme activity	3
A.G N.1	Rhodococcus strains developed in Task A must meet the following criteria: At the end of Year 1, at least one strain can utilize about 20% of one type of processed lignin. At the end of year 2, at least 3 strains can grow on lignin as sole carbon source an	The milestone has been met as evaluated by the lignin consumption as measured by Purssian blue assay	8
B.1. ML.1	Deliver more than 2 secretion system (including peptide, promoter, rbs) that enables the production of secreted proteins at more than 2% of total secreted proteins.	The milestone has been met, as reflected by the results based on enzyme activity and SDS PAGE.	4
B.2. ML.1	More than one synthetic modules designed to have cell growth on aromatic compound or lignin 3 times more than the control.	This milestone has been met by CFU count of the cells. The results confirmed over 100 times increase of cell growth	5
B.3. ML.1	>1 strain optimized for both lignin depolymerization and aromatics to lipid to reach fermentation of processed lignin as the sole carbon source at over 20% yield; 3 strains converting lignin model compounds at over 20% yield.	We do have strains that can grow on lignin as justified by cell growth data. The milestone has been met based on analysis of lignin degradation.	6
B.4. ML.1	>1 strain optimized for both lignin depolymerization and aromatics to lipid to reach fermentation of processed lignin as the sole carbon source at over 20% yield; 3 strains converting lignin model compounds at over 20% yield.	Same as above	7

Technical Progresses

- **Discovering and characterizing the enzyme-cell synergy for lignin degradation.**
- **Biological and chemical design to overcome four technical barriers to engineer an efficient lignin depolymerization functional module. The strain enables the consolidated lignin processing (CLP).**
- Designing the lipid production and aromatic compound degradation modules. Functional module integration to achieve high lipid titer.
- Designing the consolidated lignin processing for lignin-to-PHA route.

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

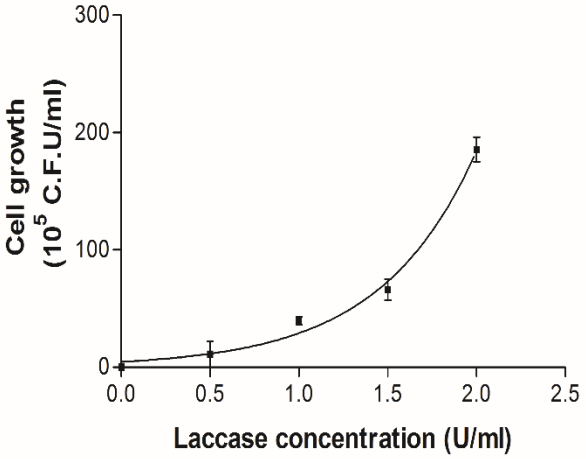
Unique Features of Laccase for Lignin Degradation

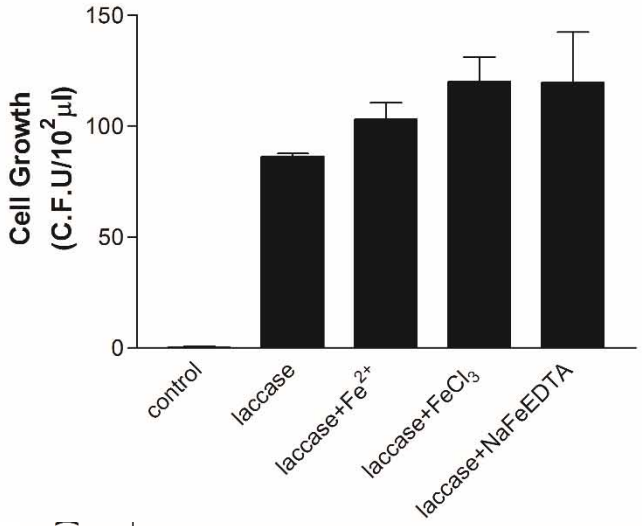


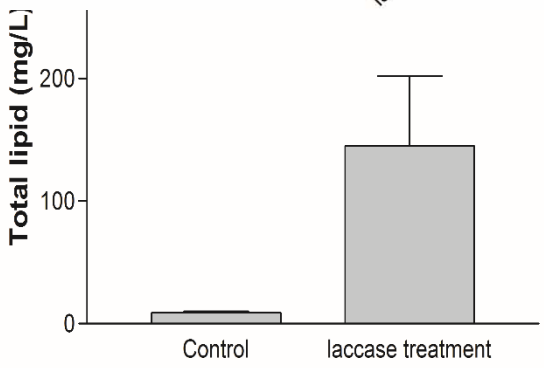
- Laccase can self-generate radicals and further utilize the radicals to catalyze downstream reactions.
- Laccase can both polymerize and depolymerize aromatics.

Scientific Questions:
Can laccase synergize with cells to promote lignin depolymerization?
How efficient is the reaction?

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 indicated that cell might consumed the low molecular weight products to promote the reaction toward lignin depolymerization.

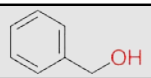
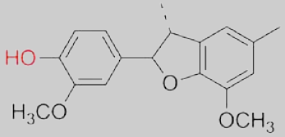
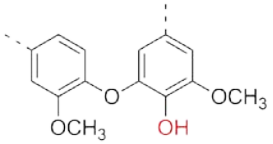
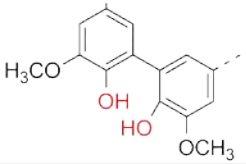
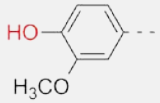
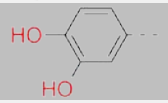
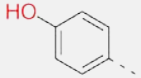
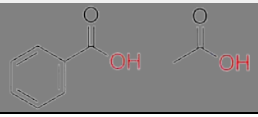
Synergy at Chemical Level

Lignin Sample	M_n	M_w	Polydispersity index
I[a]	1.73×10^3	8.08×10^3	4.66
II[b]	1.67×10^3	7.58×10^3	4.55
III[c]	2.12×10^3	2.68×10^4	12.7
IV[d]	2.15×10^3	3.30×10^4	15.3
V[e]	2.12×10^3	1.90×10^4	8.98

[a] No cell – Group I
[b] cell only – Group II
[c] Cell + Laccase – Group III
[d] Cell + Laccase + Fe – Group IV
[e] Cell + Laccase + Fe + H_2O_2 – Group V

Molecular weight of lignin increased significantly upon laccase-cell treatment. Our interpretation is that cell consumes the low molecular weight components from laccase-pretreated lignin.

Synergy at Chemical Level

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
	β-5	144.6-142.9		0.15	0.02	0.02	0.01	0.01
C_5 substituted condensed Phenolic OH	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

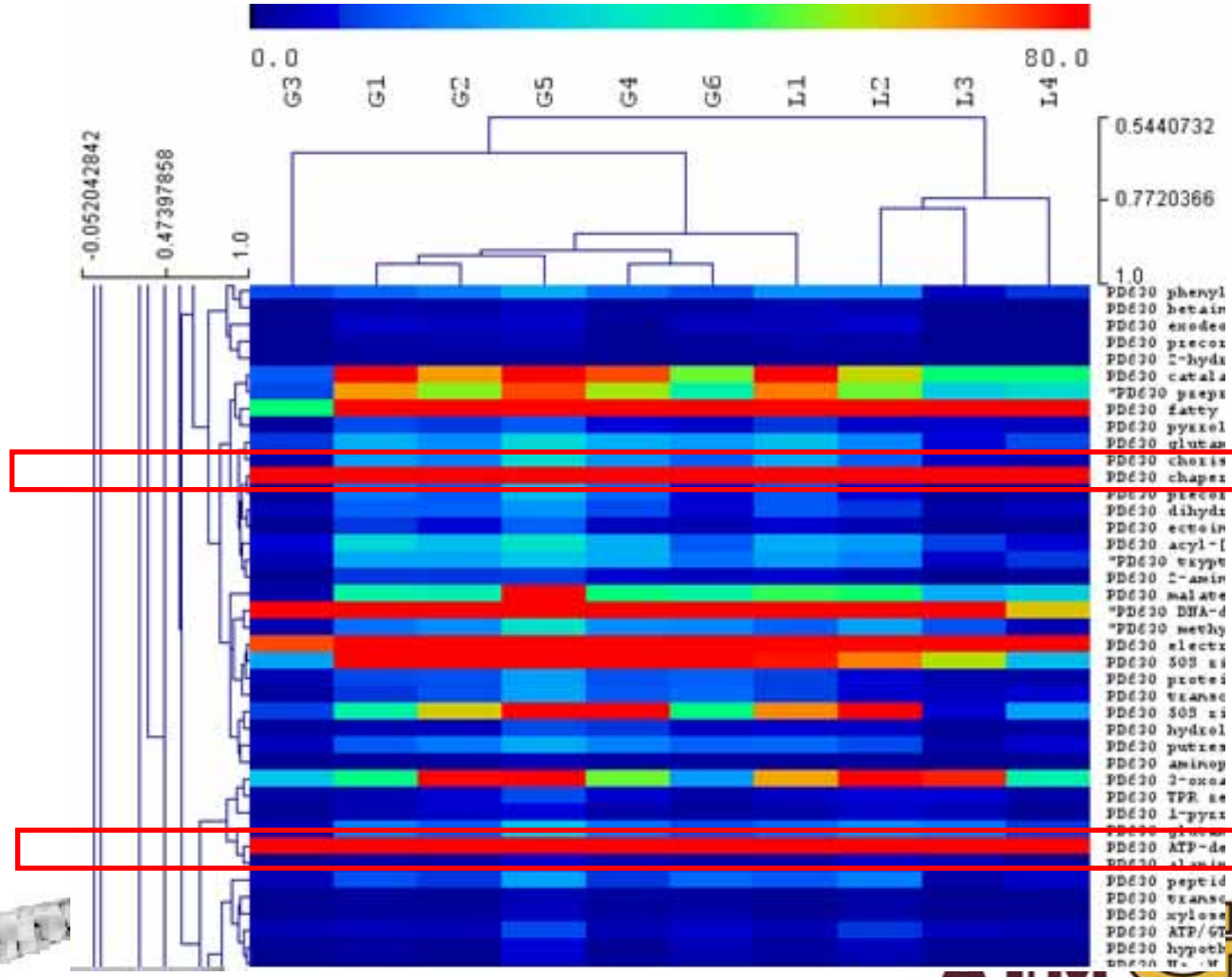
Conclusion

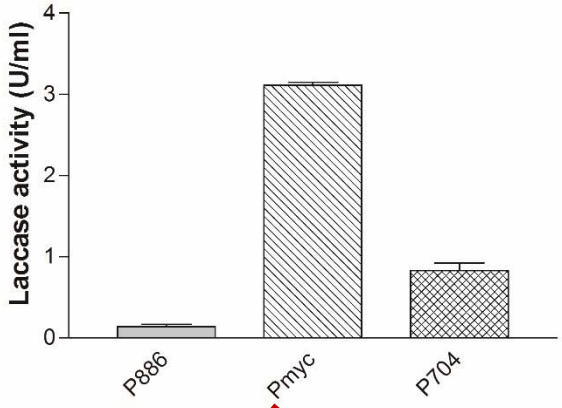
- Proper ligninase enzyme treatment will lead to significantly increased cell growth on lignin.
- Laccase can synergize with the cell to promote cell growth and lipid yield. The biological and chemical mechanisms for such synergy could be as follows:
  - Laccase and cell degrade different functional groups in lignin.
  - Laccase can degrade the most abundant groups in lignin.
  - Cell may have consumed the monomers and oligomers degraded from lignin, to promote the reaction toward depolymerization.
  - The efficient degradation of lignin by laccase may be due to the self-generated radicals.
- Laccase is an effective enzyme for engineering the consolidated lignin processing (CLP).

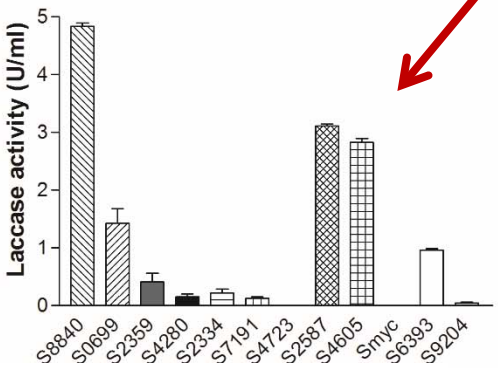
Technical Progresses – Functional Module 1: Lignin Deoplymerization -- Advanced Biological and Chemical Design toward a Consolidated Lignin Processing (CLP)

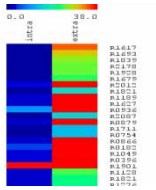
Systems Biology-Guided Design

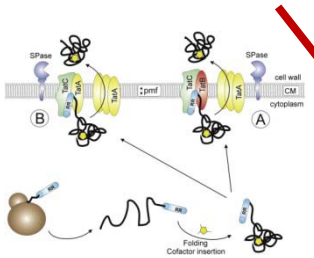
Build up the promoter pool based on proteomics analysis

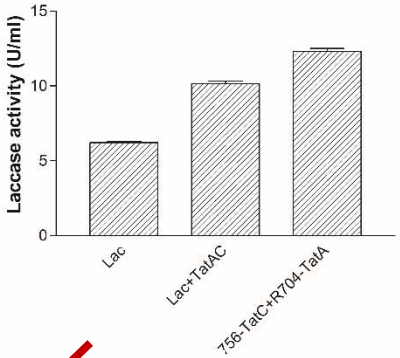


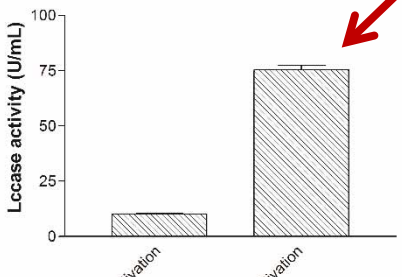












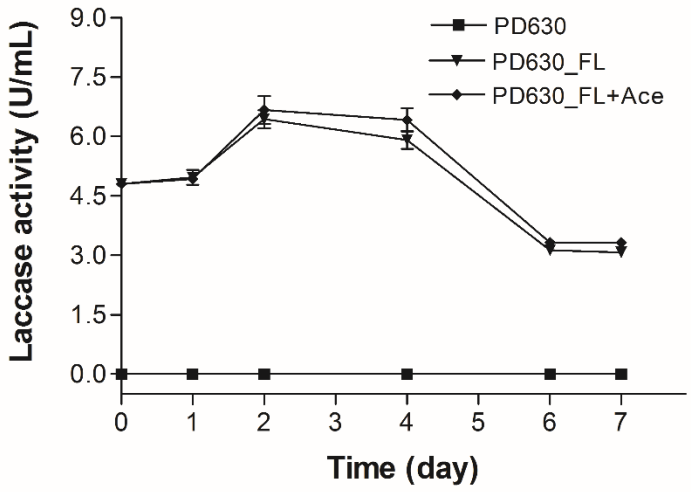
Challenge 1: Strong Promoters and RBS to Drive the Expression of the Laccase Enzyme.

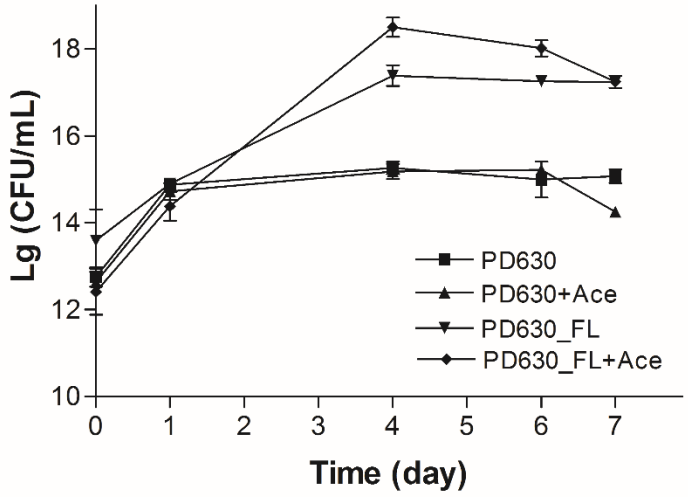
Challenge 2: Proper Secretion Peptides to Promote Efficient Secretion of Laccase

Challenge 3: Effective Secretion System to Enhance the Secretive Expression of Laccase

Challenge 4: Optimized Fermentation to Reduce Laccase Toxicity

Consolidated Lignin Conversion Integrating Chemical and BioDesign





- **The strain engineered with strong laccase expression can be used for consolidated lignin processing (CLP).**
- **The engineered strain can lead to over 1000 fold increase of cell growth.**
- **The study indicated the effectiveness of biological and chemical design in achieving consolidated lignin processing (CLP).**

Conclusions

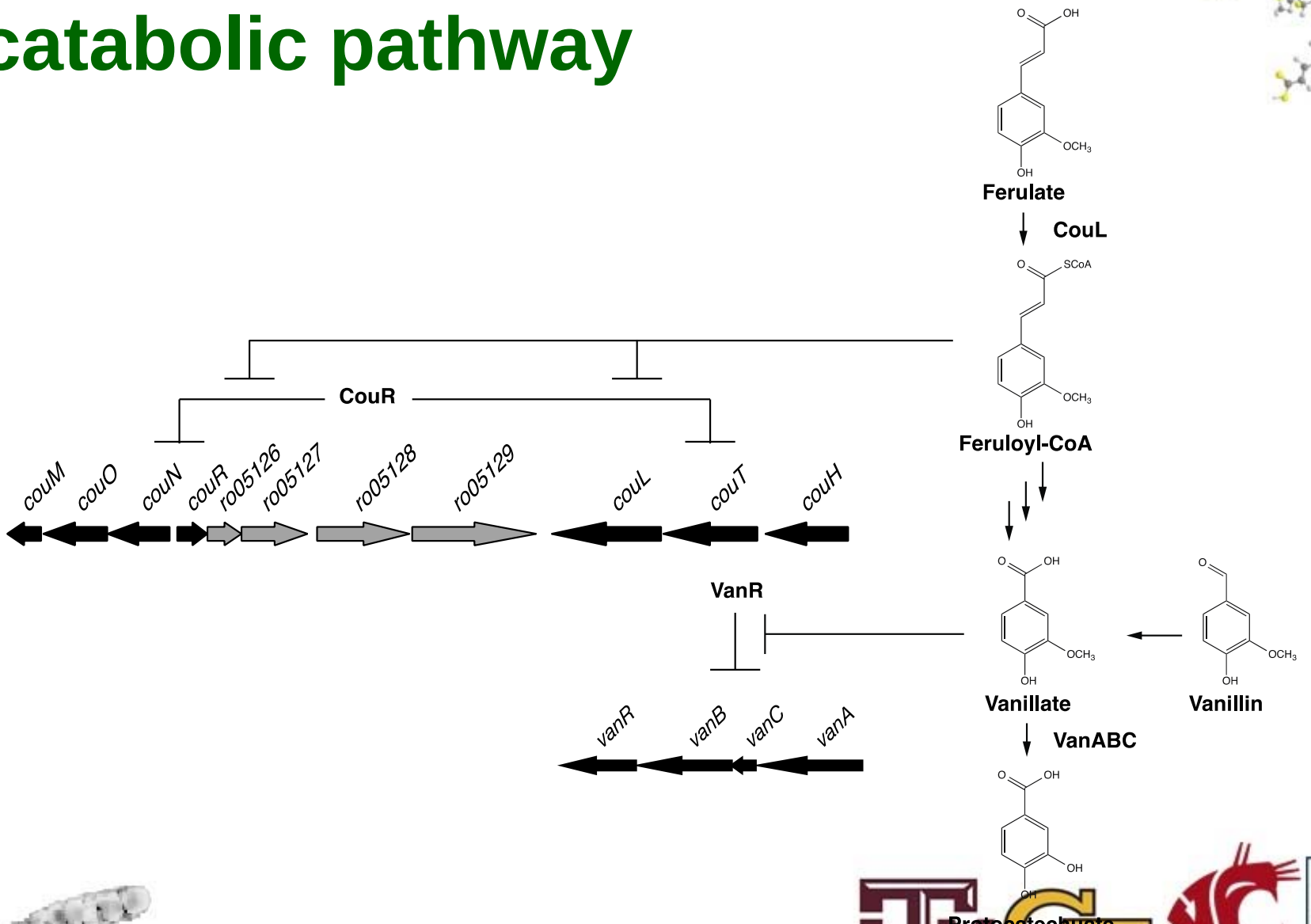
- **We have addressed four challenges to achieve laccase expression in rhodococci at 70U/ML. This is a significant technology breakthrough has profound impact for the field. The technology can be broadly applied for lignin conversion, biomass conversions, paper pulping industry, and environmental remediation. The technology principle can be applied for produce of high value proteins and protein therapeutics too. The technology is licensed and is being commercialized for biofuel applications.**

- **The rhodococci strain secreting high level of laccases can be used for Condolidated Lignin Processing (CLP) and can increase cell growth by nearly 1000 fold.**

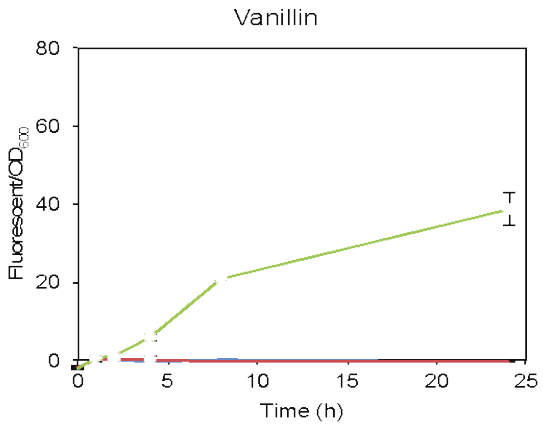
Technical Progresses – Functional Module 2: Aromatic Compound Degradation

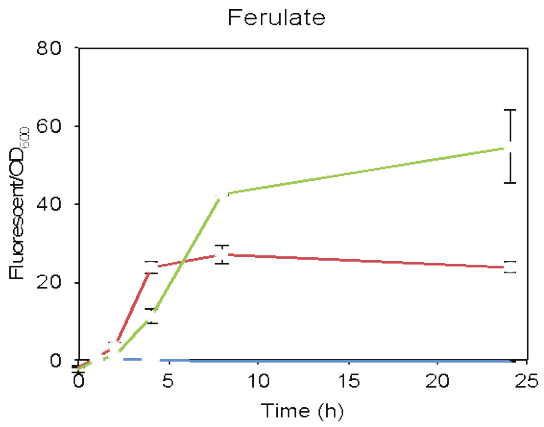
-- Vanillin and p-hydroxycinnamate catabolic pathways mapped and engineered

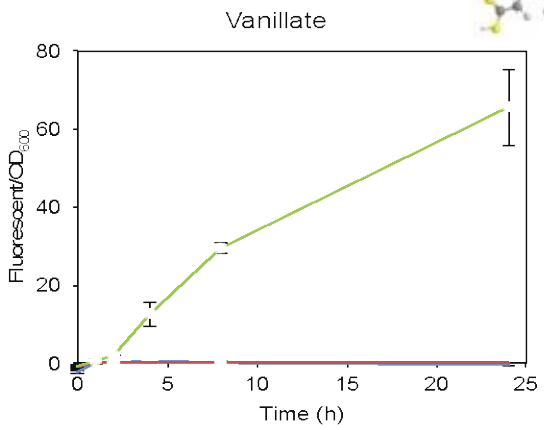
p-hydroxycinnamate catabolic pathway

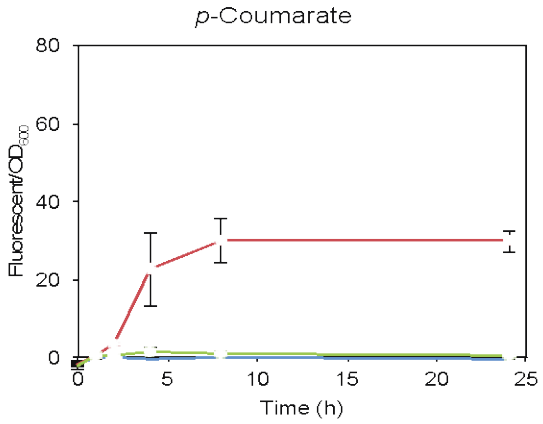


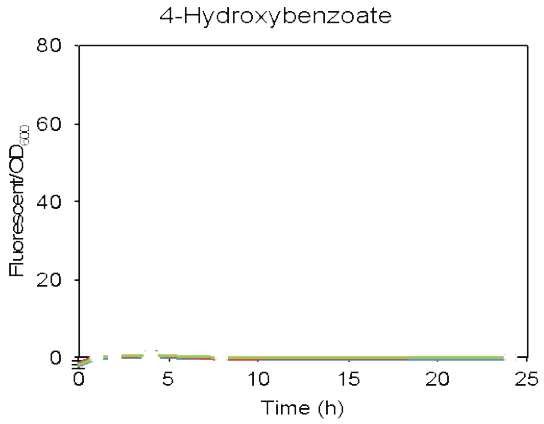
Bacterial strain development and characterization

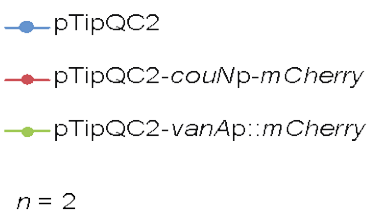












Conclusions

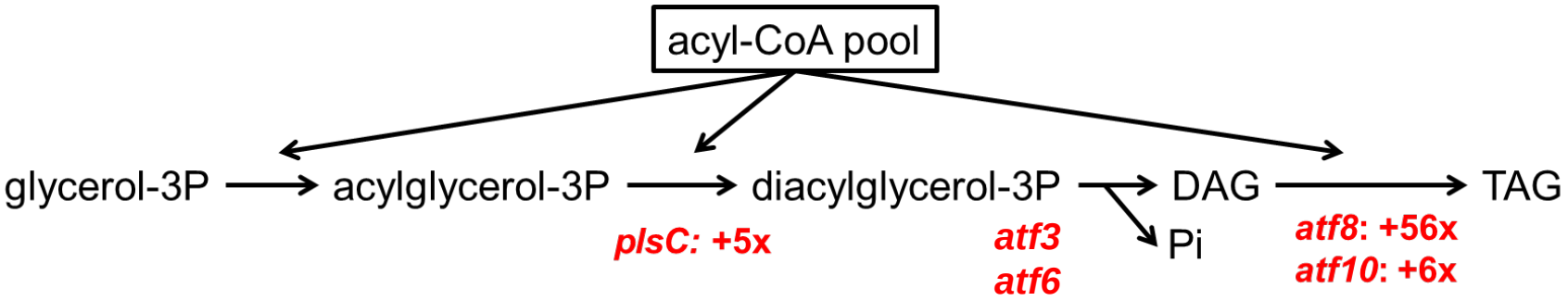
- **Vanillin and p-hydroxycinnamate catabolic pathways mapped and engineered.**
- **The results indicated that the different enzymes/pathways can be engineered to selective degrade various monomers more efficiently.**
- **The chemical pathways have precisions in terms of degrading different aromatic compounds. The speed limiting steps for different products might be different.**
- **The integration of the aromatic compound degradation functional modules don’t always provide the advantages in terms of cell growth and lipid yield. In other words, aromatic compound degradation may not be the speed-limiting step, and lignin depolymerization seems to be more speed limiting.**

Technical Progresses – Functional Module 3: Lipid Production

-- Engineering more efficient lipid production on lignin substrates in rhodococci

Targeted Genes for TAG Biosynthesis in RHA1

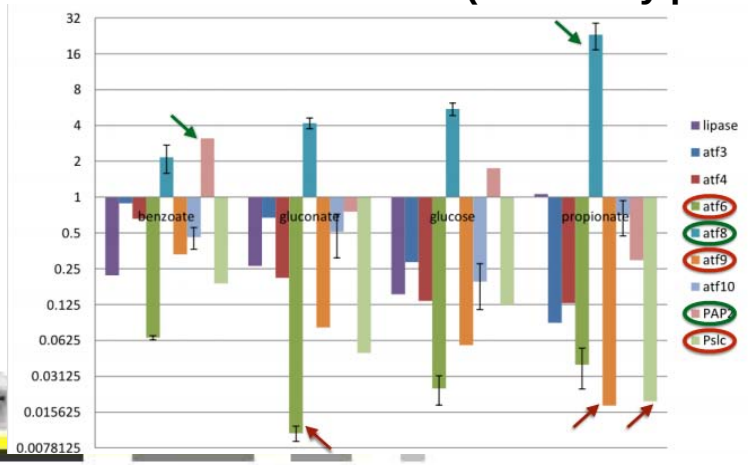
Fold-difference at stationary phase (N-limiting vs. N-excess)



atf3 and *atf6* (RHA1 homologs) up-regulated during TAG biosynthesis in *R. opacus* PD630 (Hernandez *et al.*, 2012)

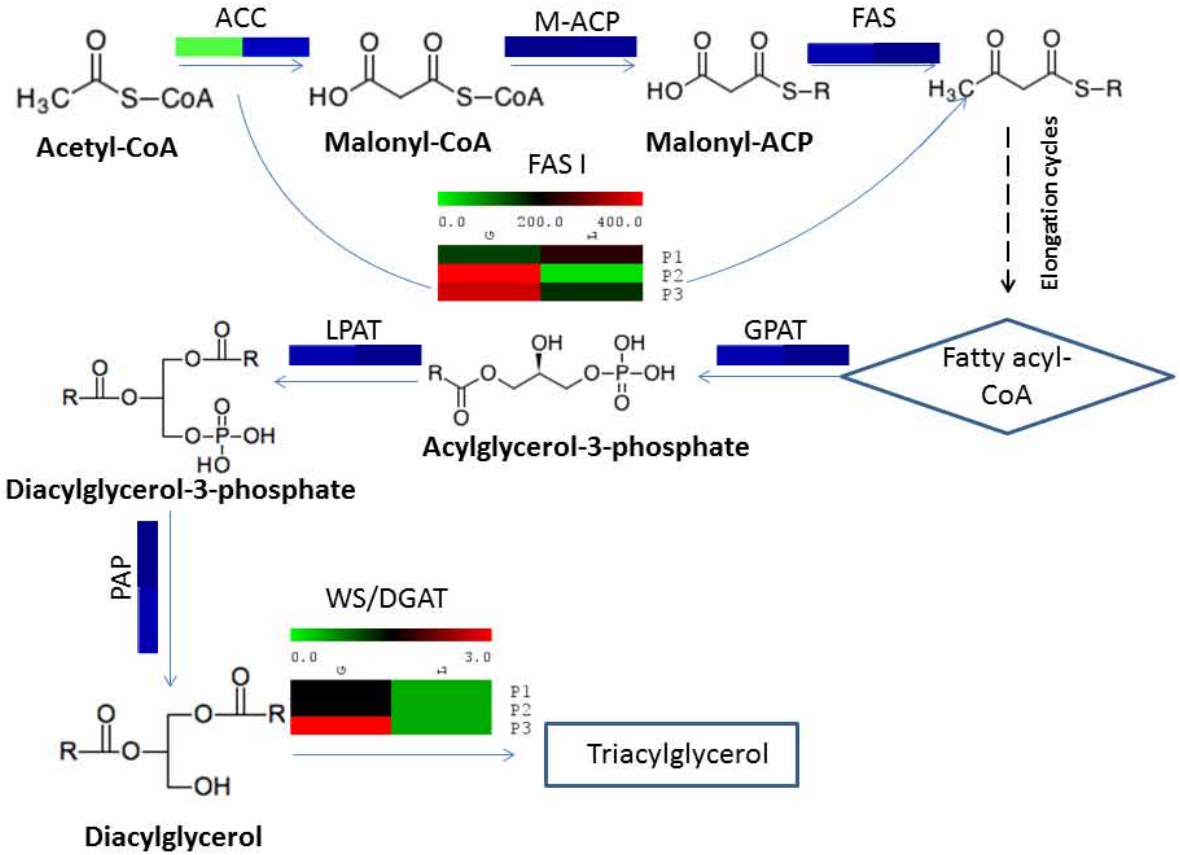
RT-qPCR analysis of gene expression

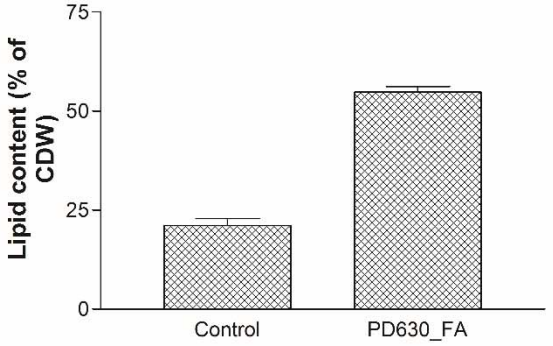
N-limitation *vs*. N-excess (Stationary phase)

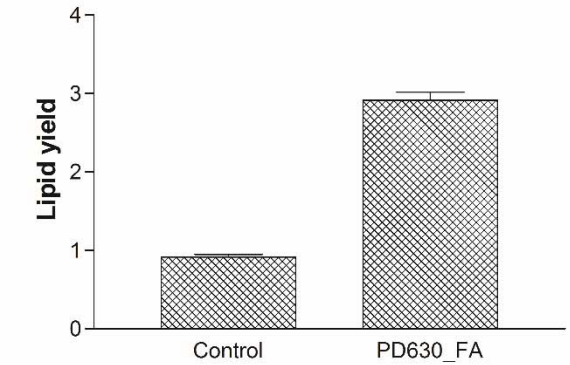


- *atf8* – the only *atf* highly up-regulated under TAG storage conditions regardless of C-source
- most tested genes more expressed under N-excess
- lipase gene down-regulated under N-limitation

Gene Discovery for Lipid Biosynthesis Module





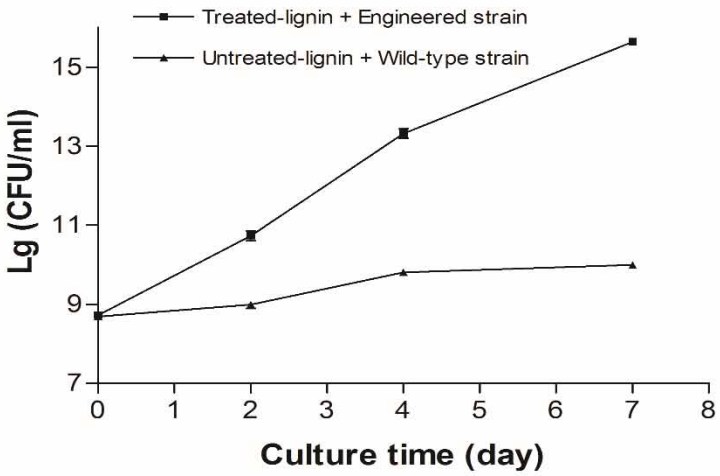


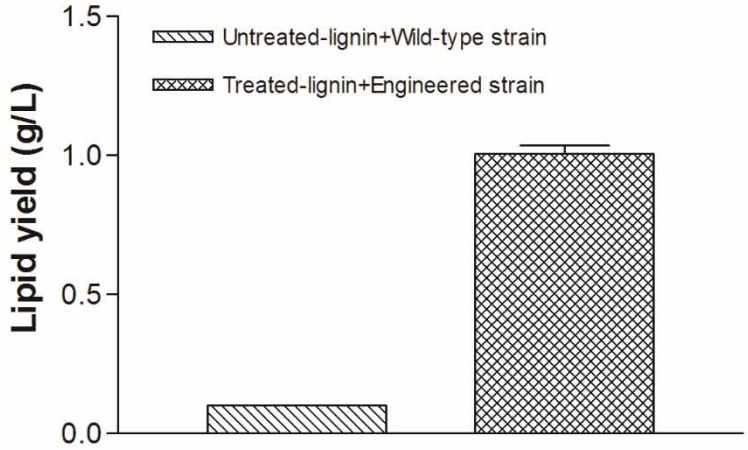
TAG biosynthesis pathway analysis based on protoemics.

Conclusions

- **Key genes for lipid biosynthesis on different C sources in rhodococci were identified based on transcriptomics and proteomics analysis.**
- **These genes include both atf catalyzing TAG biosynthesis and fas catalyzing the fatty acid biosynthesis.**
- **The over-expressing of these key genes lead to significant increase of lipid production in rhodoccoci.**

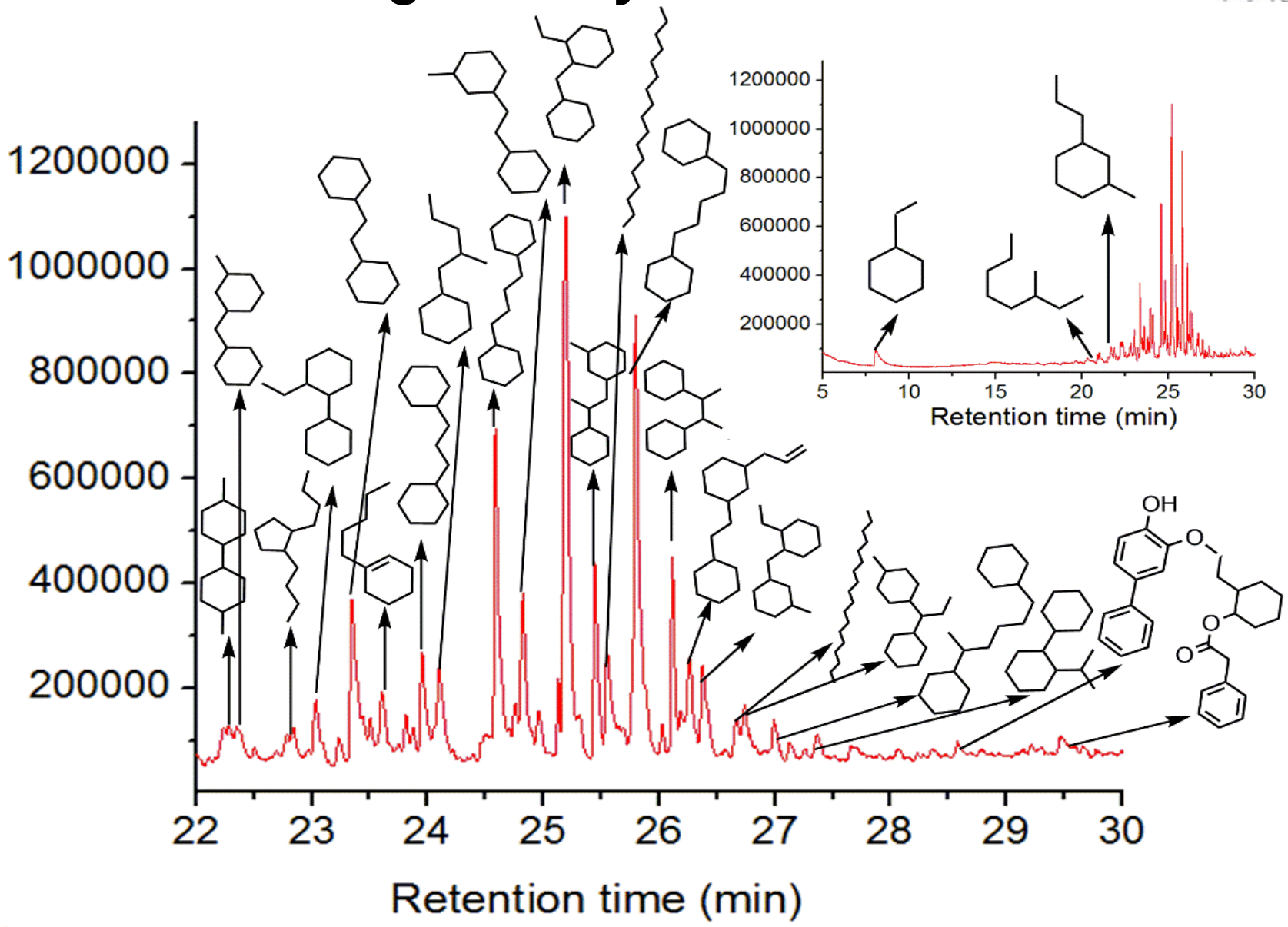
Combination of Different Functional Modules to Achieve High Lipid Titer





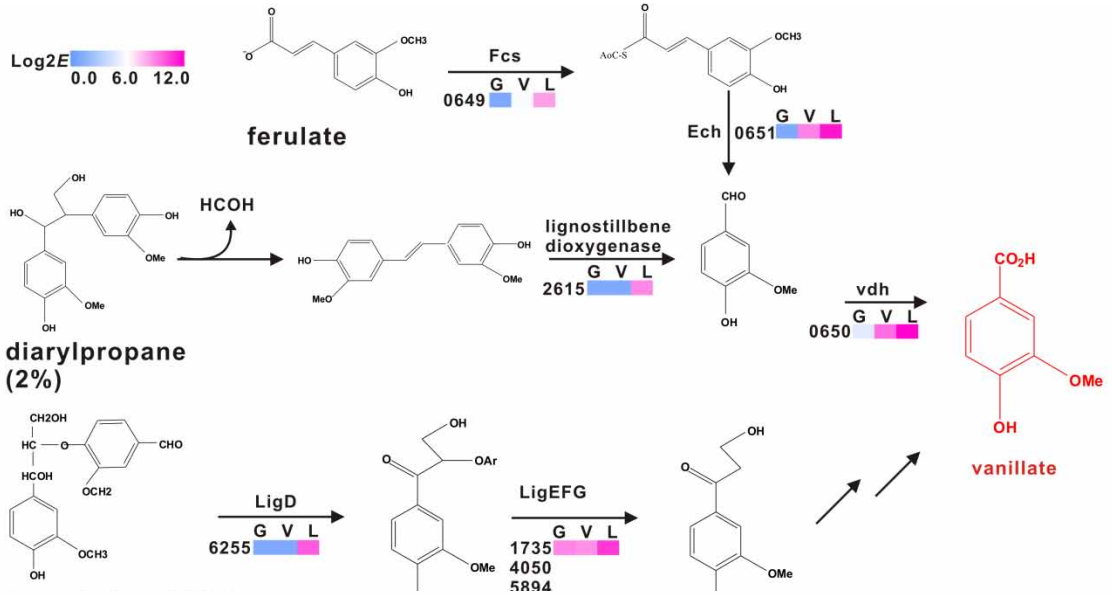
- **The three functional modules were integrated in different ways.**
- **The early data from these integration indicated that we could achieve up to 1g/L lipid yield from Kraft lignin.**
- **Further optimization can be achieved by different combination of functional modules, fermentation optimization, and various lignin substrates.**

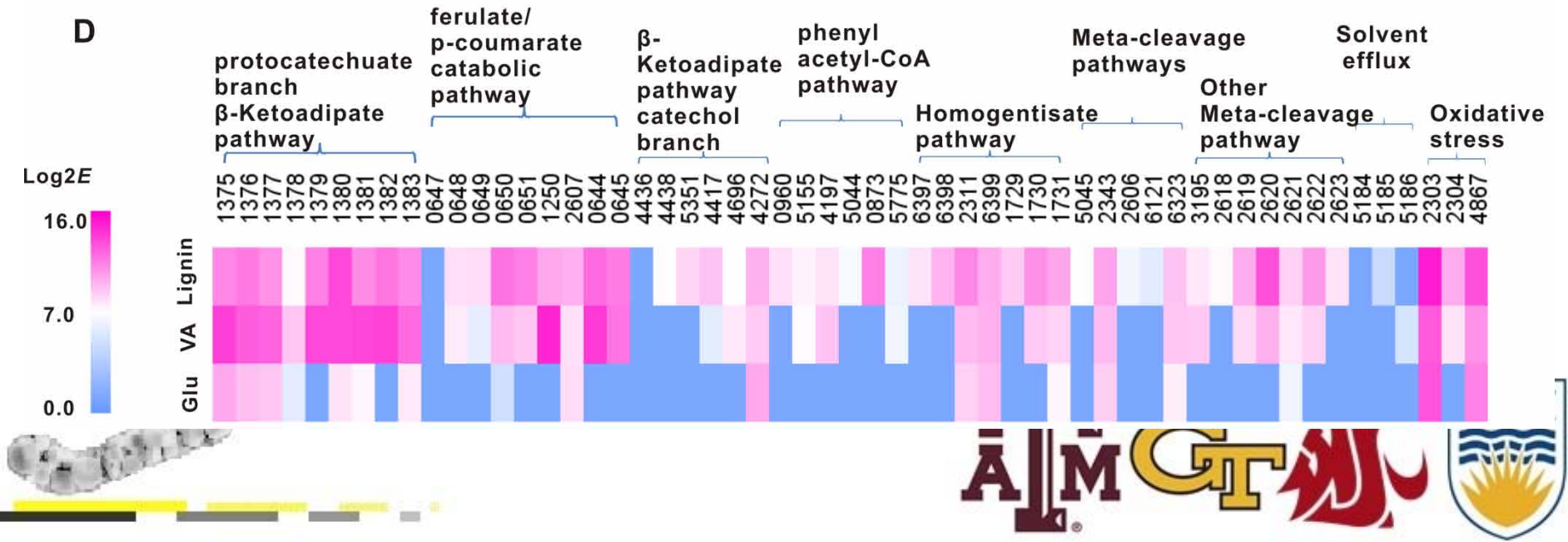
Another Perspective of Application of Biologically Solubilized Lignin – Hydrocarbons from HDO



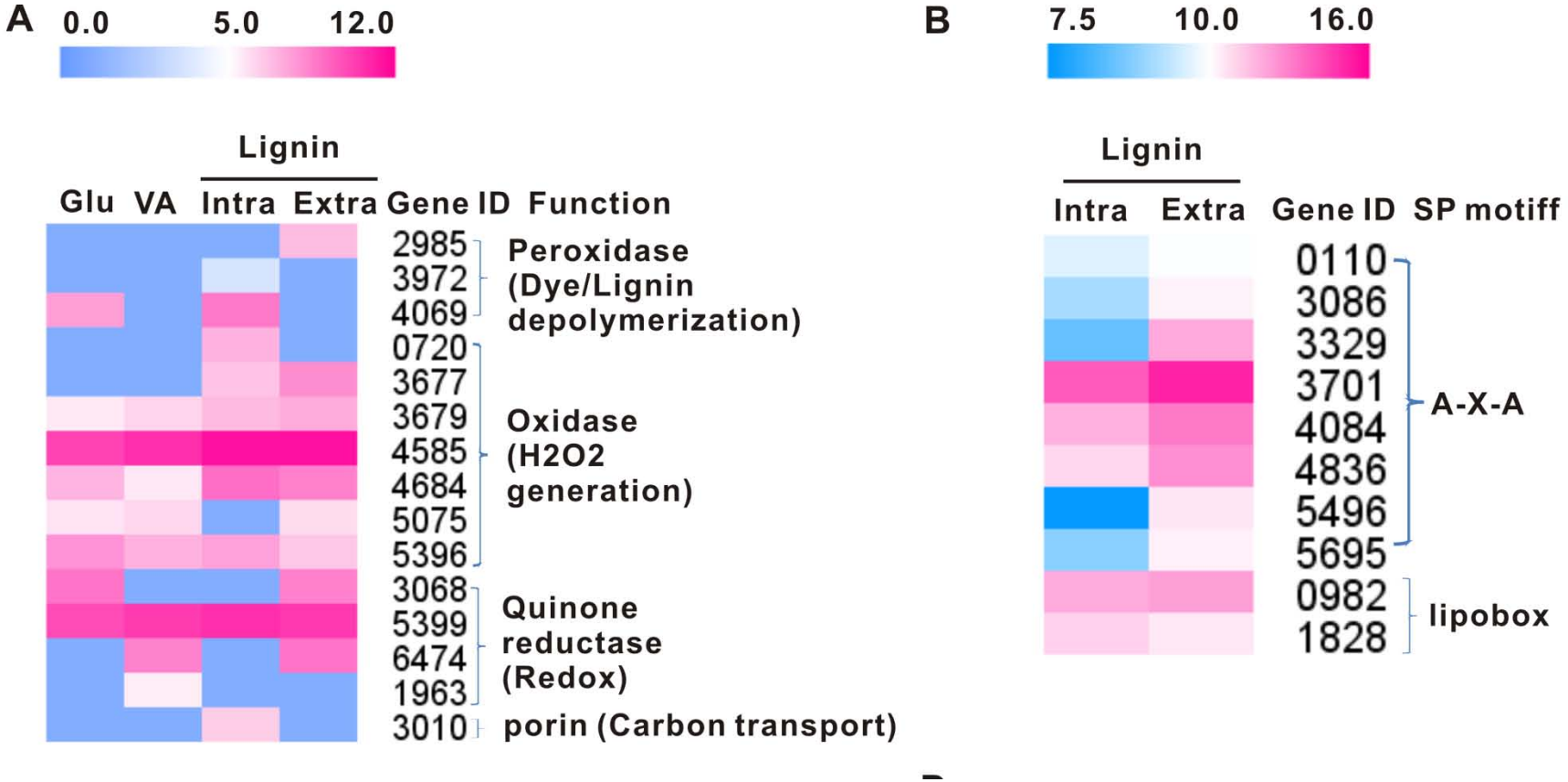
Technical Progresses
-- Consolidated Conversion of Lignin for *P. putida*

Overview of Proteomics



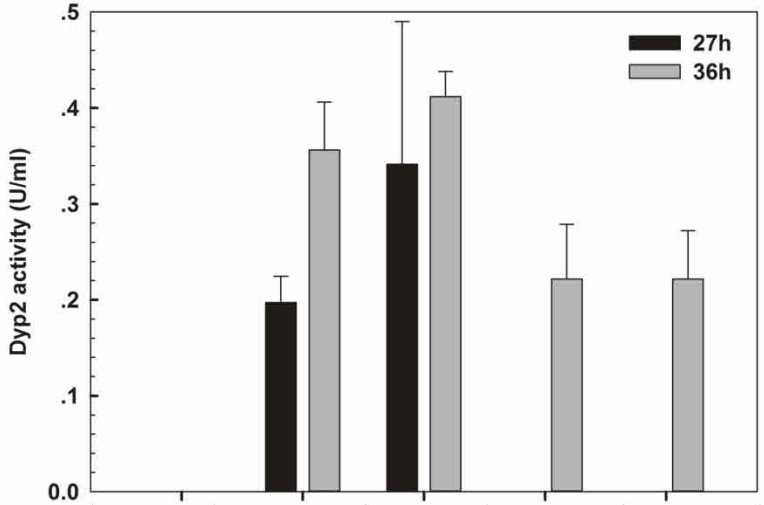


Design of Lignin Depolymerization Modules



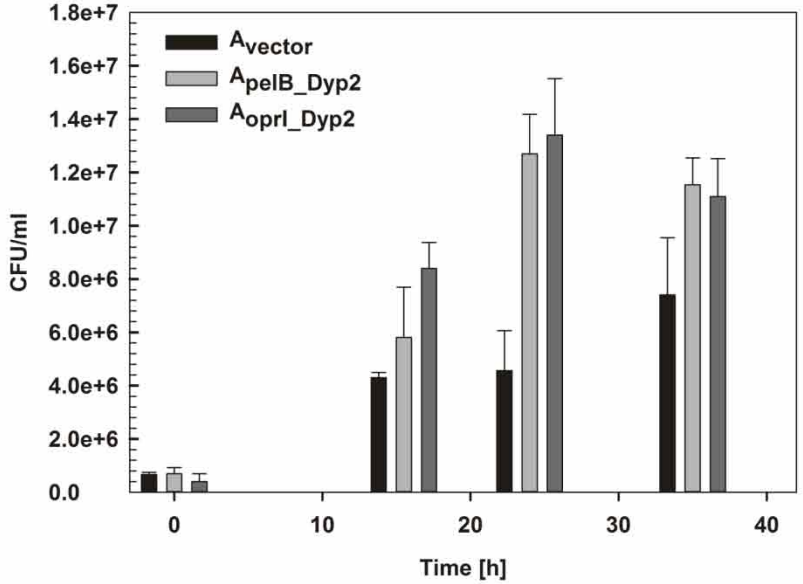
Design of Lignin Depolymerization Modules

C

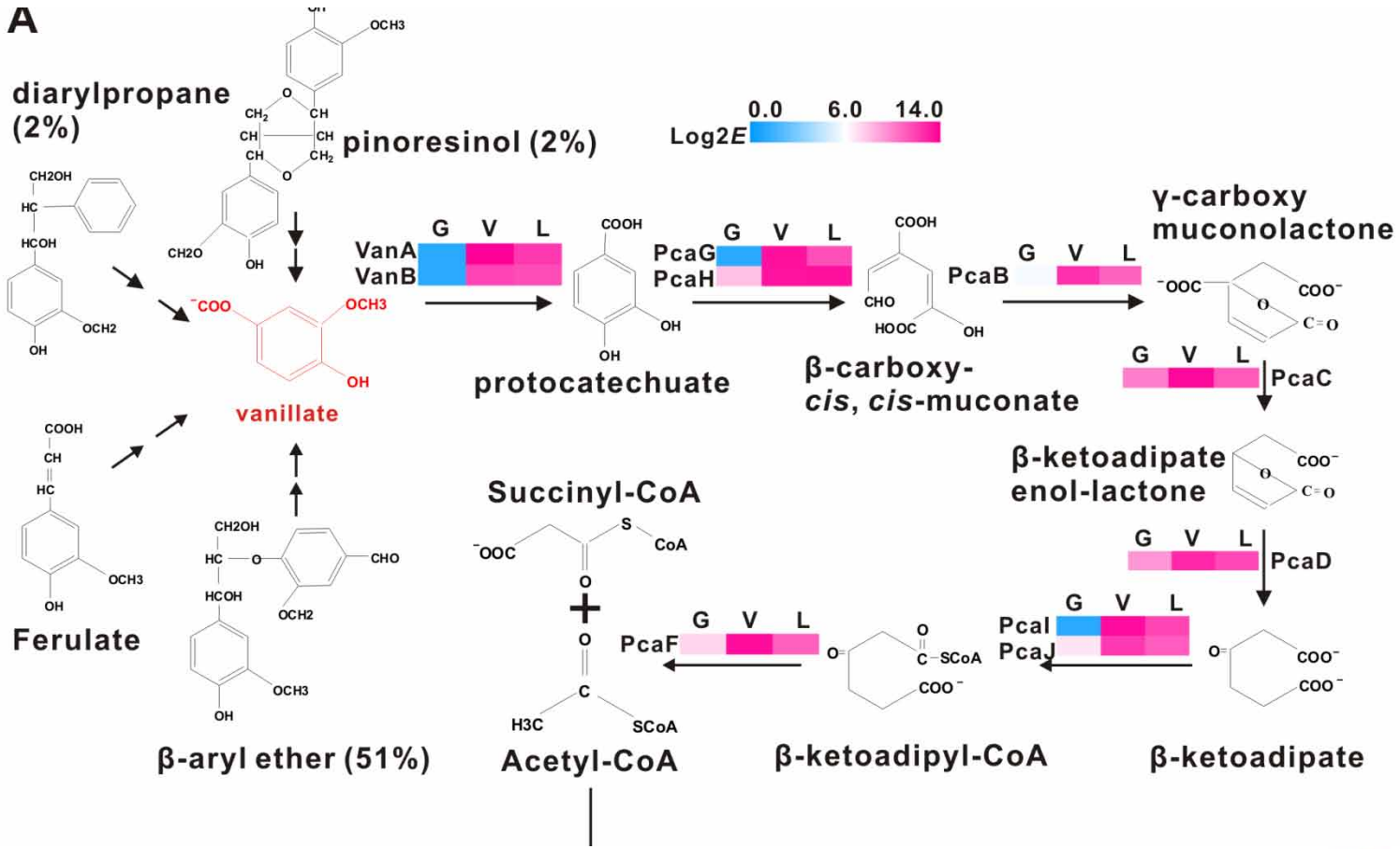


Plasmid	P1099	P1099 _Dyp2	P1099 _Dyp2	P1099 _Dyp2	P1099 _Dyp2
SP gene ID	-	0982	pelB	0110	3701
SP motiff	-	L-A-X-G-C-X	A-X-A	A-X-A	A-X-A
Leader peptidase	-	II	I	I	I

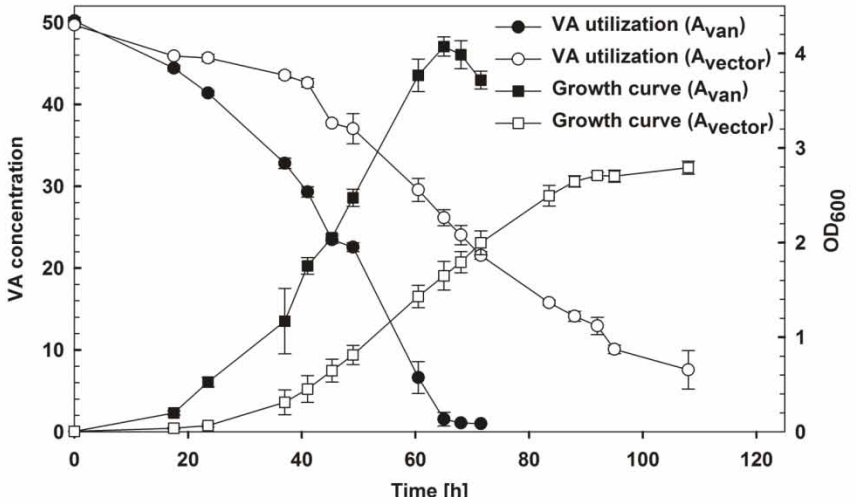
D

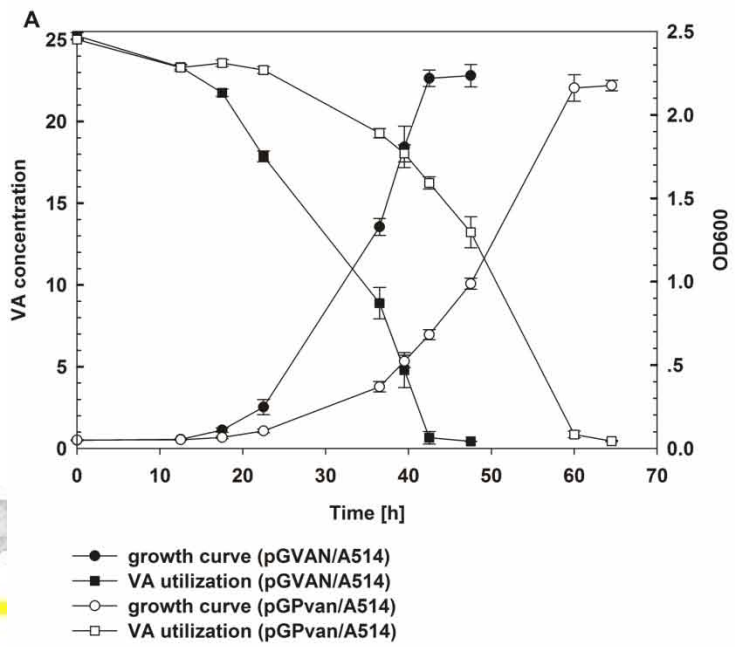


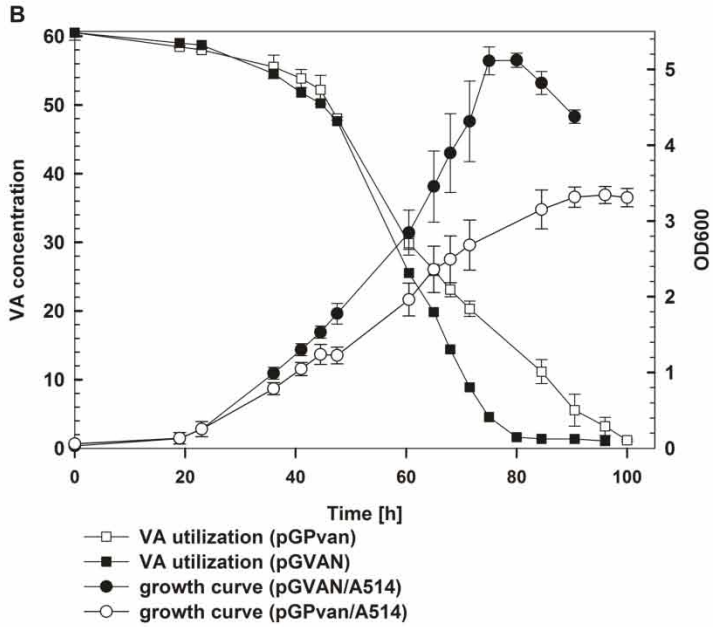
Design of Aromatic Compound Catabolism Module



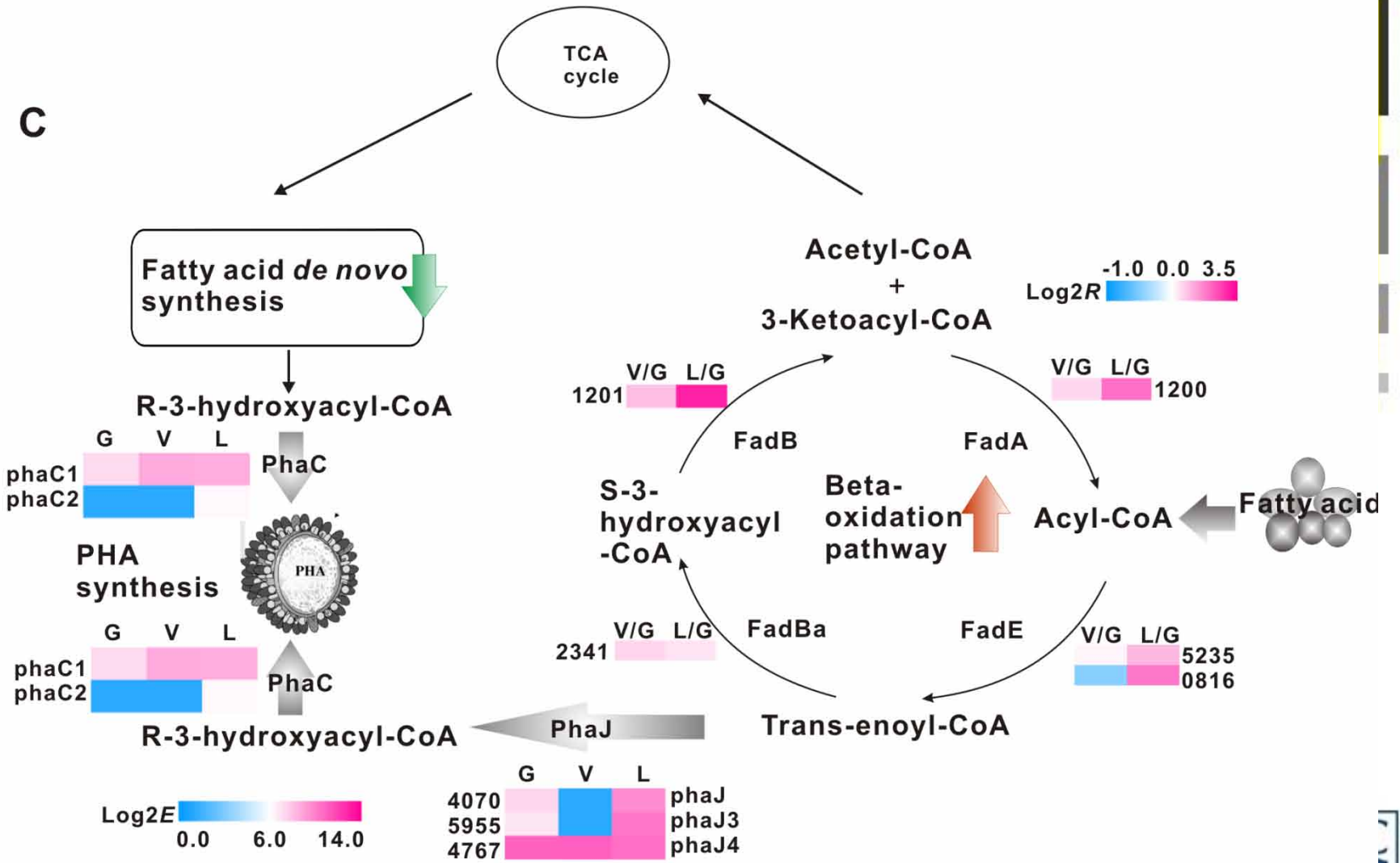
Design of Aromatic Compound Catabolism Module



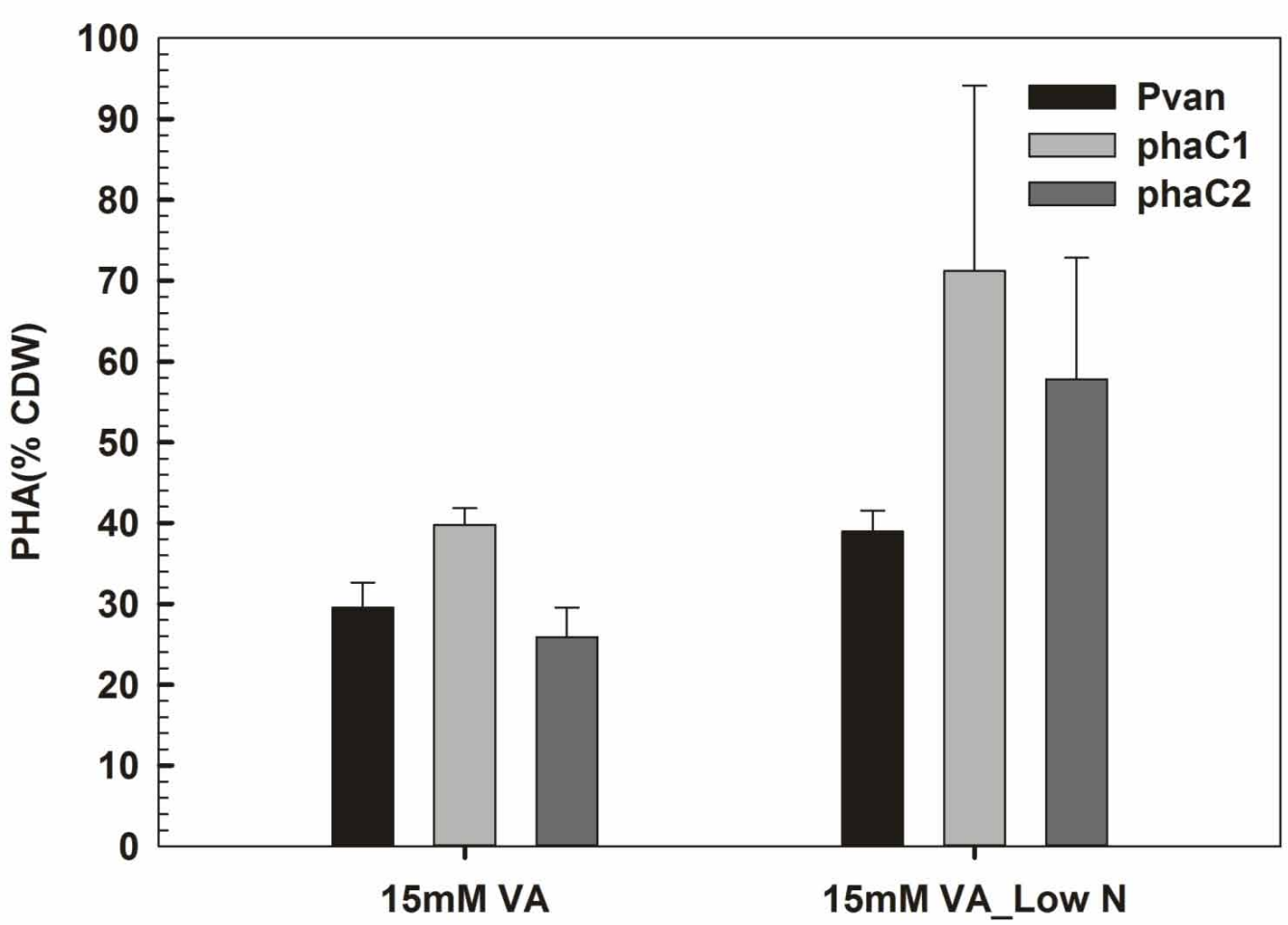




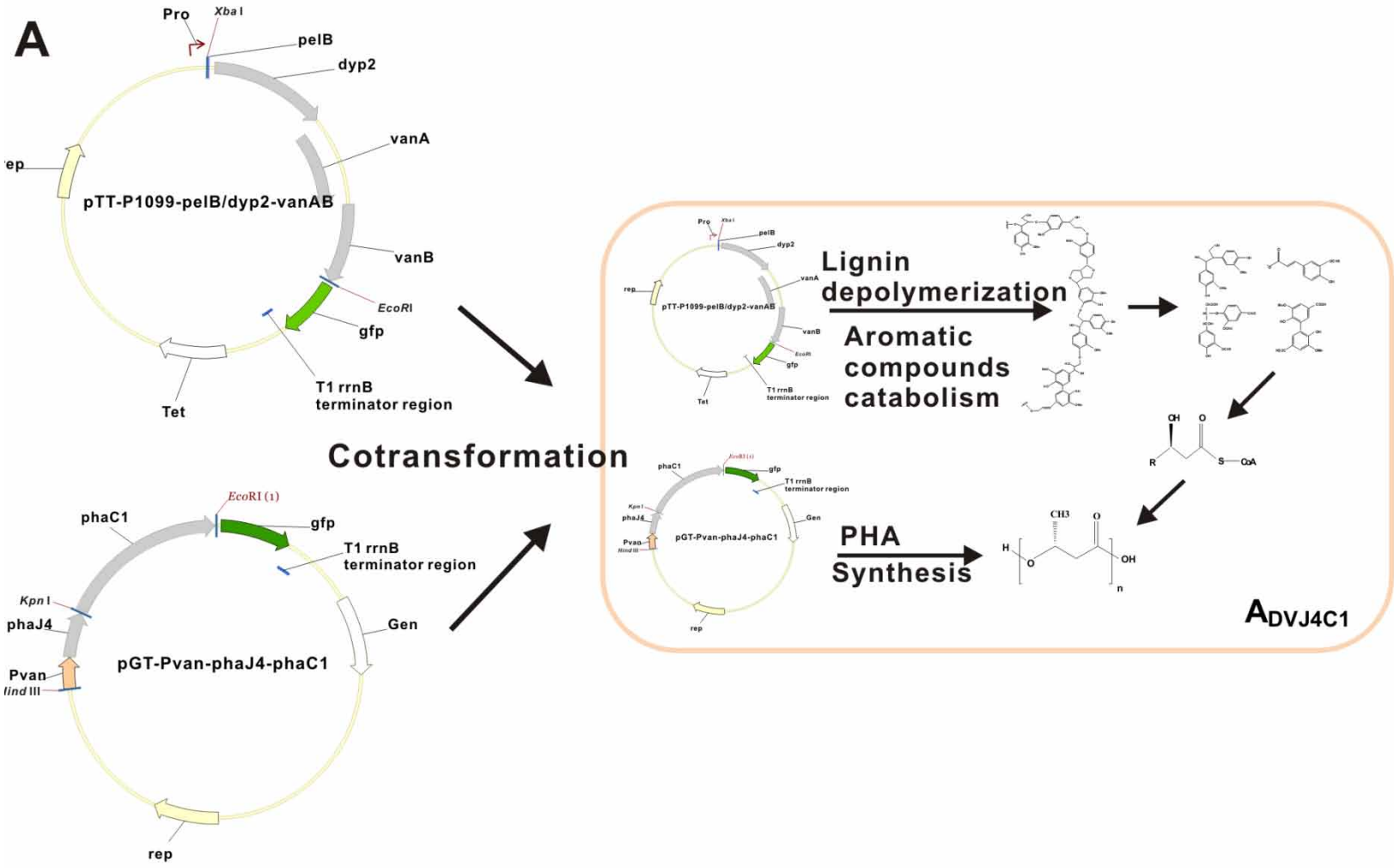
Design of PHA Production Module



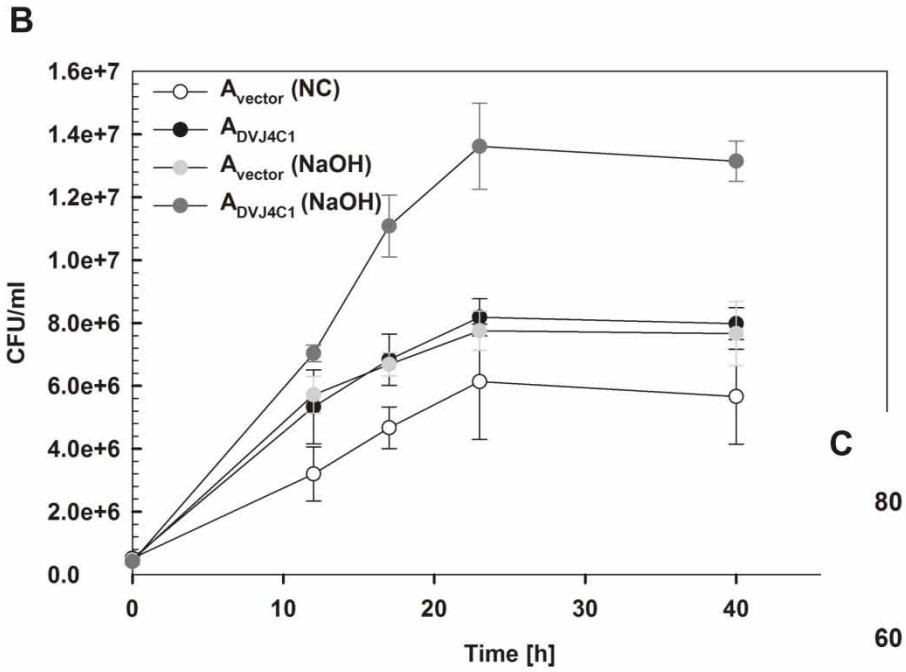
Design of PHA Production Module

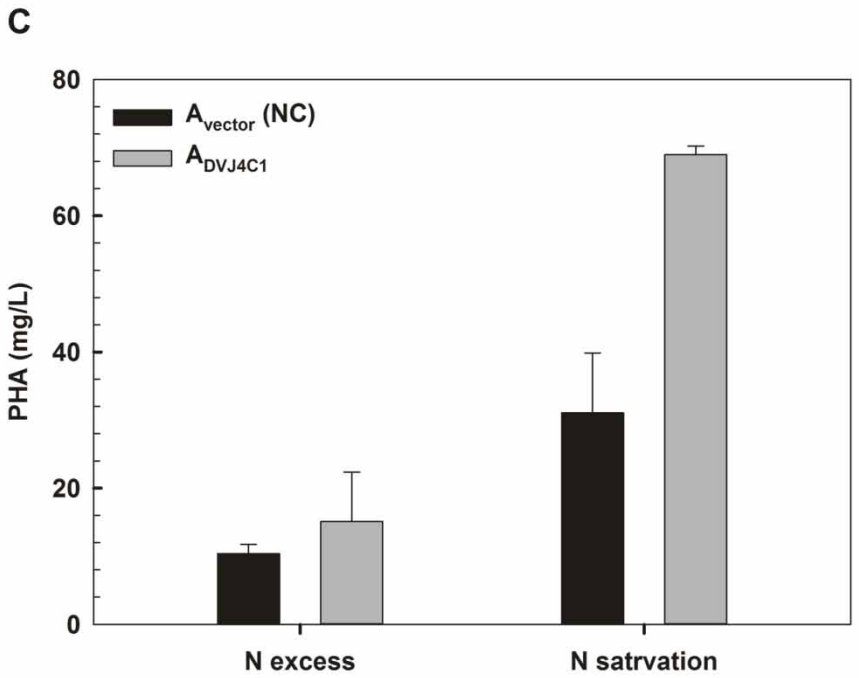


System Integration for Lignin to Bioplastics



System Integration for Lignin to Bioplastics

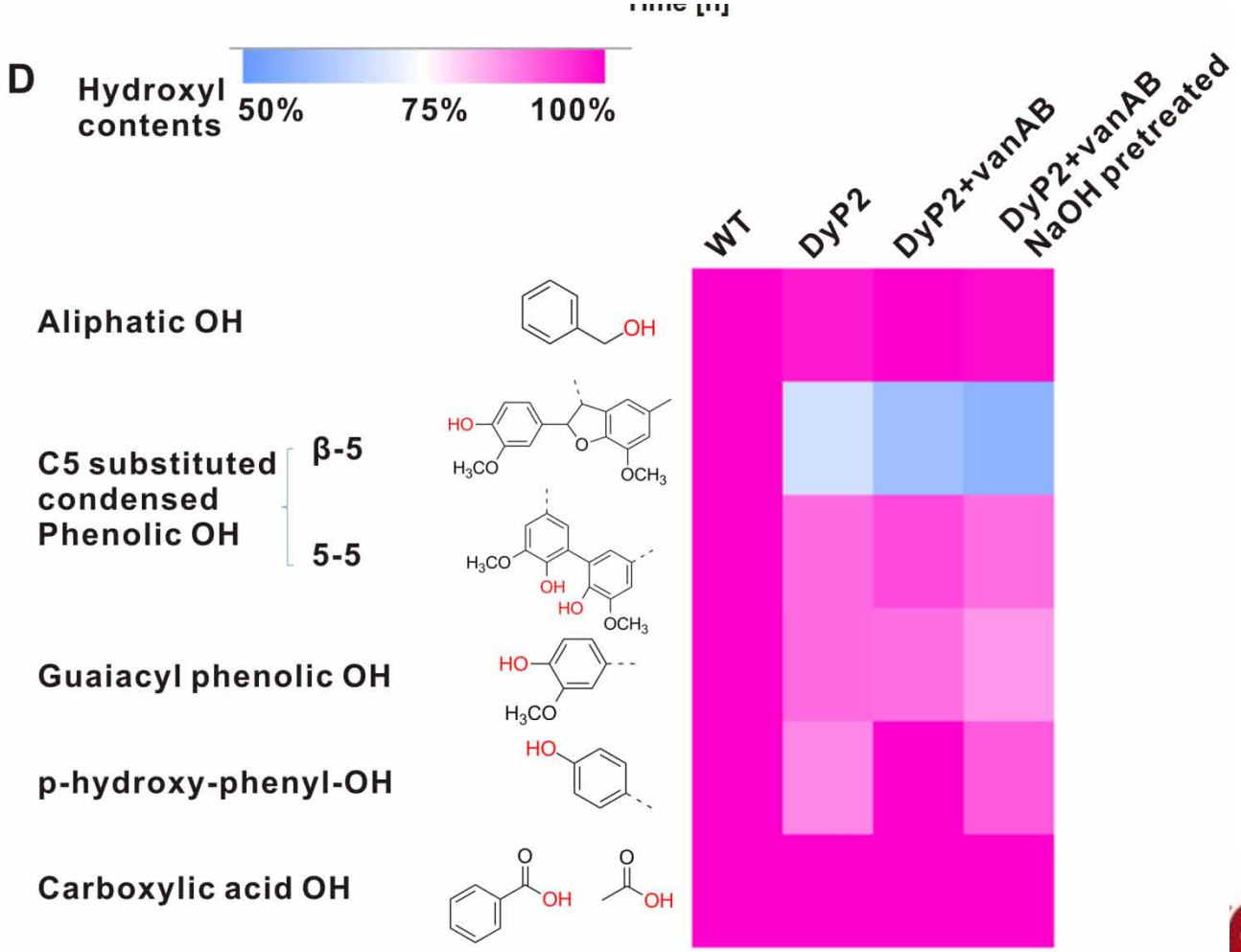




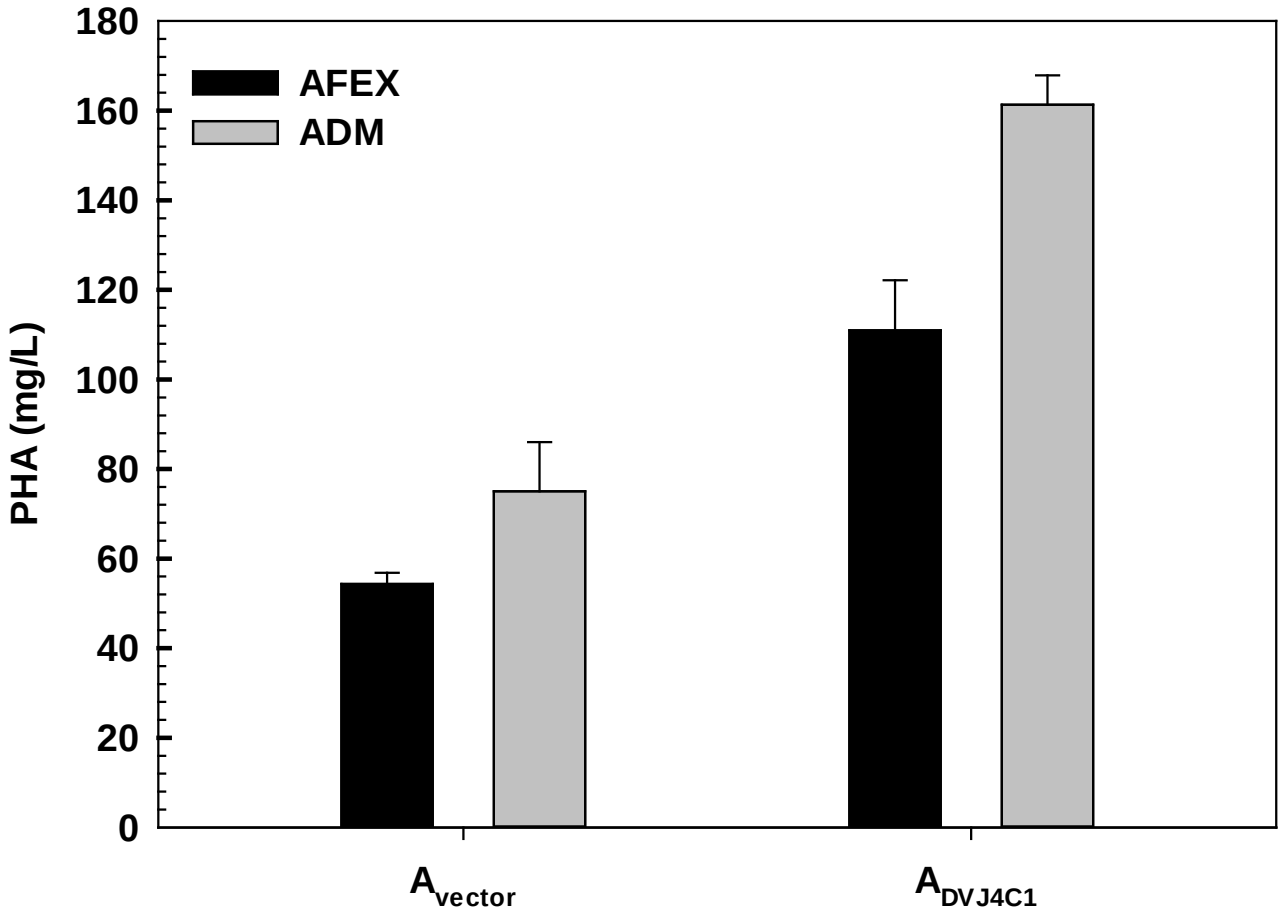
MCL Bioplastics are the Major Products

PHA substrate	Strains	Culture	PHA Composition (mol%)					
			3HHx (C6)	3HO (C8)	3HD (C10)	3HDD (C12)	3HTD (C14)	3HHD (C16)
Vanillate	A_{vector}	Low N (65mg/L)	7.06±2.68	19.04±3.48	33±4.52	30.85±1.11	ND	10.05±2.04
	A_{JC1}		3.22± 0.14	30.14±2.7	2.49±0.07	15.13±3.24	ND	49.02±4.45
	A_{JC2}		ND	38.93±2.41	ND	53.06±4.29	ND	8.01±0.21
	A_{vector}	High N (1g/L)	ND	ND	34.78±2.91	65.22±1.24	ND	ND
	A_{JC1}		ND	ND	ND	59.30±1.10	38.78±0.87	1.92±0.24
	A_{JC2}		ND	ND	27.02±3.33	ND	72.98±2.64	ND
Lignin	$A_{vectors}$	Low N (65mg/L)	3.36±1.87	ND	ND	96.64±1.87	ND	ND
	$A_{JC1DYP2}$		4.29±0.63	15.16±2.72	ND	25.66±12.79	54.89±9.92	ND
	Avectors	High N (1g/L)	15.32±3.63	19.16±4.32	ND	50.05±5.35	15.46±3.61	ND
	$A_{JC1DYP2}$		22.64±13.8	5.12±2.21	ND	57.23±5.08	24.12±19.5	ND

Lignin Degradation



PHA Titer for Different Biorefinery Waste Stream



Conclusion

1. A *P. putida* strain with strong aromatic compound and lignin degradation capacity has been identified
2. Comparative genomics revealed lignin and aromatic compound degradation mechanisms – coordinative pathways
3. Based on the systems biology analysis, we have designed three functional modules to both validate the molecular mechanisms and enable the lignin depolymerization, aromatic compound utilization, and PHA production.
4. The integration of these functional modules have enabled lignin the PHA route for mcl PHA production.
5. The biorefinery waste seem to perform better than kraft lignin for conversion.

Technical Accomplishments

- **All major technical milestones were met. We are working on achieving final project target yield.**
- **>1g/L lipid titer on lignin represents more than 30 times increase of previous published state-of-the-art on EOL lignin. There is no report so far on lipid production from Kraft lignin.**
- **Cell growth increased about 1000 times on Kraft lignin, which is also a significant improvement over the previous reports on other substrates.**
- **Fermentation for lignin-to-lipid route is at four days, and lignin-to-PHA route at less than 2 days. The optimized feedstock load so far is about 2% (20g/L). We are further optimizing conditions to achieve the project target yield.**
- **Nine manuscripts published or submitted, two more to be submitted, and more underway.**
- **Two comprehensive provisional patent profilios filed. The commercialization partner has been identified and is aggressively seeking funding for scale up.**

Publication List

1. Wells T, Wei Z, and **Ragauskas AJ**, Bioconversion of Lignocellulosic Pretreatment Effluent via Oleaginous Rhodococcus opacus DSM 1069, Biomass and Bioenergy, 2015, 72: 200-205.
2. Wei Z, Zeng G, Kosa M, Huang D, **Ragauskas AJ**, Pyrolysis Oil-Based Lipid Production as Biodiesel Feedstock by *Rhodococcus opacus*, Applied Biochemistry and Biotechnology, 2015, 175:1234–1246.
3. Wei Z, Zeng G, Huang F, Kosa M, **Ragausaks AJ**, Bioconversion of oxygen-pretreated Kraft lignin to microbial lipid with oleaginous Rhodococcus opacus DSM 1069, submitted.
4. Xie S, Qin X, Cheng Y, Laskar D, Qiao W, Sun S, Reyes L, Wang X, Dai Y, Sattler S, Kao K, **Yang B,** Zhang X, and **Yuan JS**, "Simultaneous Conversion of All Cell Wall Components by Oleaginous Fungus without Chemi-physical Pretreatment," Green Chemistry, 2014. DOI: 10.1039/C4GC01529K
5. Jeon J, Zhang L, Laskar L, Lemmon J, Choi D, , Nandasiri M, Hashmi A, Xu J,. Motkuri R, Fernandez C, Liu J, Lutkenhaus J, Tucker M, McGrail P, **Yang B,** and Nune K**,** Controlling Porosity in Lignin-Derived Nanoporous Carbon for Supercapacitor Applications, ChemSusChem, 8(3): 428–432, 2015.
6. Yan L, Zhang L, and **Yang B***. Enhancement of Total Sugar and Lignin Yields through Dissolution of Poplar Wood by Hot Water and Dilute Acid Flowthrough Pretreatment, Biotechnology for Biofuels, 2014, 7:76 doi:10.1186/1754-6834-7-76.
7. Laskar D, Tucker M, Chen X, Helms D, and **Yang B**. "Noble-Metal Catalyzed Hydrodeoxygenation of Biomass-Derived Lignin to Aromatic Hydrocarbons," Green Chemistry, 2014, 16 (2), 897 – 910. DOI: 10.1039/c3gc42041h.
8. Lin L, Cheng Y, Pu Y, Sun S, Li X, Pierson EA, Gross DC, **Dai SY, Ragauskas AJ, Yuan JS**, Systems Biology-Guided Biodesign of Consolidated Lignin Conversion, Submitted.
9. Zhao C, Xie S, Pu Y, Zhang R, Huang F, **Ragauskas AJ, Yuan JS**, Synergistic Enzymatic and Microbial Conversion of Lignin for Lipid, Submitted.
10. Xie S, Lin F, Pu Y, Wang X, Sun S, Li M, Dai SY, **Ragauskas AJ, Yuan JS,** Advanced Biological and Chemical Design for Lignin Bioconversion. In preparation.
11. Amara S, Seghezzi S, Diaz-Salazar C, **Eltis LD**. 2015. Atf8, a key diacylglycerol acyltransferase in the biosynthesis of triacylglycerols by Rhodococcus jostii RHA1. In preparation for J. Bacteriol.

Relevance

- The research provided a potentially effective approach to convert lignin to lipid as biofuel precursor, which well addresses the MYPP goal to improve biorefinery efficiency and cost effectiveness.

- We expect that the outcome will improve biorefinery sustainability and cost effectiveness, and thus addresses the need of BETO, the MYPP goals, and the challenges in biofuel industry.

- 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.

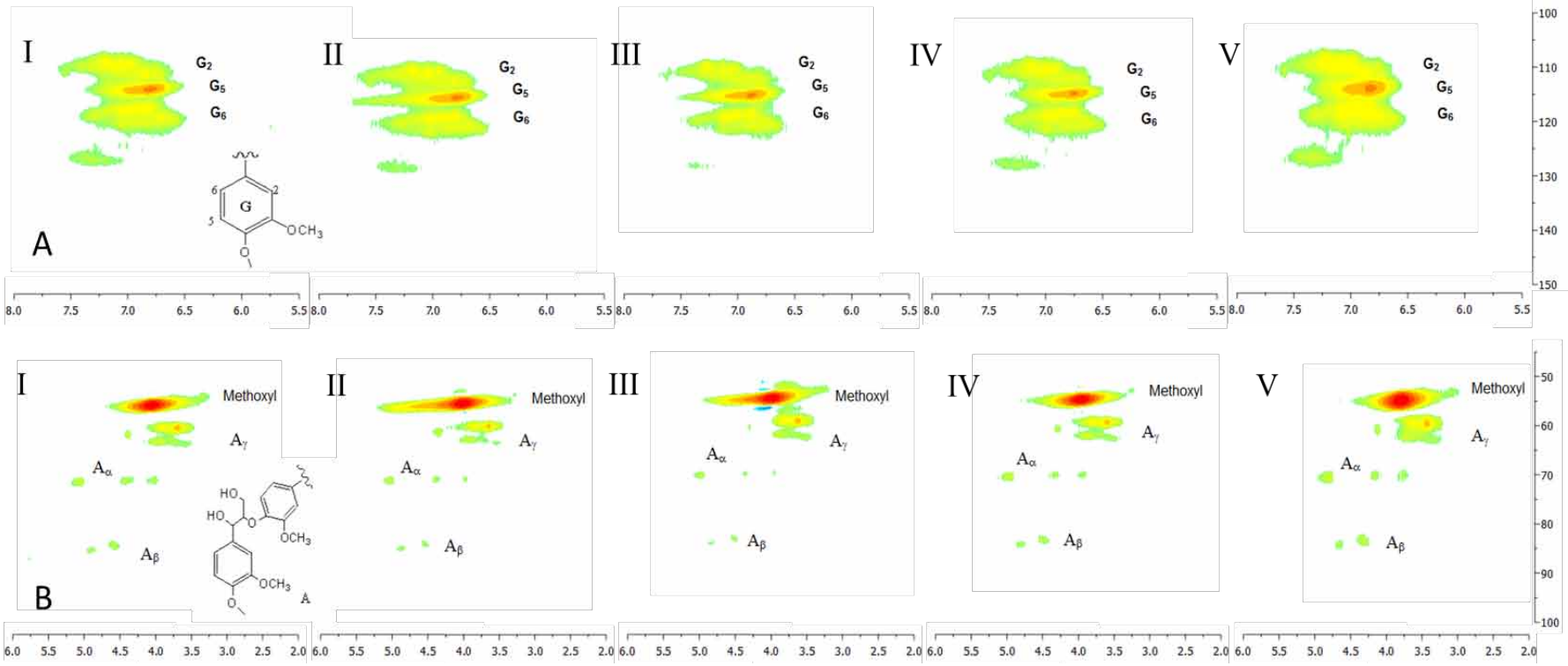
- The technology has been licensed and will be scaled up for commercialization as solutions for biorefinery.

Future Work

- We have made significant technical breakthroughs in ligninase expression. This will open a new avenue on bioprocessing.
- Different combination of functional modules to optimize the lignin-to-lipid conversion. This includes integration of different functional modules into a super strain or utilization of multiple strains system to improve yield.
- Fermentation optimization will be carried out to identify the proper oxygen, carbon, nitrogen and other conditions for lignin-to-lipid conversion and lignin depolymerization. Fedbatch fermentation will be considered.
- Biological conversion seems to utilize biorefinery slurry better, assuming the carbohydrate component in the slurry. We will carry out fermentation of different biorefinery waste stream.
- Comprehensive NMR, GC/MS, and GPC analysis will be carried out to analyze the aforementioned conditions to guide the further optimization and the integration of chemical treatment to further achieve higher yield. The reiterative design will help to improve the lipid yield further.
- The goal is to achieve 5 to 10 g/L lipid titer.

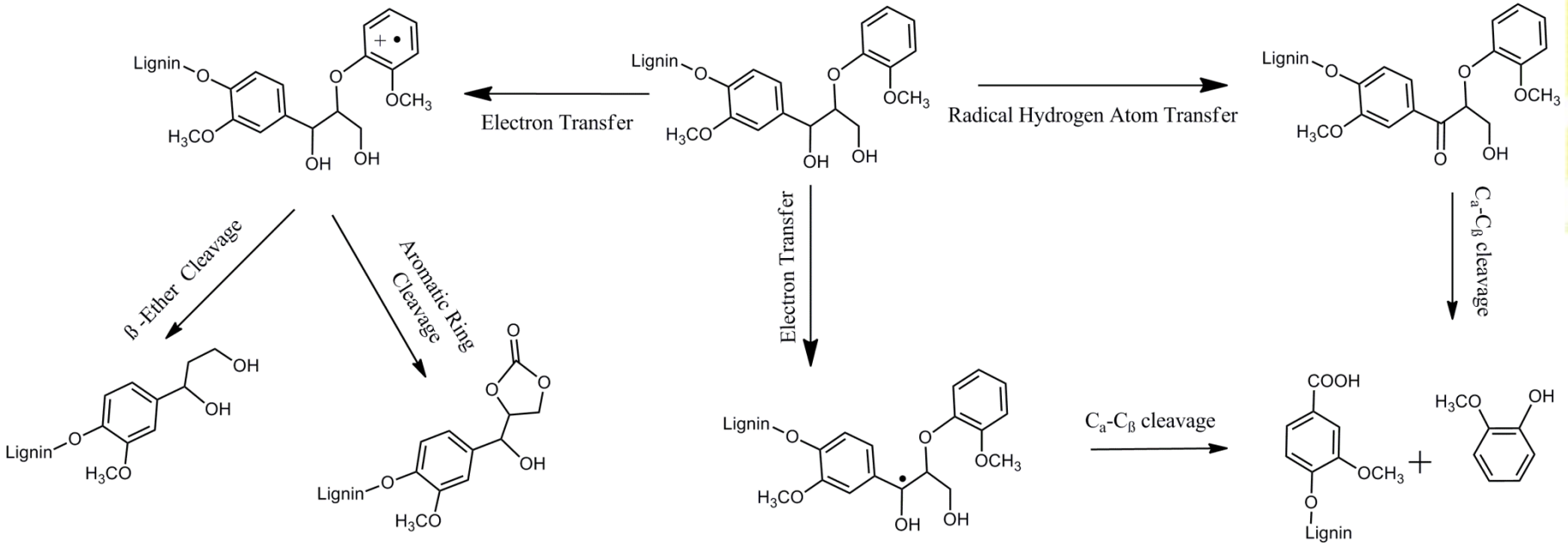
Additional Slides

Synergy at Chemical Level

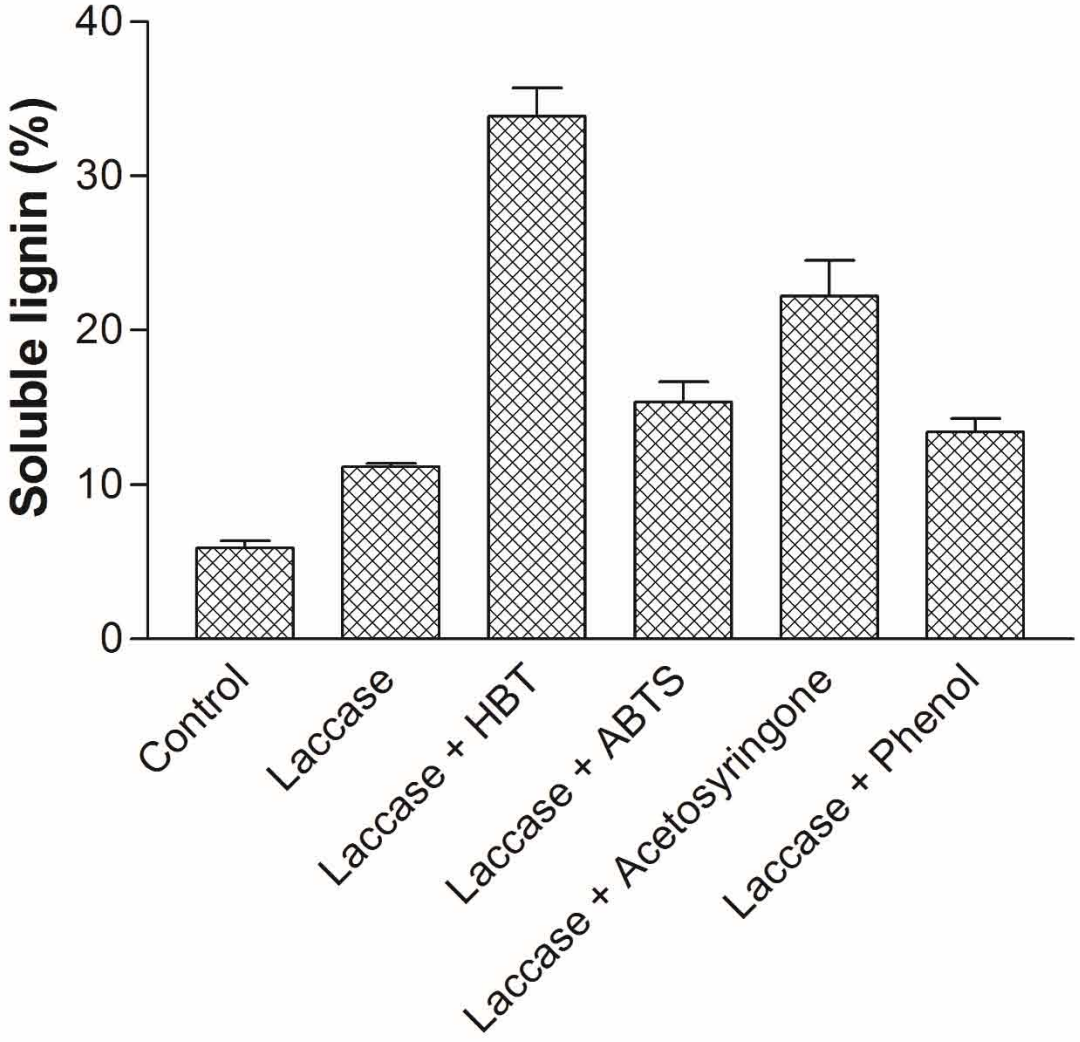


Technical Progresses
-- Advanced Chemical Design to Promote Laccase Depolymerization of Lignin

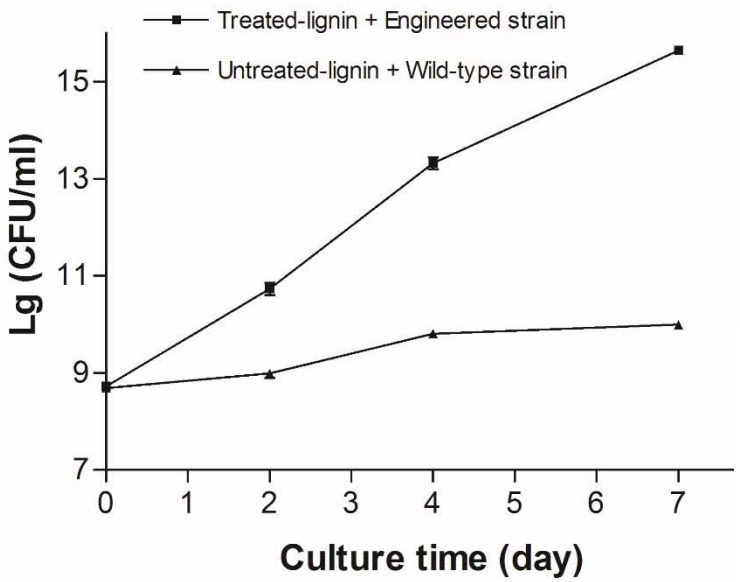
Synergistic Enzyme and Electron Mediator Function for Lignin Depolymerization

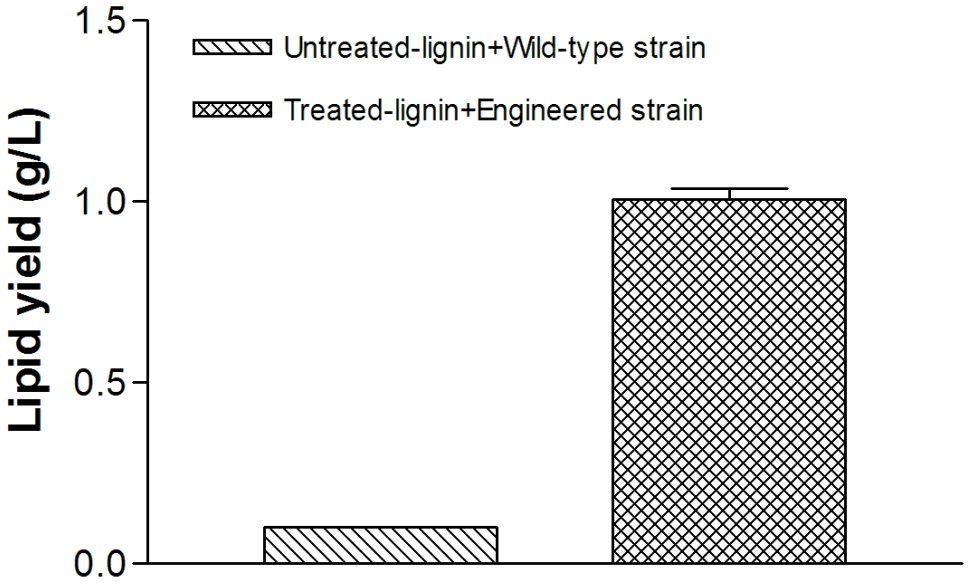


Laccase as the Main Driving Force for Improvement

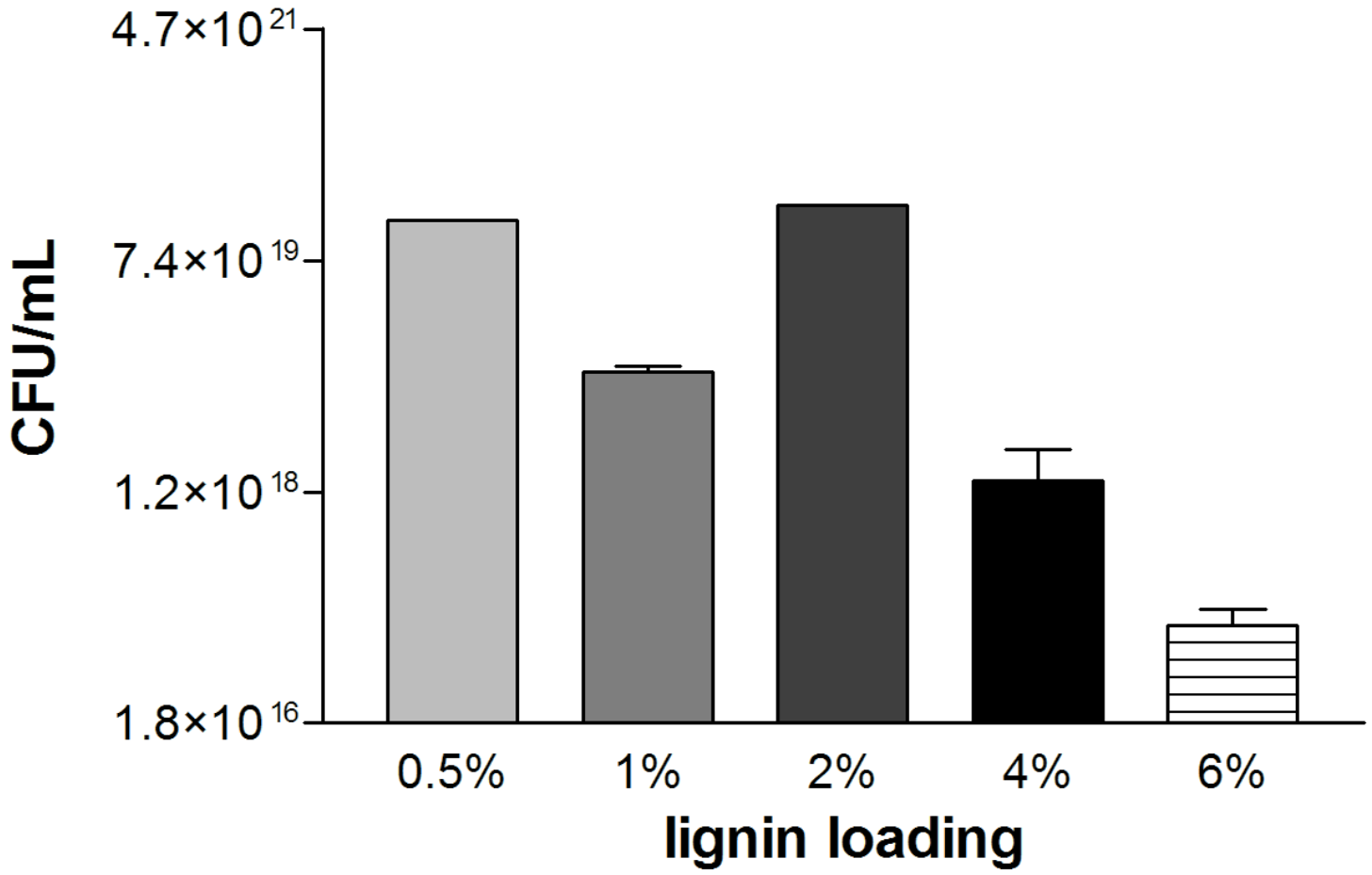


Highest Lipid Titer Achieved





Consolidated Lignin Conversion Integrating Chemical and BioDesign



Nitrogen Conditions