Biodesign of Plants and Microbes for Sustainable Production of Fuels and Chemicals

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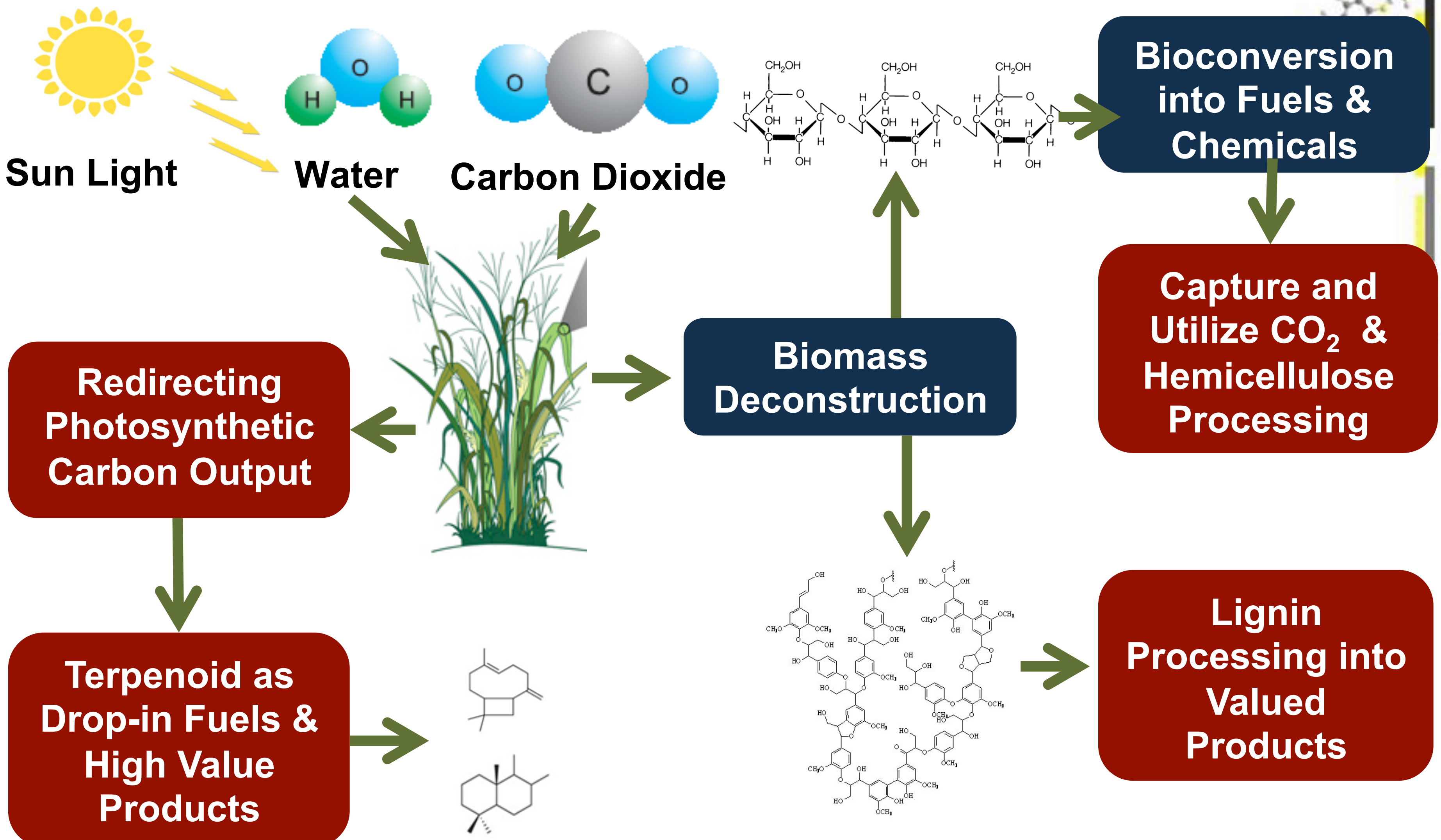
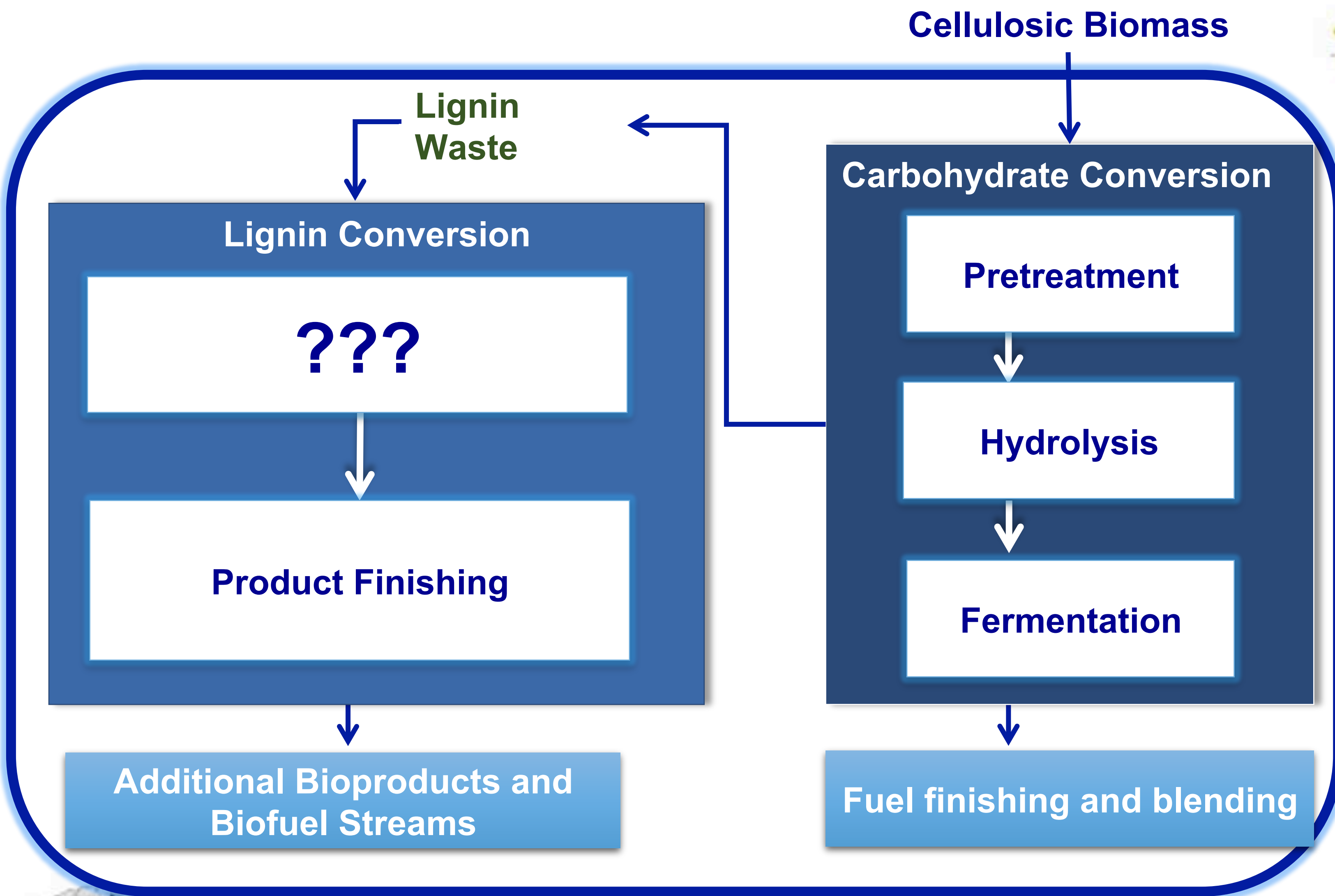
Challenges for Sustainable Fuels & Chemicals



Sustainability and Cost Effectiveness

Lignin Streams to Enable Integrated Biorefinery



Lignin Recalcitrance is Crucial for Land Plants

Species	CAD	CCoA MT	4CL	CCR	PAL	C4H	HCT	CO MT	C3H	F5H	Total
O. tauri	3	1	0	0	0	0	0	0	0	0	4
O. RCC809	2	1	0	0	0	0	0	0	0	0	3
O. lucimarinus	3	1	0	0	0	0	0	0	0	0	4
Phaeodactylum	1	1	1	2	0	0	0	0	0	0	5
Thalassiosira	0	0	1	0	0	0	0	0	0	0	1
Chlamydomonas	4	2	0	4	0	0	0	0	0	0	10
Laccaria bicolor	2	1	5	0	2	0	0	0	0	0	10
Volvox	3	2	0	1	1	0	0	0	0	0	7
Physcomitrella	4	2	11	7	14	4	4	3	1	0	50
Spike moss	18	8	26	29	2	2	6	28	2	0	121
Arabidopsis	9	7	13	7	4	1	1	16	3	2	63
Medicago	21	4	10	18	4	1	6	26	1	3	93
Sorghum	14	7	15	44	8	3	4	41	2	3	141
Rice	5	11	16	55	14	4	9	38	1	3	157
Poplar	21	7	22	40	6	3	7	35	4	4	149
Total	110	55	120	207	55	18	37	187	14	15	818

Xu, et al. BMC Bioinformatics, 2009, 10(Suppl 11):S3

Technical Barriers and Considerations

You can make anything but money from lignin:

1. **Recalcitrance of lignin**
2. **Metabolic limits for conversion**
3. **Titer, productivity, and cost-effectiveness**
4. **Market compatibility**
5. **Different product streams need to be available**

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

Nature Systems for Lignin Degradation

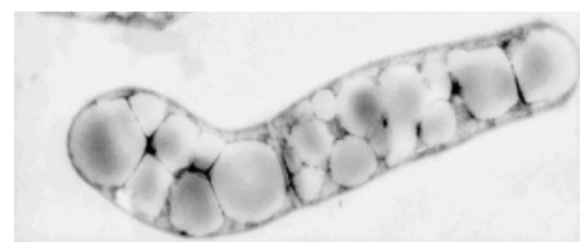
Termite

Long horned beetle

White rot fungi

Pseudomonas putida

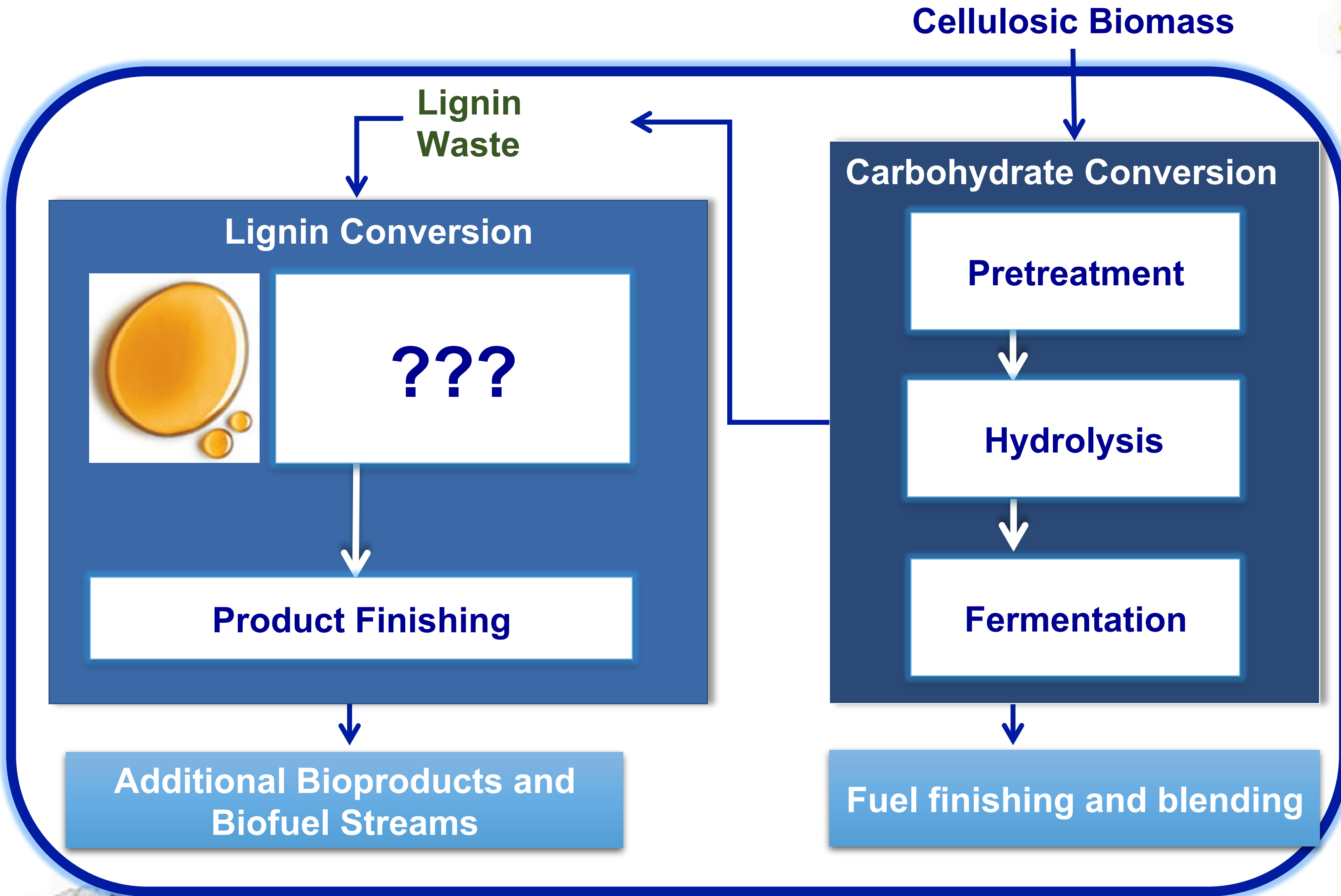
Streptomyces sp.



Rhodococcus sp.

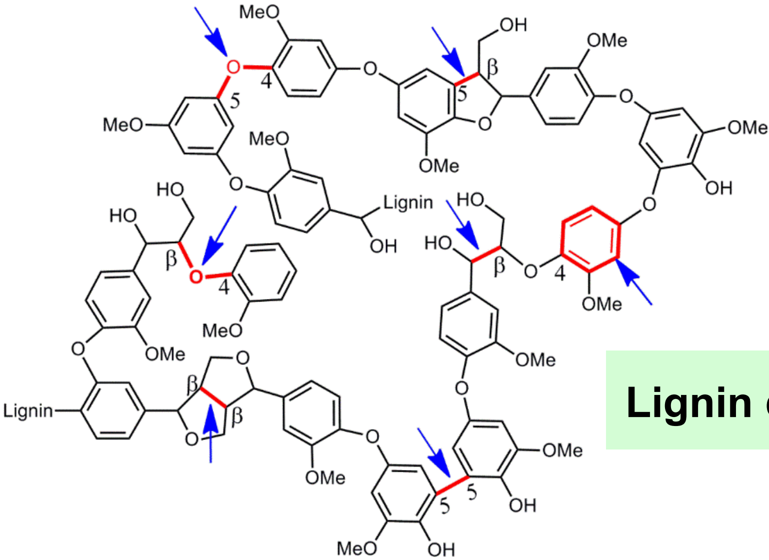
Xie, et al. Curr Opinion of Biotech, 2014, 27:195-203

Lignin Streams to Enable Integrated Biorefinery

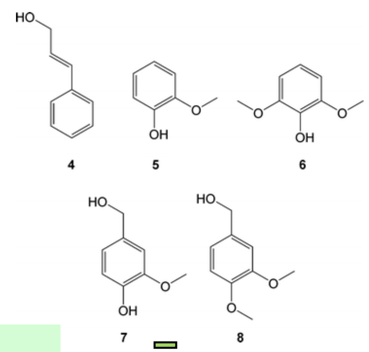


Xie, et al. Industrial Biotechnology 12 (3), 161-167

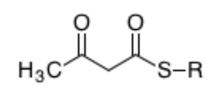
Challenges for Lignin Bioconversion



Lignin depolymerization



Aromatic compound utilizition capacity



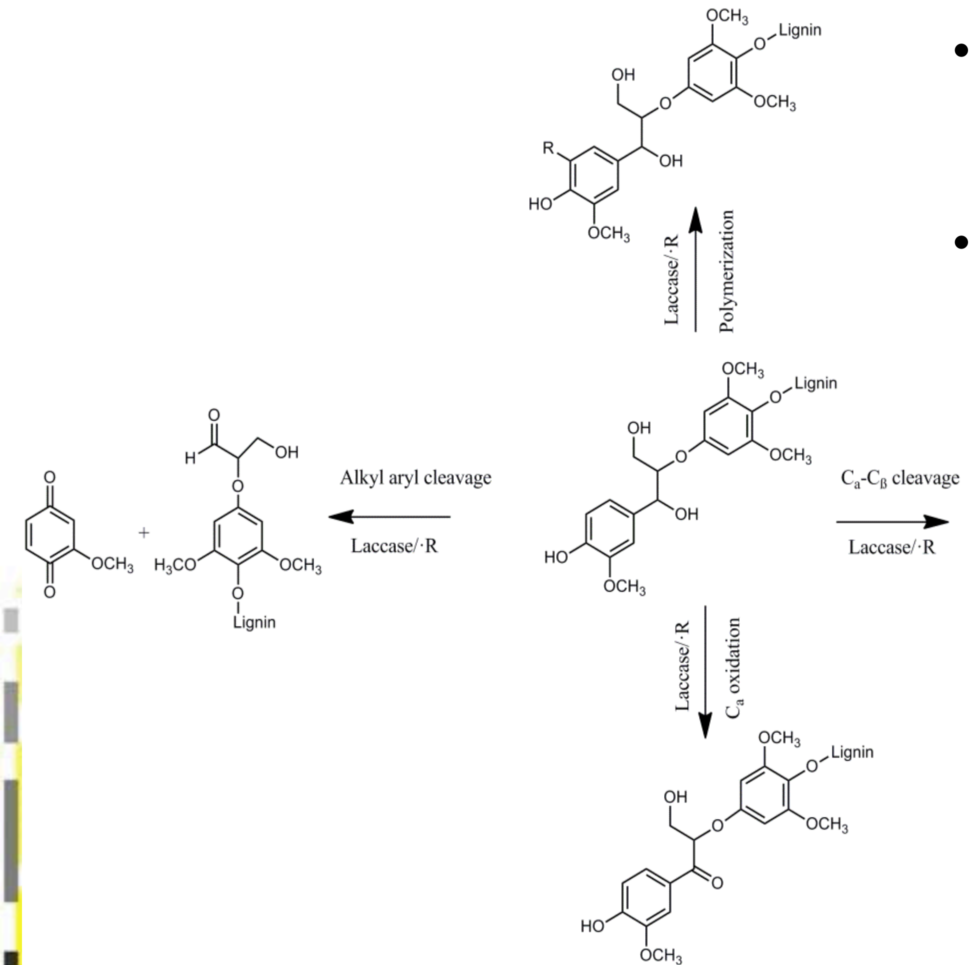
Lipid accumulation capacity

- Redox reactions instead of hydrolysis, requiring electron donor.
- Diverse chemical linkages.
- Diverse aromatic compound monomers
- Diverse chemical intermediates

Lipid Production

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

Laccase for Lignin Degradation

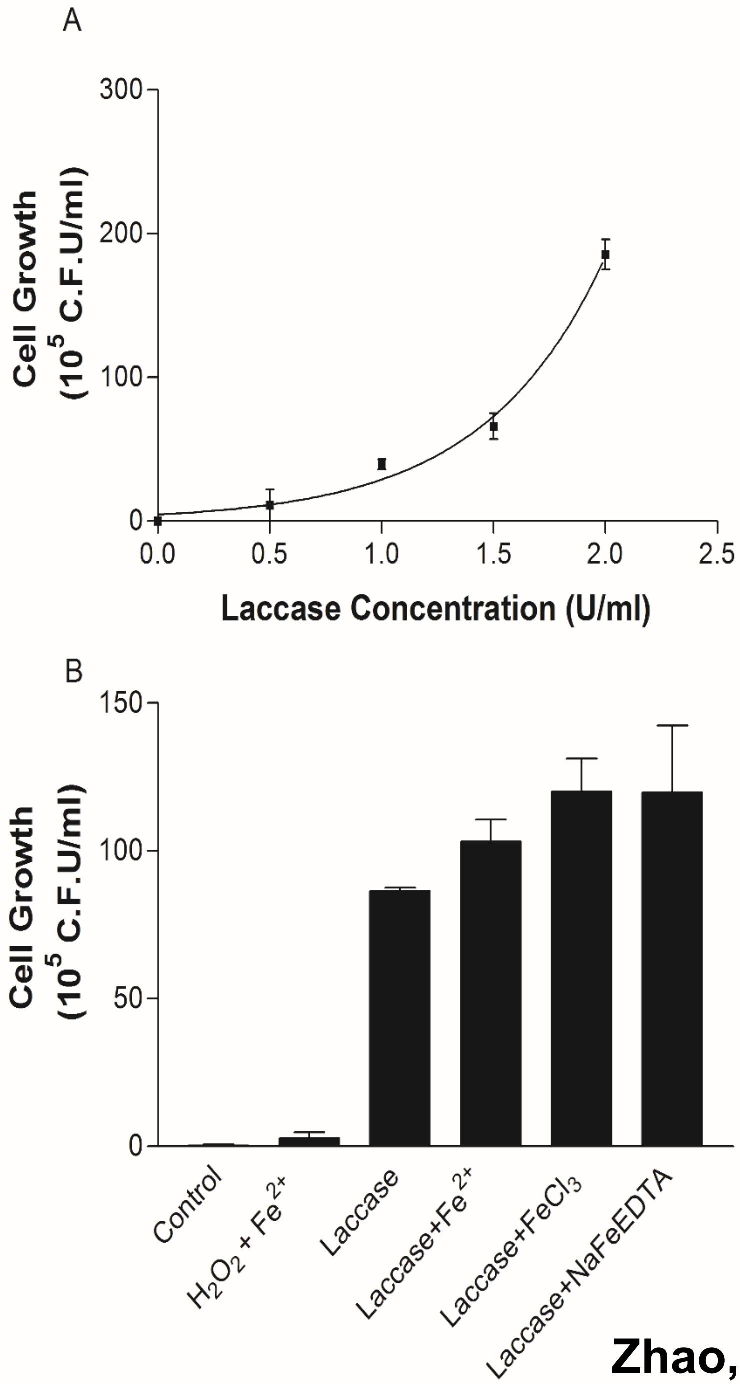


- Laccase can self-generate radicals and further utilize the radicals to catalyze downstream reactions.
- Laccase can both polymerize and depolymerize aromatics.

Scientific Questions:

- Can laccase synergize with cells to promote lignin depolymerization?
- How efficient is the synergy?

Zhao, et al., *Green Chemistry*, 2016, 18, 1306-1312.

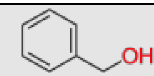
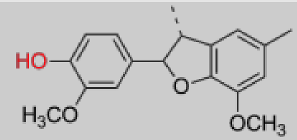
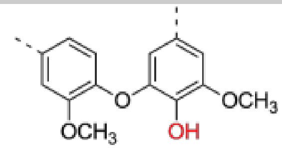
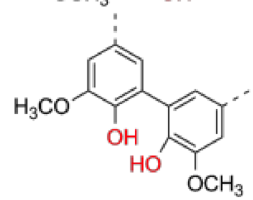
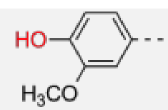
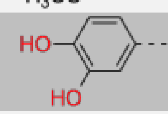
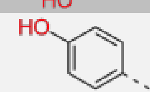
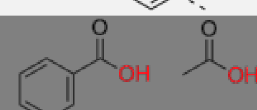


Laccase and Cell Synergy

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

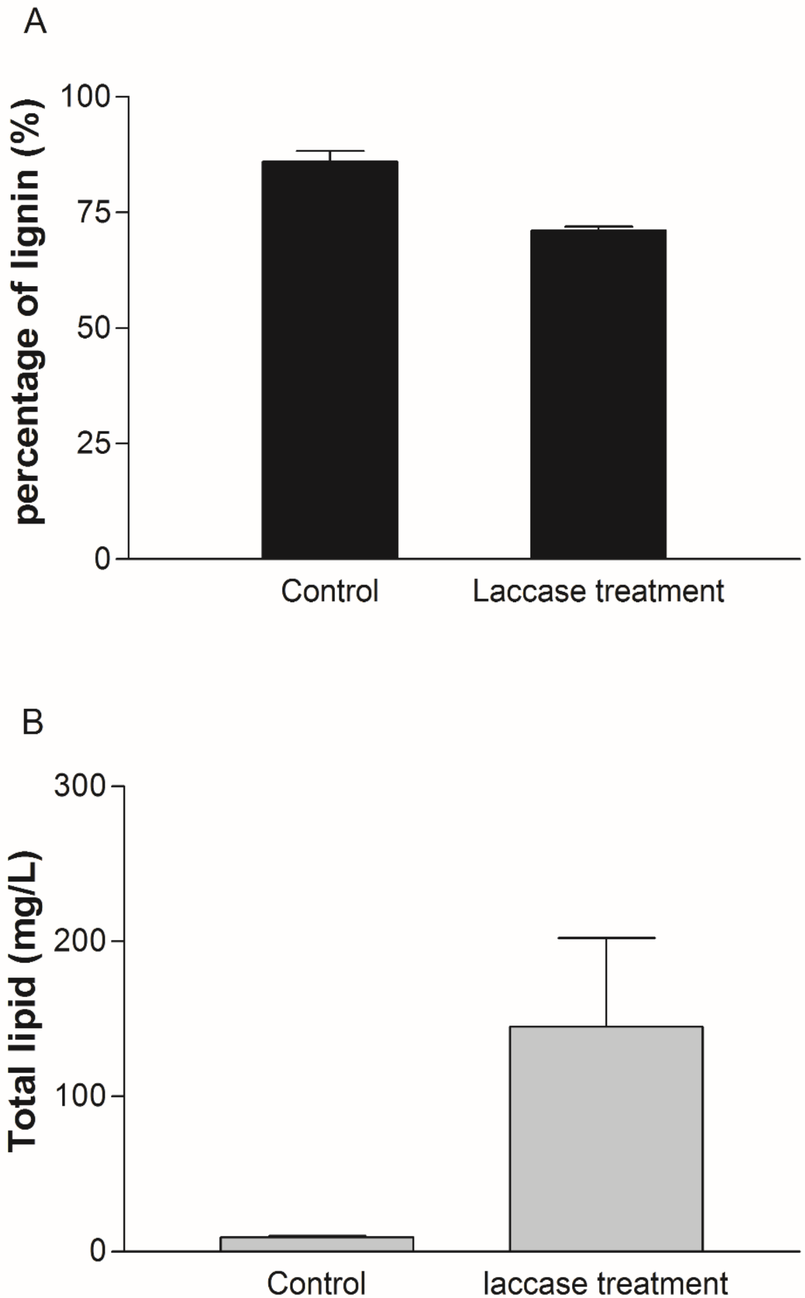
Zhao, et al., *Green Chemistry*, 2016, 18, 1306-1312.

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
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

Zhao, et al., *Green Chemistry*, 2016, 18, 1306-1312.

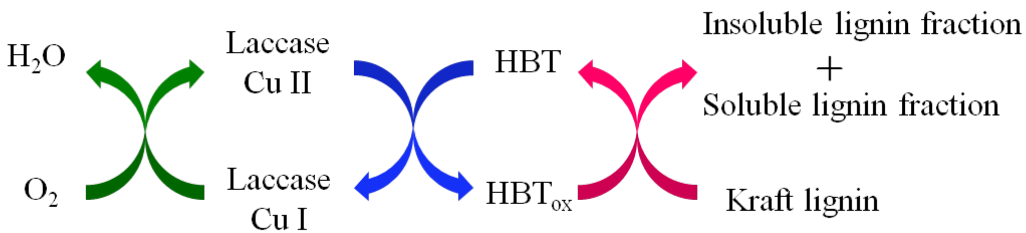
Increase of Lipid Yield by SDF

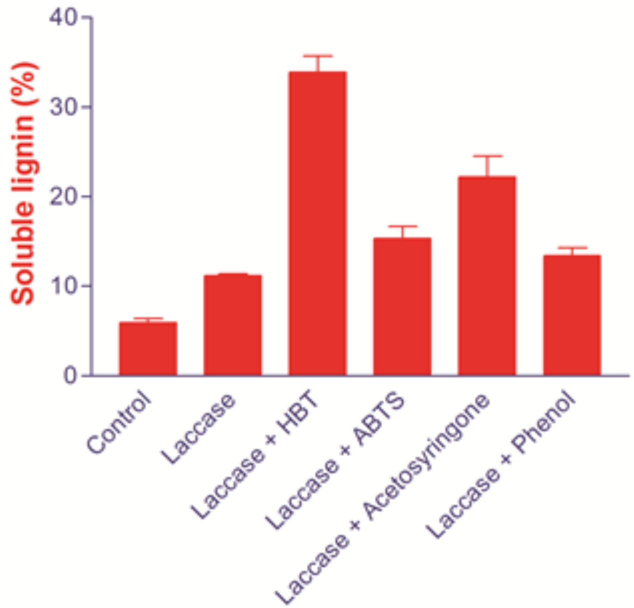


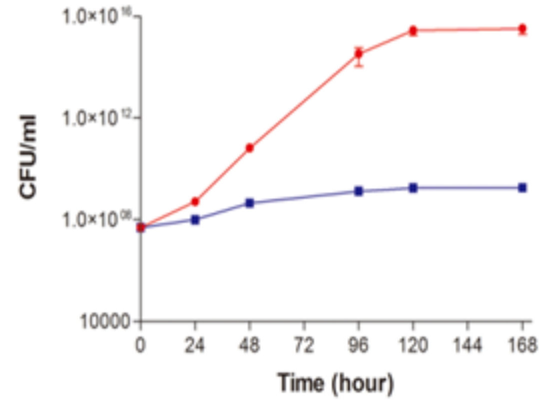
- The Prussian blue assay confirmed the decrease of lignin content.
- The lipid yield increased for about 17 fold in the cells with laccase treatment at 1 U/mL.

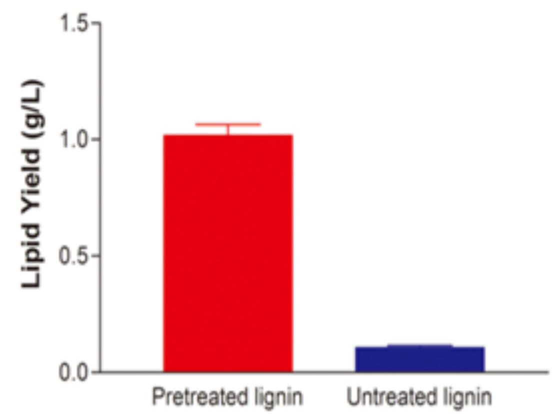
Zhao, et al., *Green Chemistry*, 2016, 18, 1306-1312.

Lignin Depolymerization and Solubilization by Laccase-Mediator system





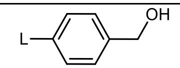
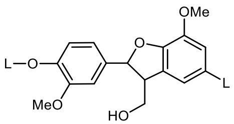
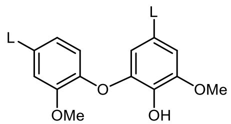
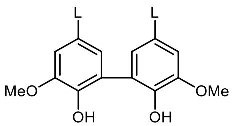
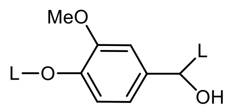
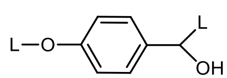
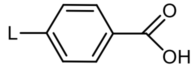




Laccase-mediator system has enhanced redox transfer in lignin depolymerization, released >35% of lignin, and enabled >1g/L titer in conversion.

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)

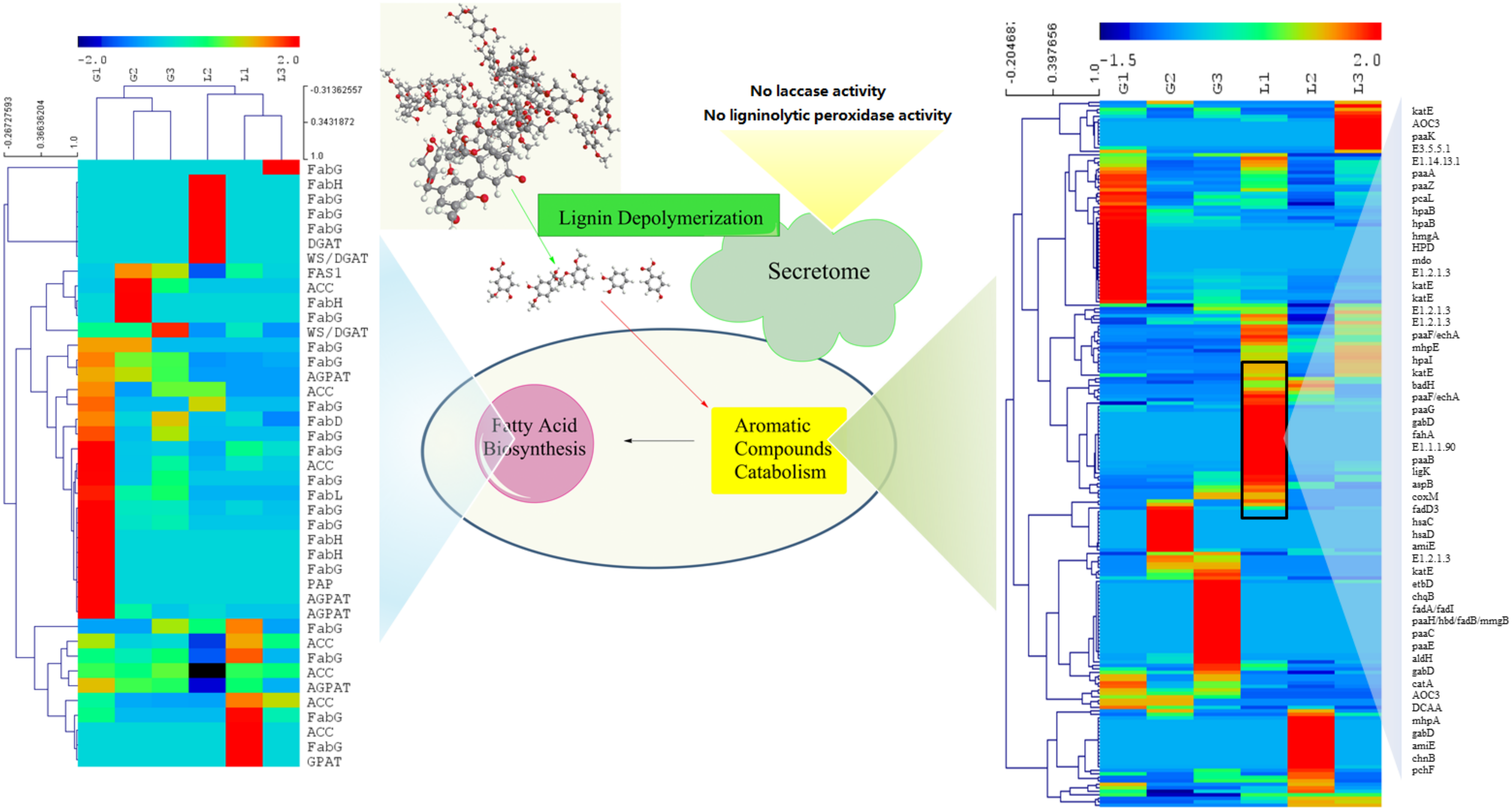
Summary

- Ligninase like laccase can synergize with the cell to promote cell growth and lipid yield on lignin. The biological and chemical mechanisms for such synergy is 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.

- Laccase is an effective enzyme for engineering the efficient lignin bioconversion.

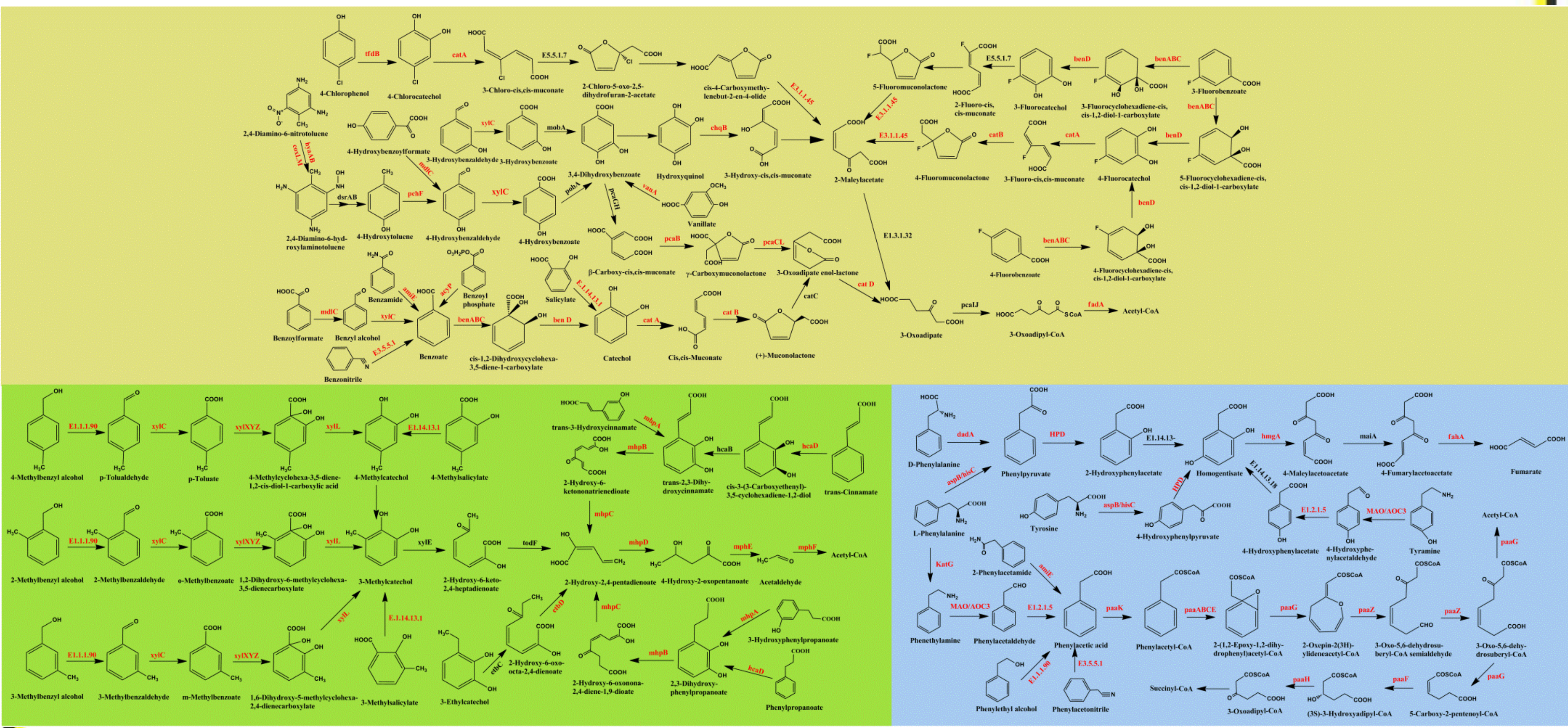
- Enzyme mediator system is even more effective in lignin degradation

Systems Biology Analysis of Lignin Degradation in *R. opacus*



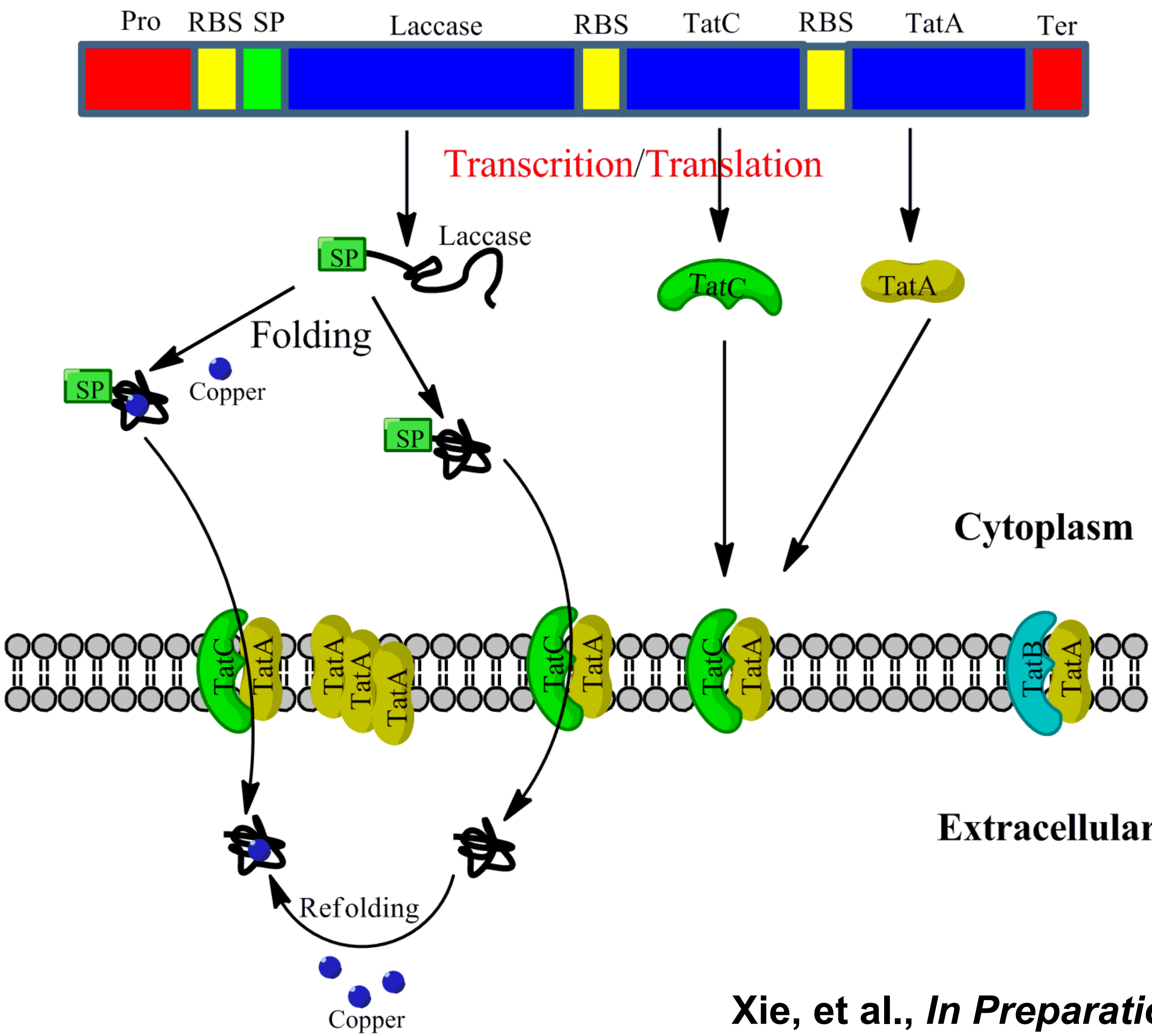
Xie, et al., *In Preparation*.

Aromatic Compounds Catabolism Capacity as Revealed by Proteomics



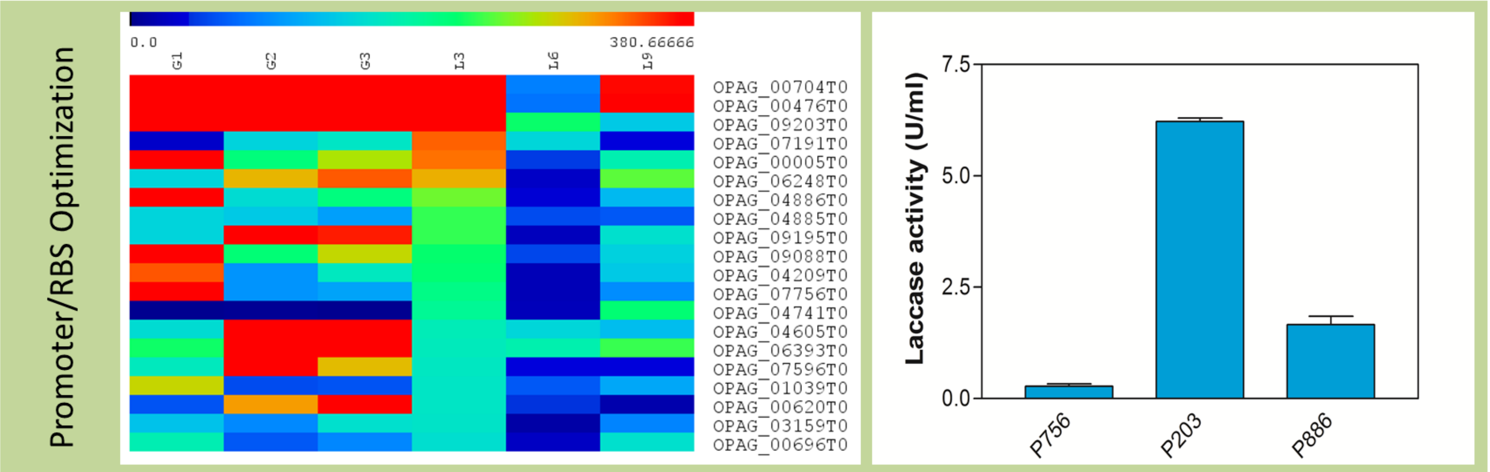
Xie, et al., *In Preparation*.

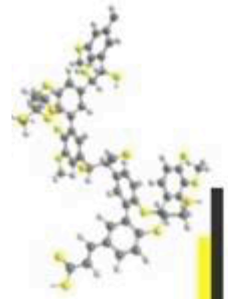
Building Lignin Depolymerization Module



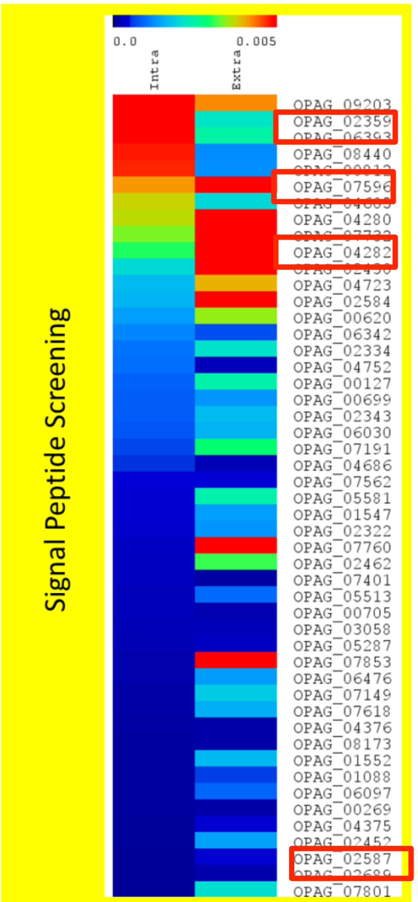
Xie, et al., *In Preparation*.

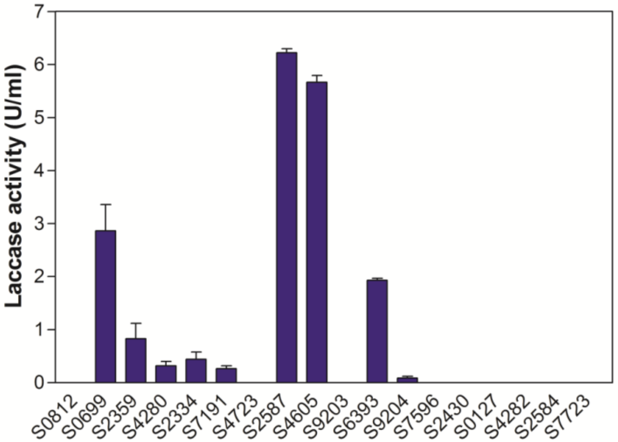
Step 1

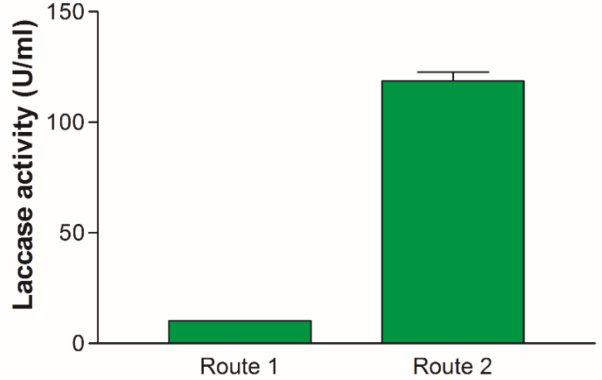


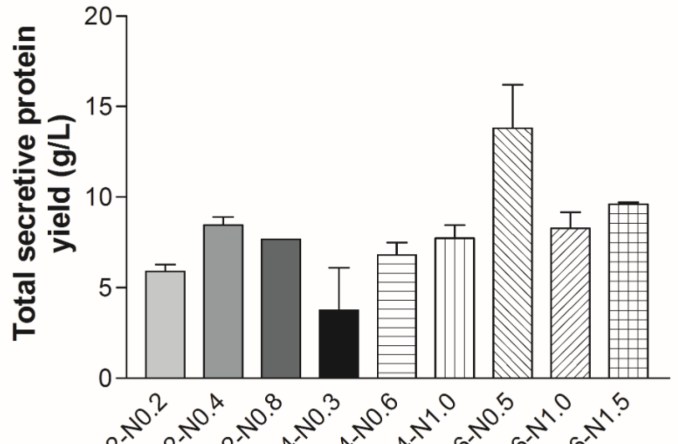


Step 2



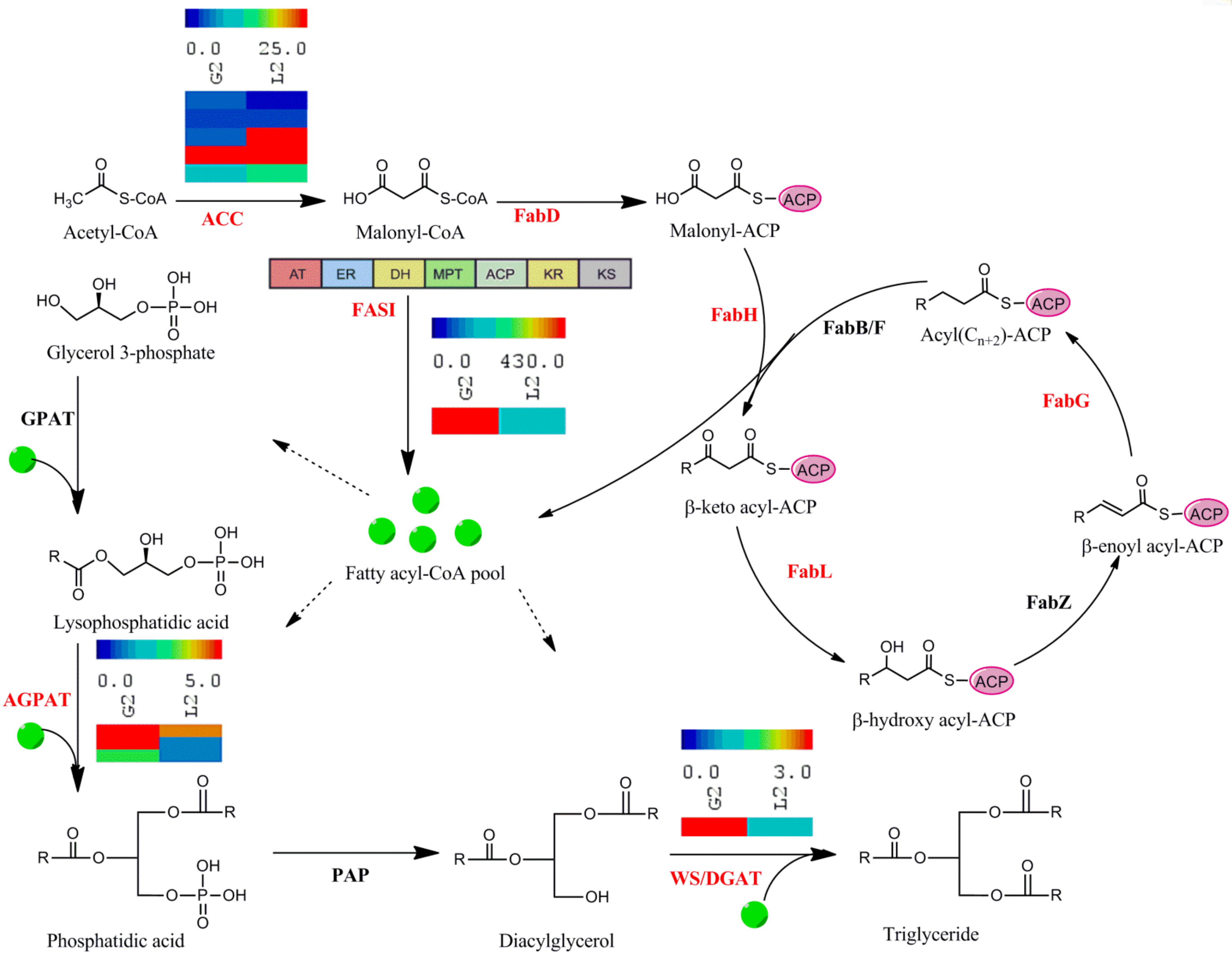




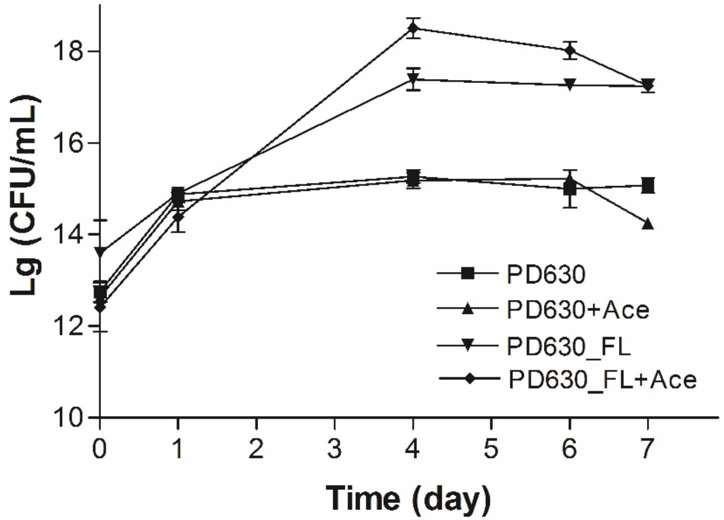


Proteomics-Guided Synthetic Design

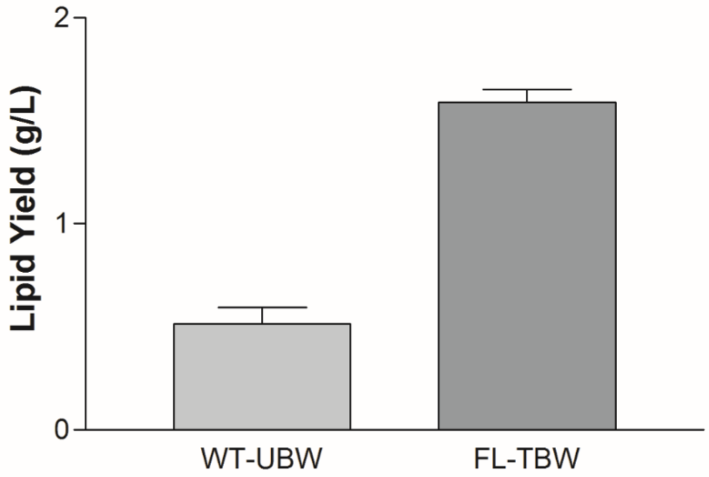
Systems Biology-Guided Optimization of Lipid Production

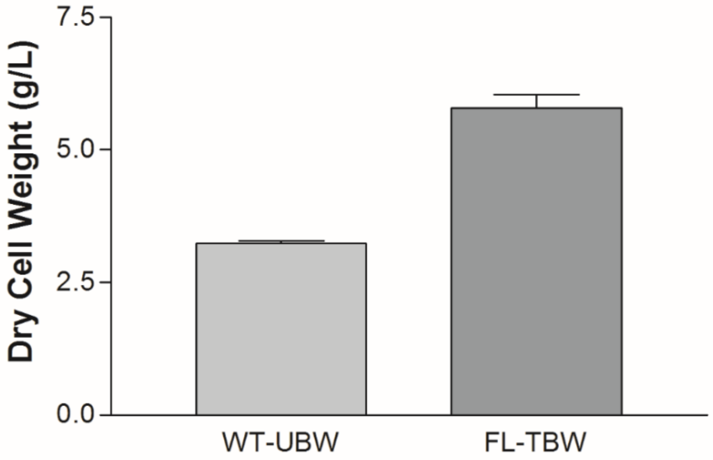


Integration of Functional Modules Leads to Record Lipid Yield from Lignin



The bacterial dry biomass weight and lipid yield grown on biorefinery waste. WT-UBW: wild type strain grown on untreated biorefinery waste; FL-TBW: engineered strain grown on pretreated biorefinery waste.





Xie, et al., *In Preparation*.

Summary

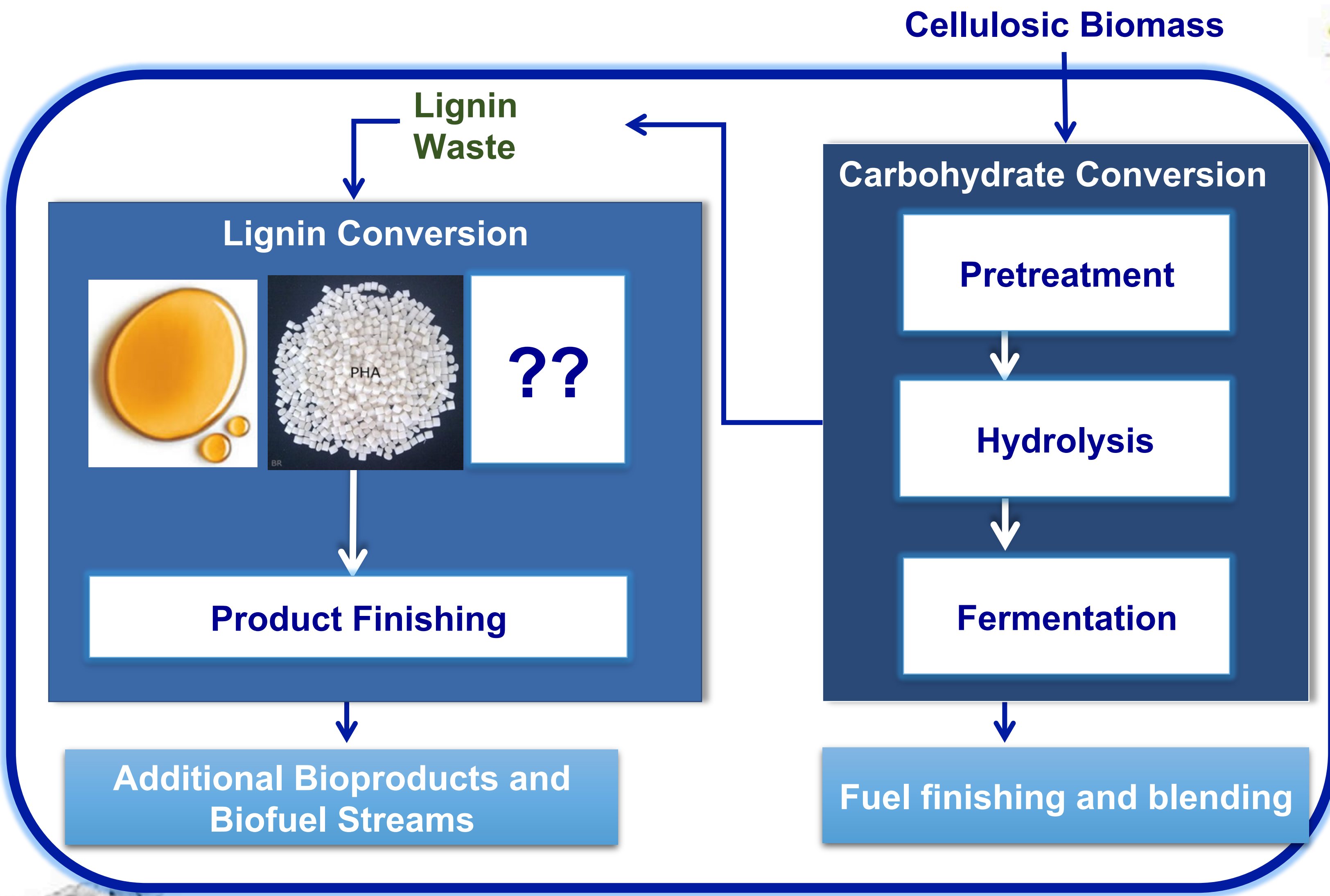
1. Four steps systems biology guided biodesign has enabled *R. opacus* PD630 to produce laccase at >120 U/ml and >15g/L. The study demonstrated that Gram positive bacteria can be engineered to secretively expression and produce large quantity of protein with broad applications in bioeconomy

2. Systems biology analysis revealed that enzymes important for lipid accumulation. The optimization of fatty acid biosynthesis pathway in *R. opacus* PD630 could significantly improve its lipid accumulation.

3. The integration of the secretive laccase system and fatty acid biosynthesis two modules led to record level production of lipid from lignin. We are close to commercial relevant yield through these designs.

Xie, et al., *In Preparation*.

Lignin Streams to Enable Integrated Biorefinery



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

Reverse Design of NBUS for Lignin Fuels & Products

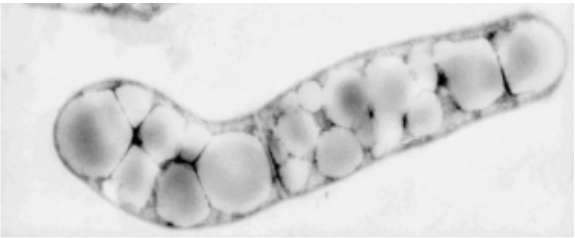

Termite


Long horned beetle

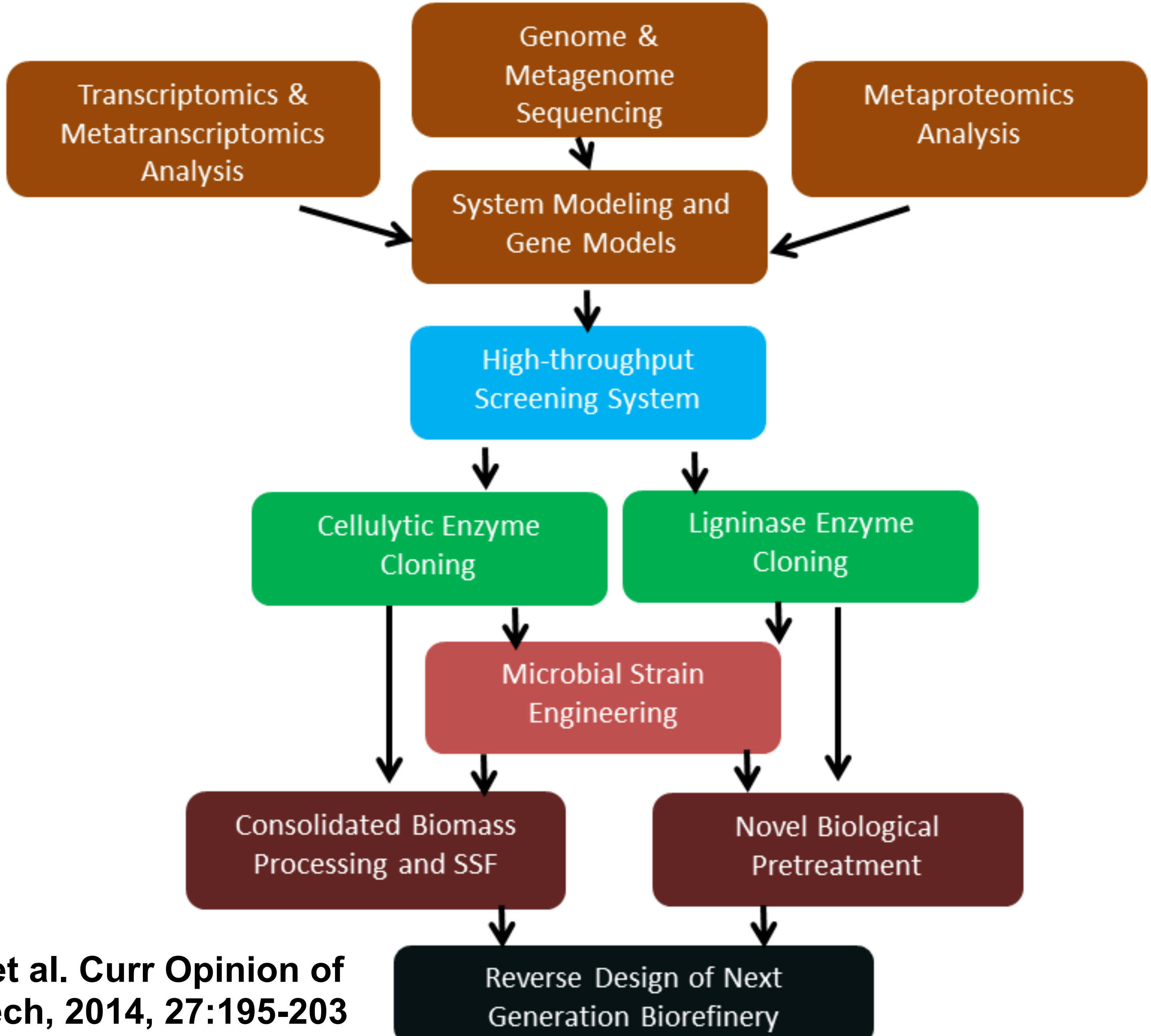

White rot fungi


Pseudomonas putida


Streptomyces sp.

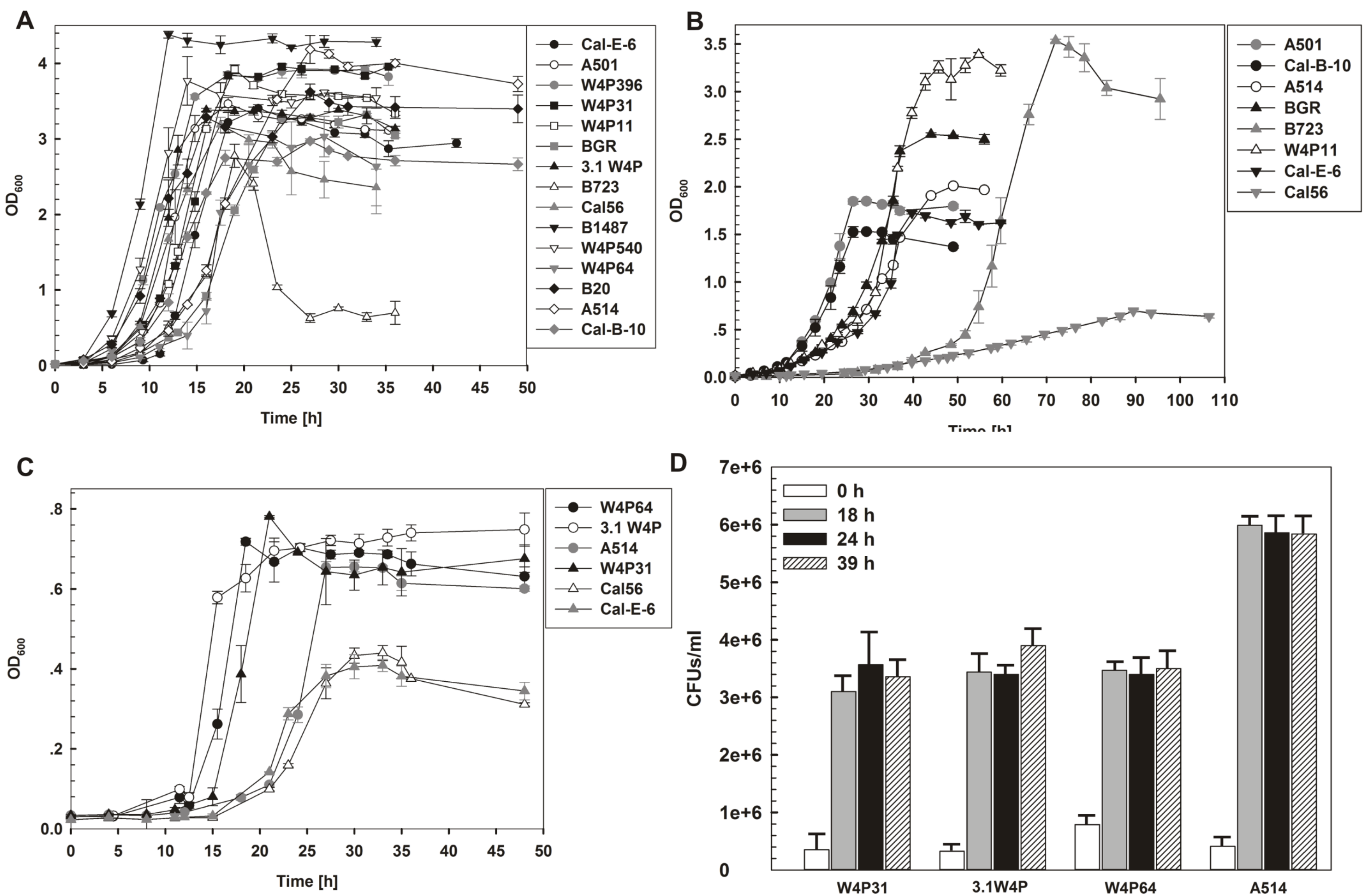

Rhodococcus sp.

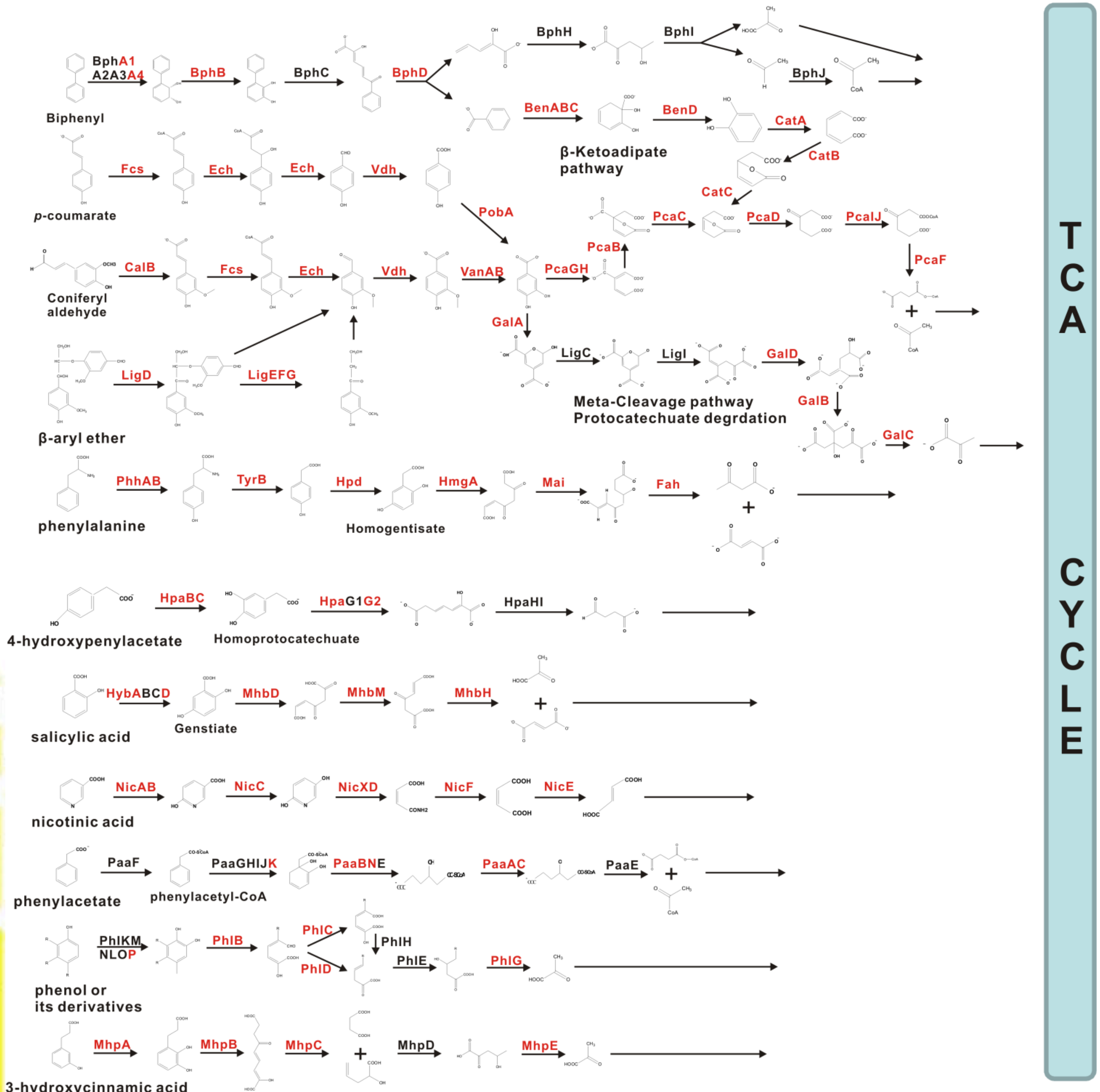
Xie, et al. Curr Opinion of Biotech, 2014, 27:195-203



Xie, et al. Curr Opinion of Biotech, 2014, 27:195-203

Discovering a Lignin-Utilization *P. putida* Strain

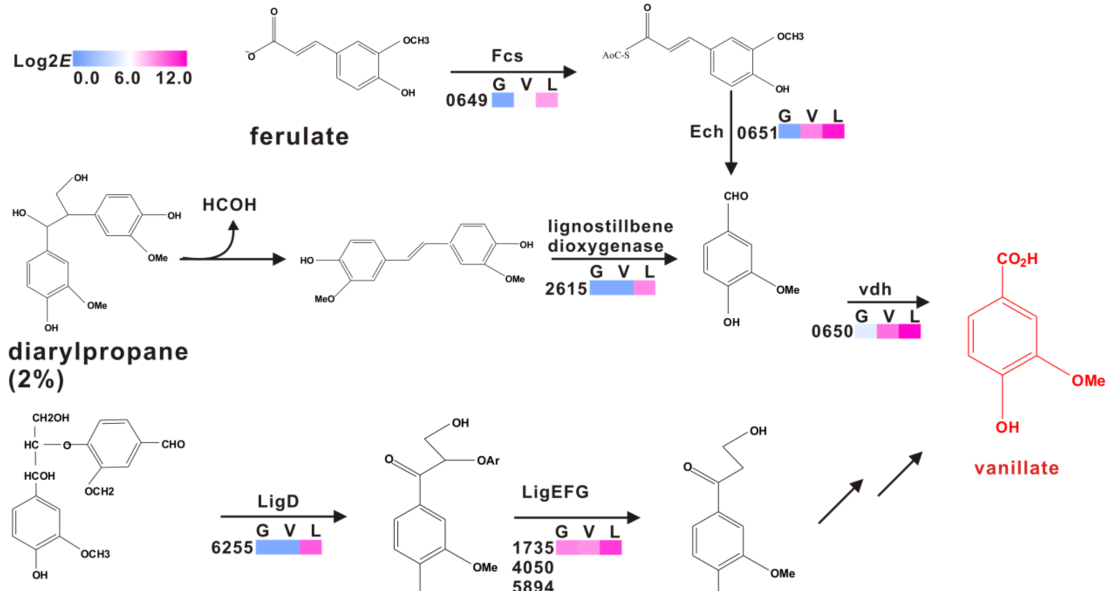


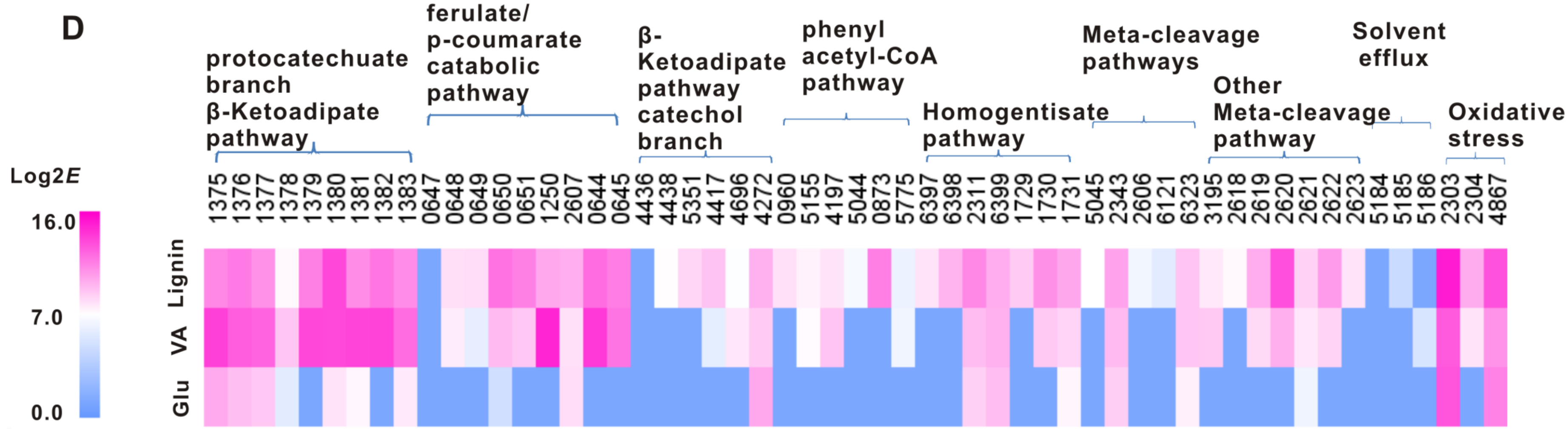


Broad Aromatic Compound Degradation Pathway as Revealed by Genome Sequencing

Lin, et al., Green Chemistry, 2016, 18: 5536-5547.

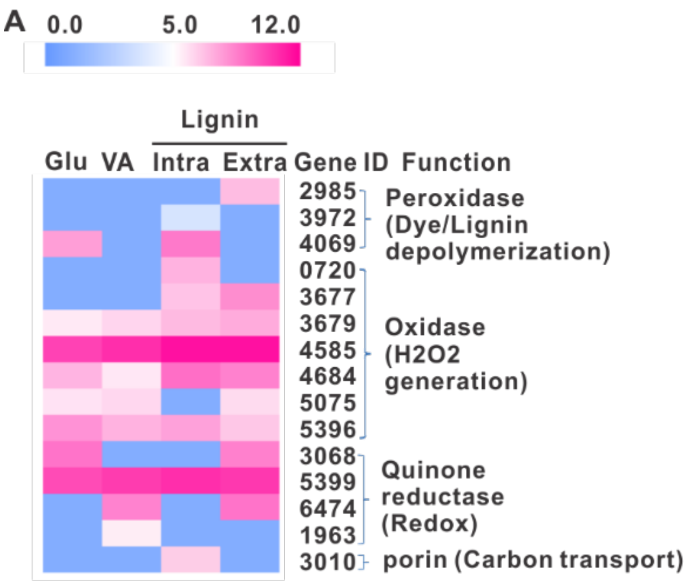
Overview of Proteomics

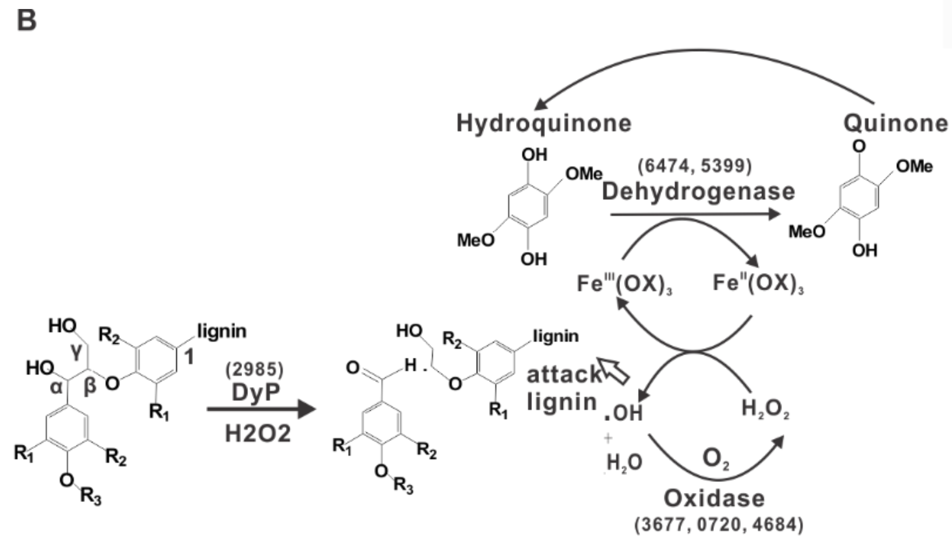


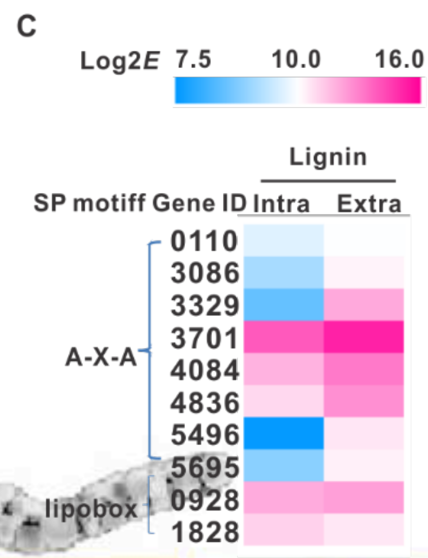


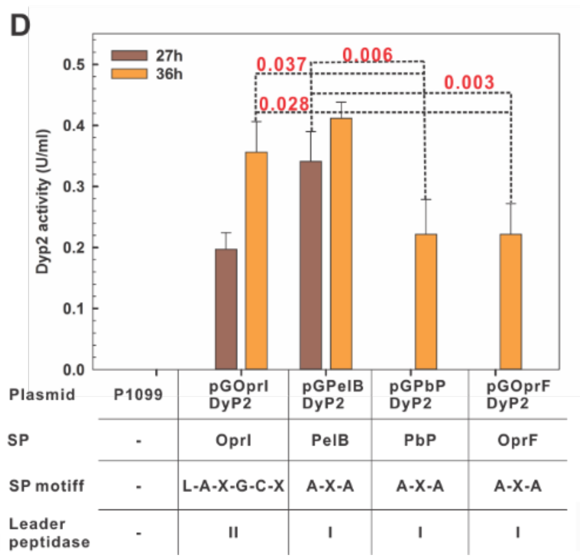
Lin, et al., Green Chemistry, 2016, 18: 5536-5547.

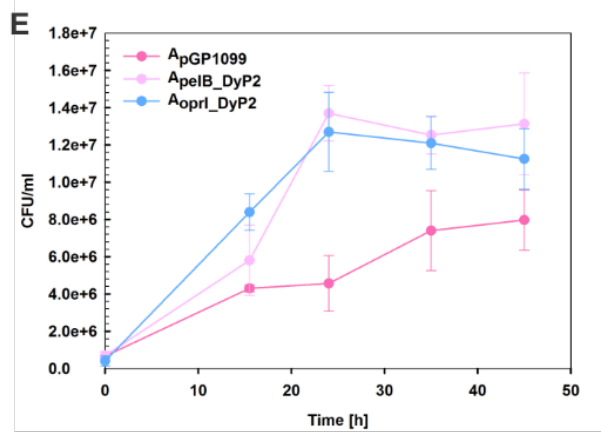
Design of Lignin Depolymerization Modules





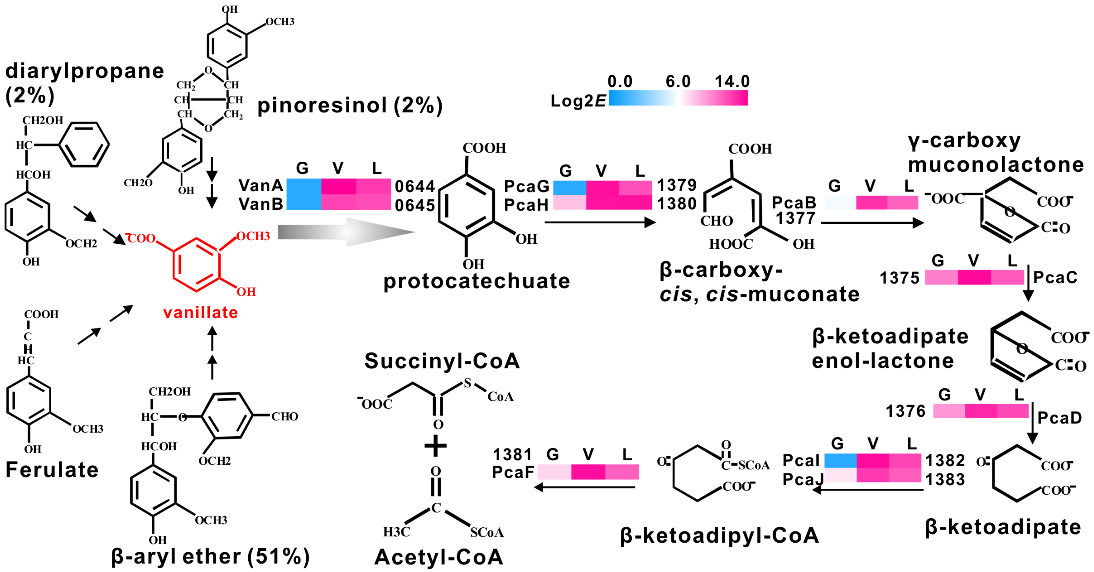


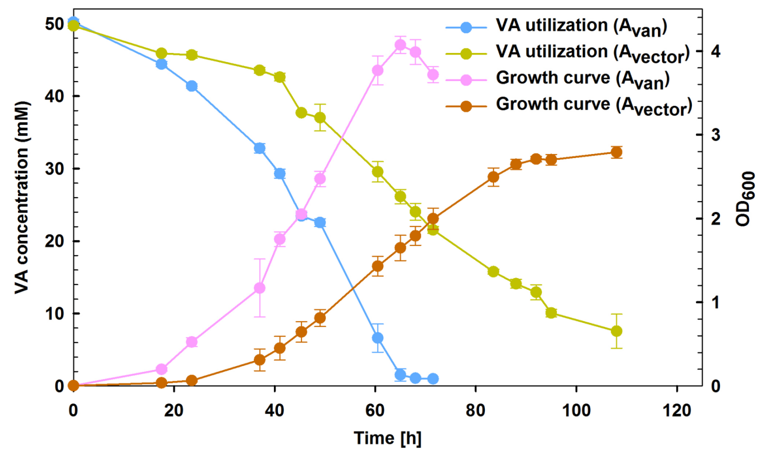


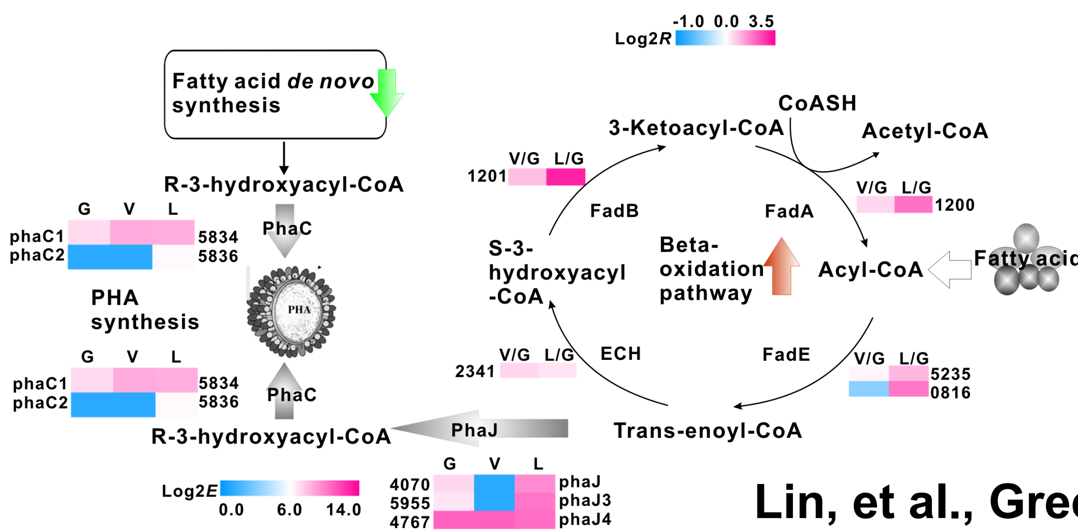


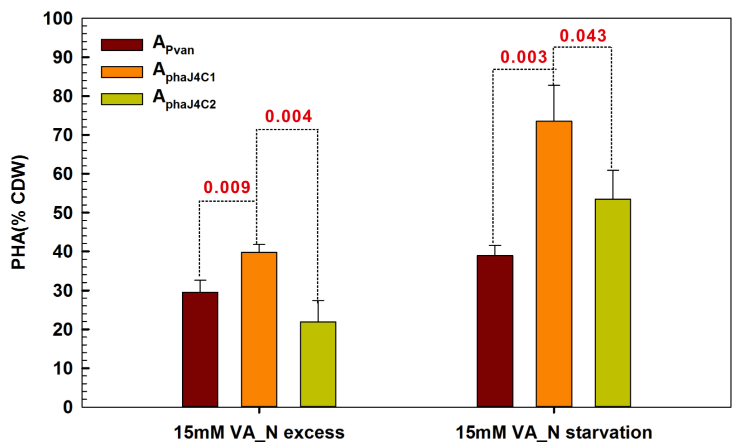
Lin, et al., Green Chemistry, 2016, 18: 5536-5547.

Design of Aromatic Compound Catabolism and PHA Modules



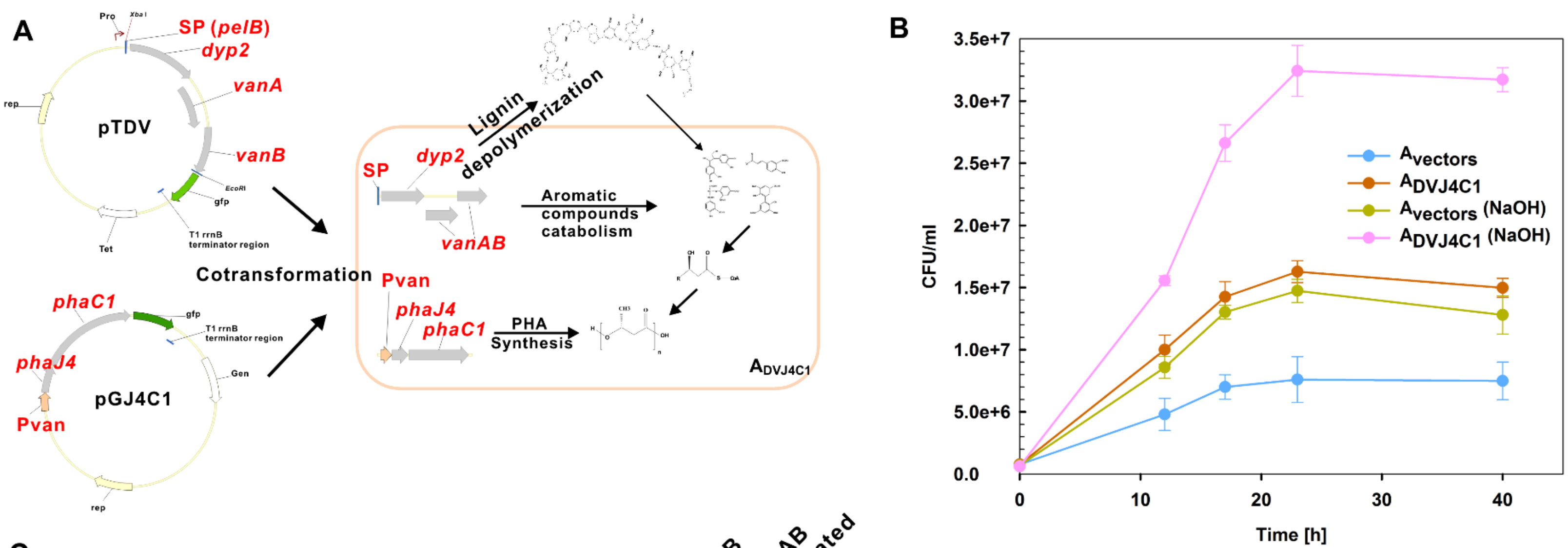


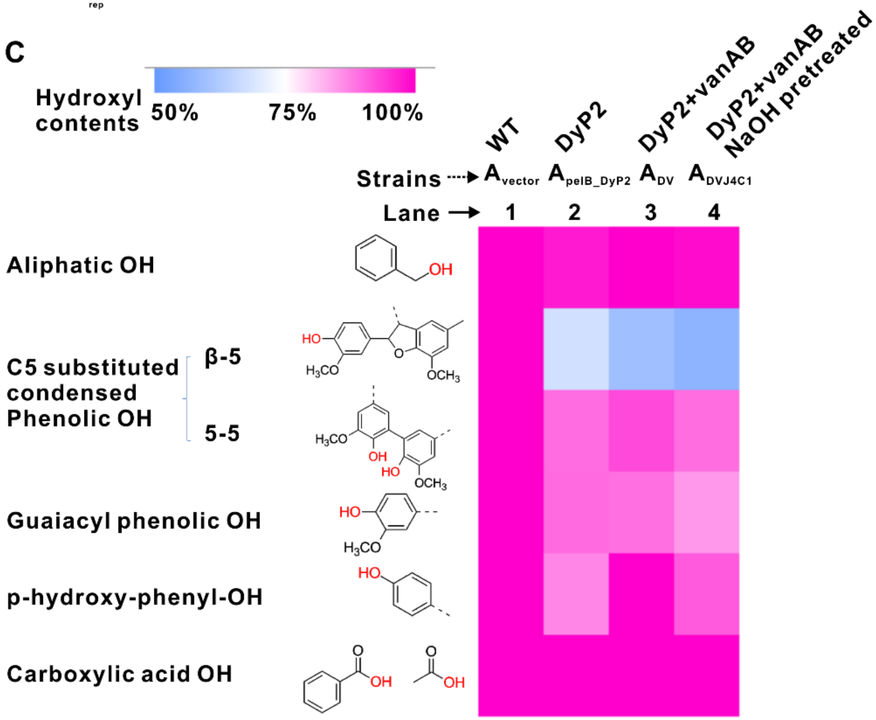


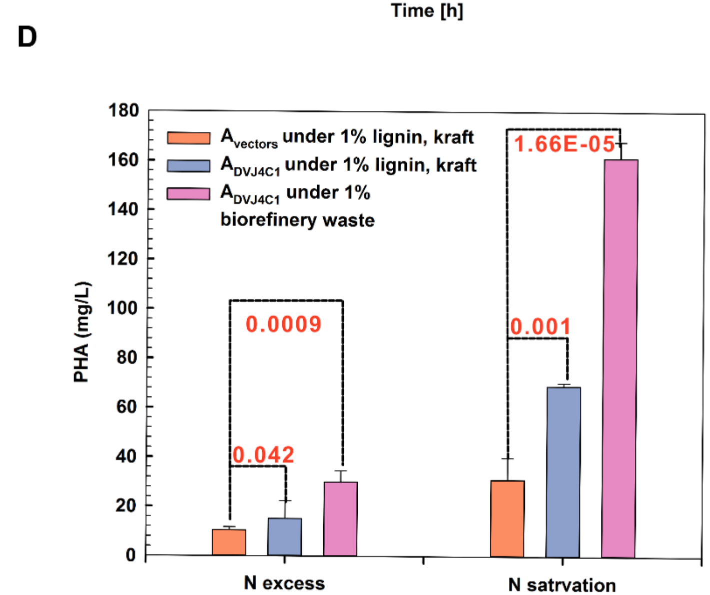


Lin, et al., Green Chemistry, 2016, 18: 5536-5547.

System Integration for Lignin to Bioplastics







Lin, et al., Green Chemistry, 2016, 18: 5536-5547.

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 consolidated lignin conversion to PHA with increased yield.

Lin, et al., Green Chemistry, 2016, 18: 5536-5547.

Project Relevance: Multistream Integrated Biorefinery (MIBR) for Biomanufacturing

Lignocellulosic biomass → Biological conversion → Lignin containing Biorefinery wastes

Fractionation

Soluble lignin (low molecular weight)

Fermentation

Lipid & Biodiesel

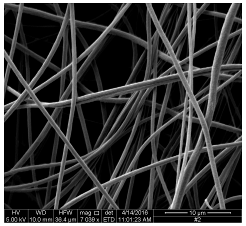
Separation → Asphalt Binder Modifier

HDO → Jet fuel

Insoluble lignin (high molecular weight)

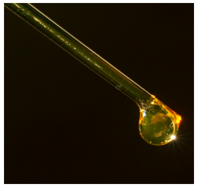
Material Synthesis

Carbon fibers



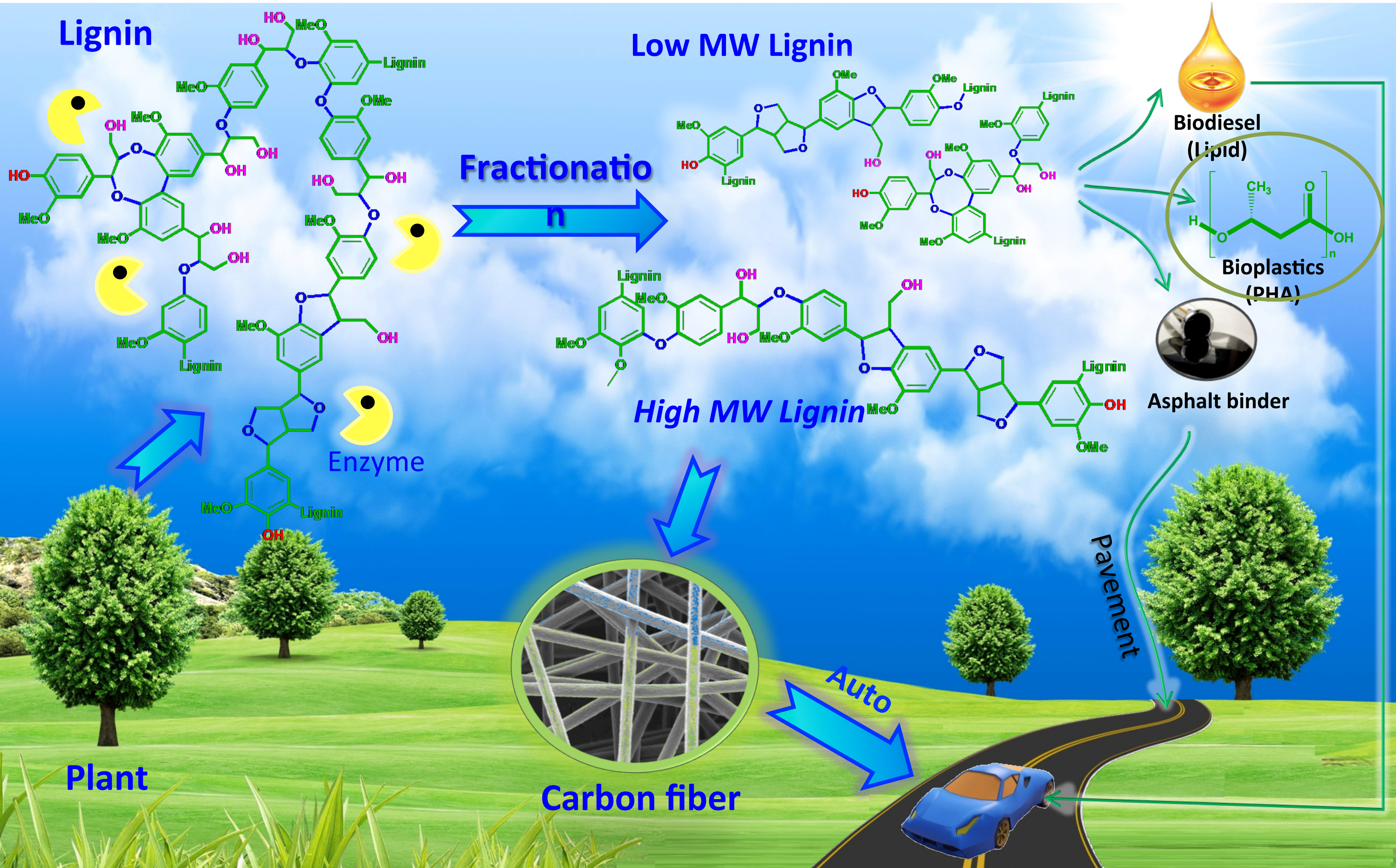
Li et al., Green Chemistry, 2017, In press

Xie et al., ACS Sus. Chem. Eng, In Press



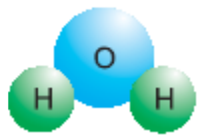
Wang et al., Green Chemistry, 2016, 18, 2802-2810.
Wang et al., Green Chemistry, 2015,17, 5131-5135.
Laskar et al., Green Chemistry, 2014, 16 (2), 897 – 910.

Relevance – Enabled Multistream Integrated Biorefinery (MIBR)

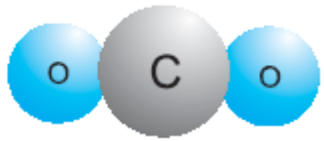


Challenges for Sustainable Fuels & Chemicals

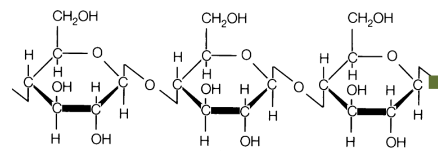
Sun Light



Water



Carbon Dioxide



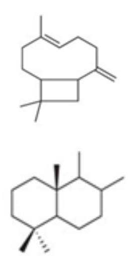
Bioconversion into Fuels & Chemicals

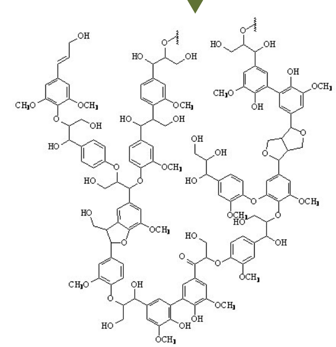
Capture and Utilize CO_2 & Hemicellulose Processing

Biomass Deconstruction

Redirecting Photosynthetic Carbon Output

Terpenoid as Drop-in Fuels & High Value Products



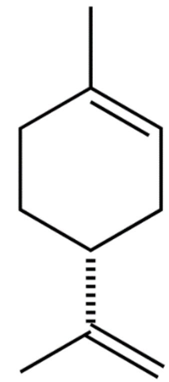


Lignin Processing into Valued Products

Sustainability and Cost Effectiveness

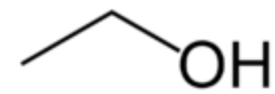
Terpenes for Fuels and Chemicals

- Thermophysical and thermochemical properties of target fuel molecules
  - Energy Density
  - Cloudy Points
  - Others
  - ‘Drop in’ biofuels
  - Minimal hydrogen consumption during cracking
- Diverse product stream amenable to different markets – squalene, astaxanthin, taxadiene, etc.

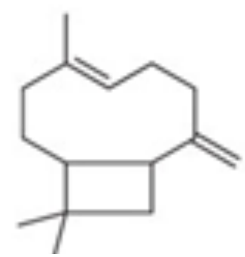


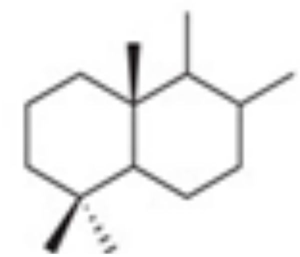
Energy Density
45 MJ/Kg
38 MJ/L

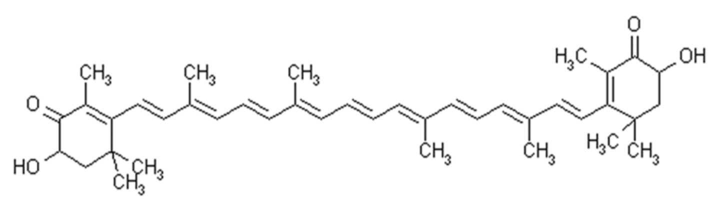
Ethanol



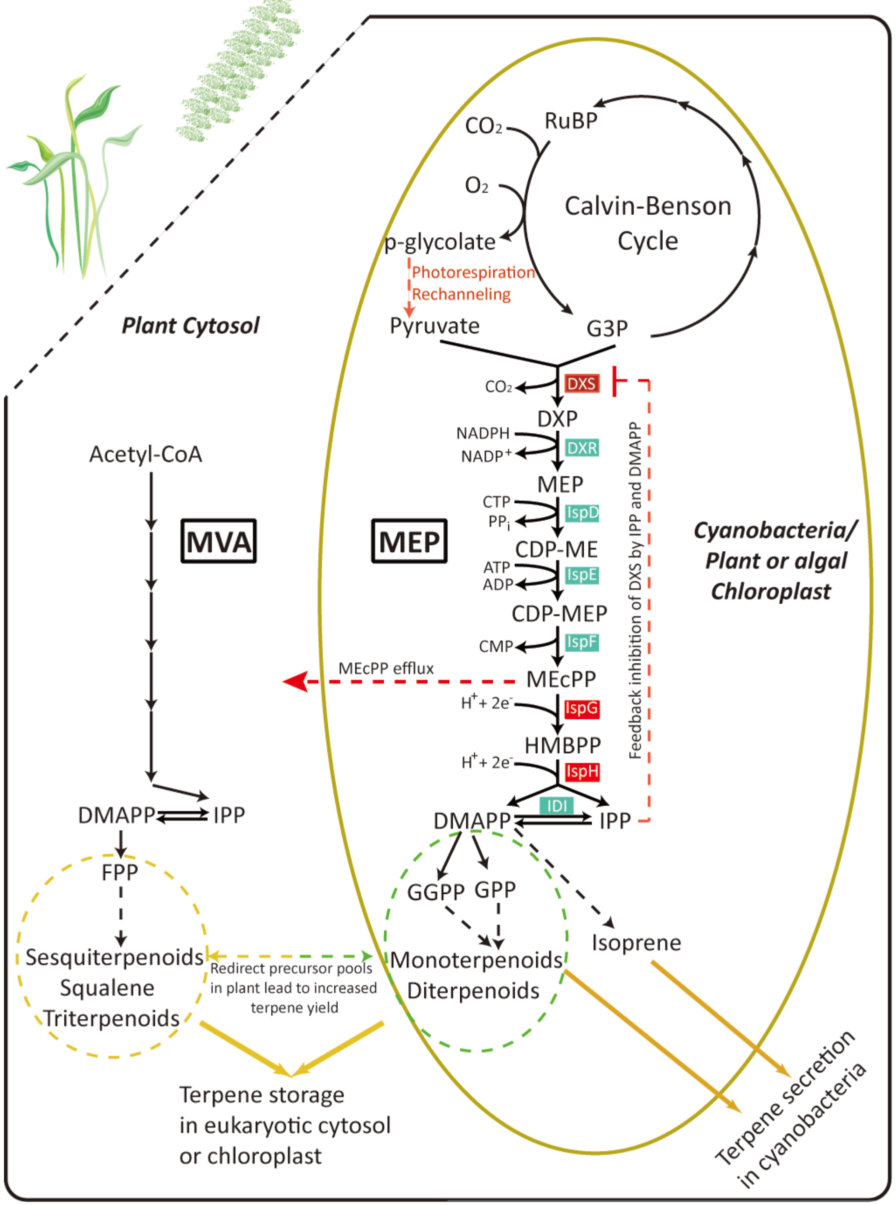
Energy Density
30 MJ/Kg
24 MJ/L







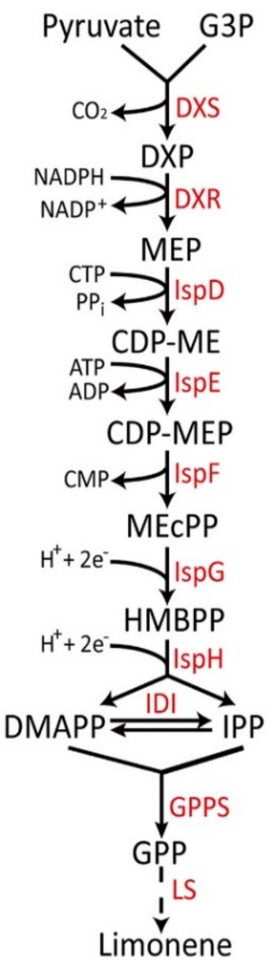
Scientific Challenges



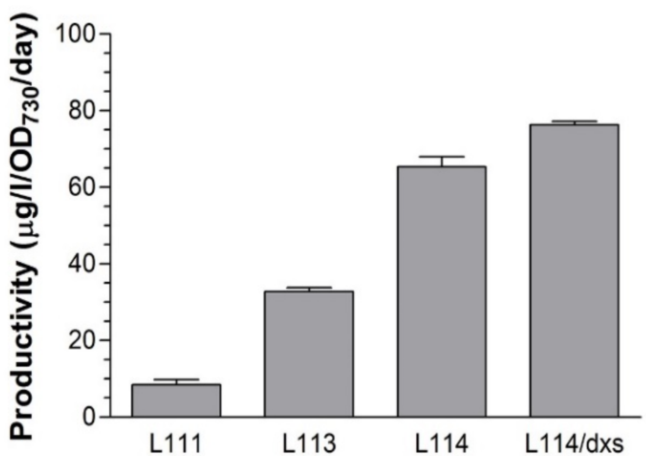
- **Scientifically, perfect unconventional sink!**
- **Low carbon partition**
- **Limited by carbon fixation in photosynthesis**
- **Limited by extensive feedback and downstream consumption of the compounds.**

Enhancing Cyanobacterial Terpene Flux

a

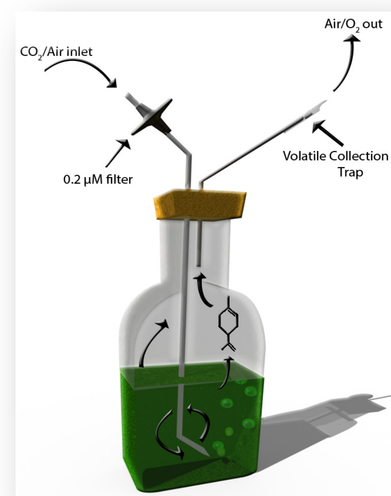


b



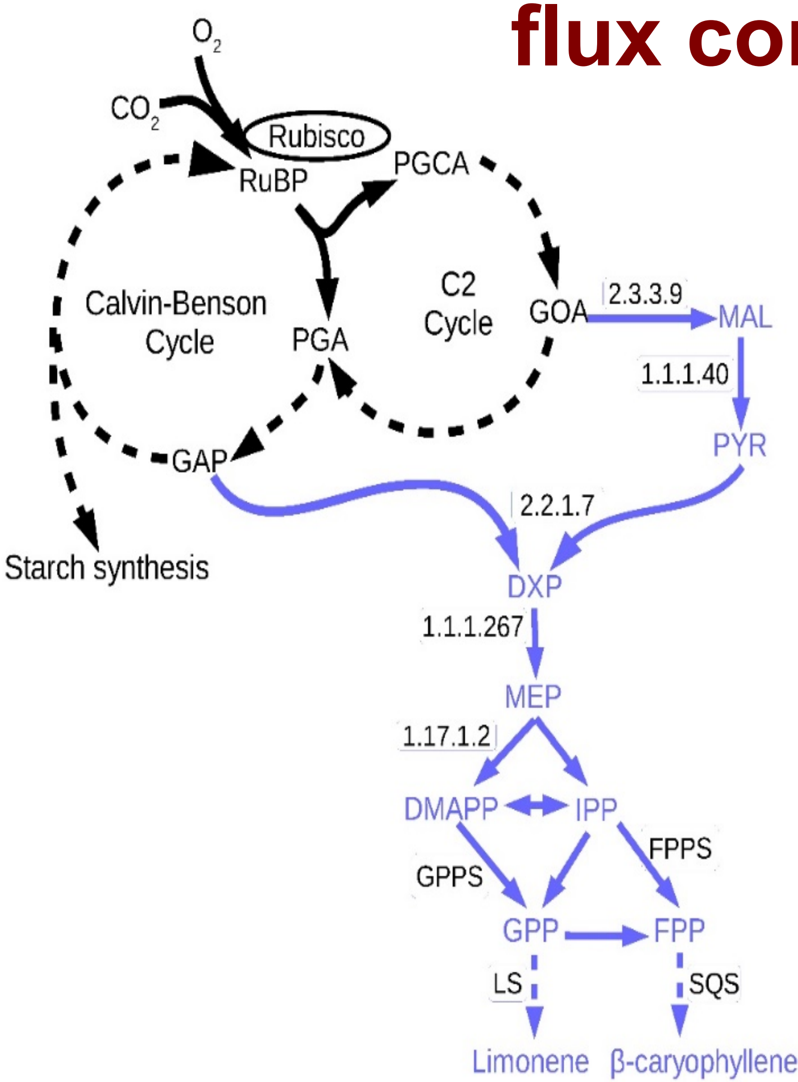
L111: *Ptrc:ls*
L113: *Ptrc:ls* (synthetic RBS)
L114: *Ptrc:ls/gpps*
L114/dxs: *Ptrc:ls/gpps; $P_L$lacO$_1$:dxs*

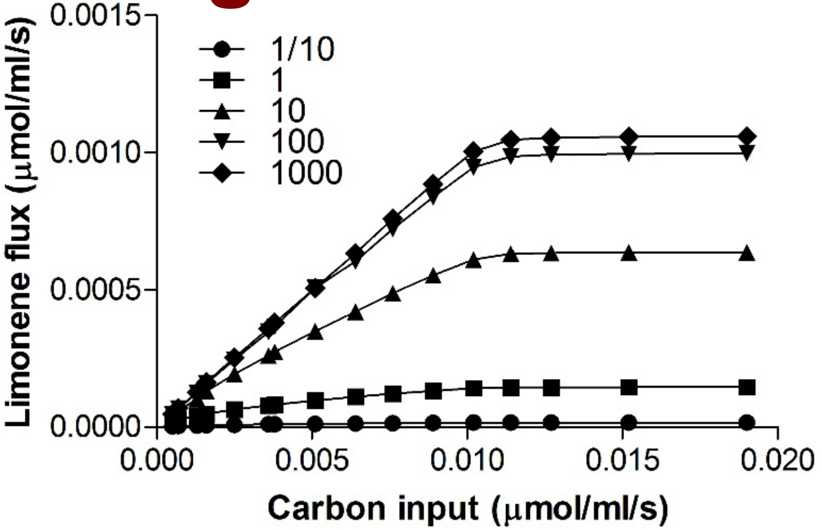
- Metabolic rigidity limits carbon partition into MEP derived terpenes
- *Synechococcus elongatus*
- Limonene synthase (LS) from *mentha spicata*
- Geranyl pyrophosphate synthase (GPPS) from *Abies grandis*
- 1-deoxy-D-xylulose 5-phosphate synthase (DXS) from *Botryococcus braunii*



Wang X, et al. (2016) Proc Natl Acad Sci U S A. 113(50): 14225.

Computational modeling reveals a key flux controlling node

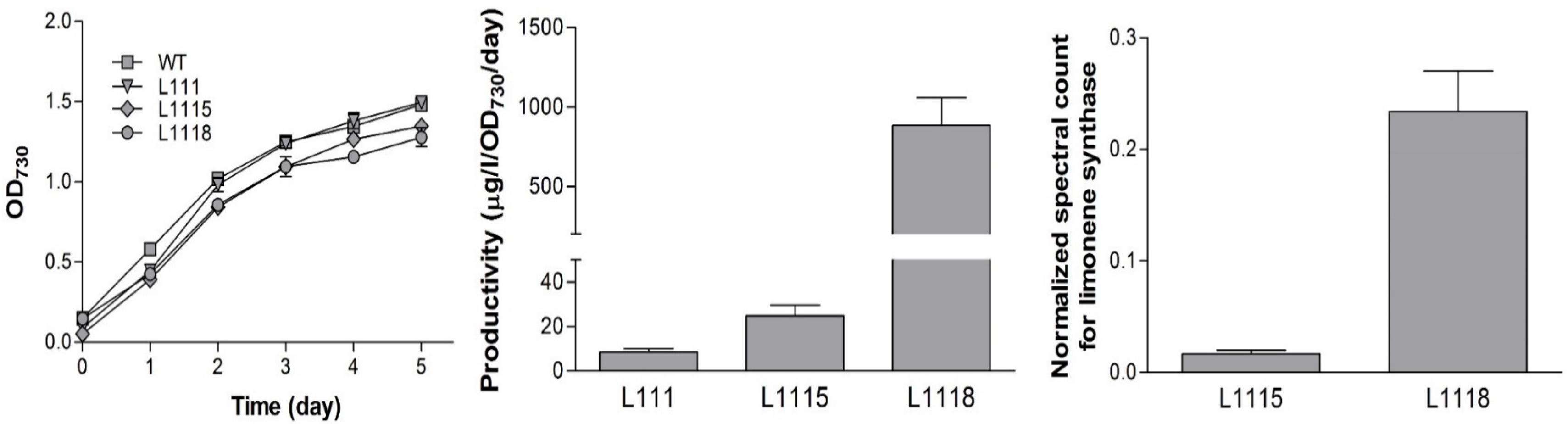




- Model based on a photosynthesis kinetics model by adding MEP pathway kinetics
- Carbon input controlled by pyruvate generation
- Limonene synthase activity was simulated

Wang X, et al. (2016) Proc Natl Acad Sci U S A. 113(50): 14225.

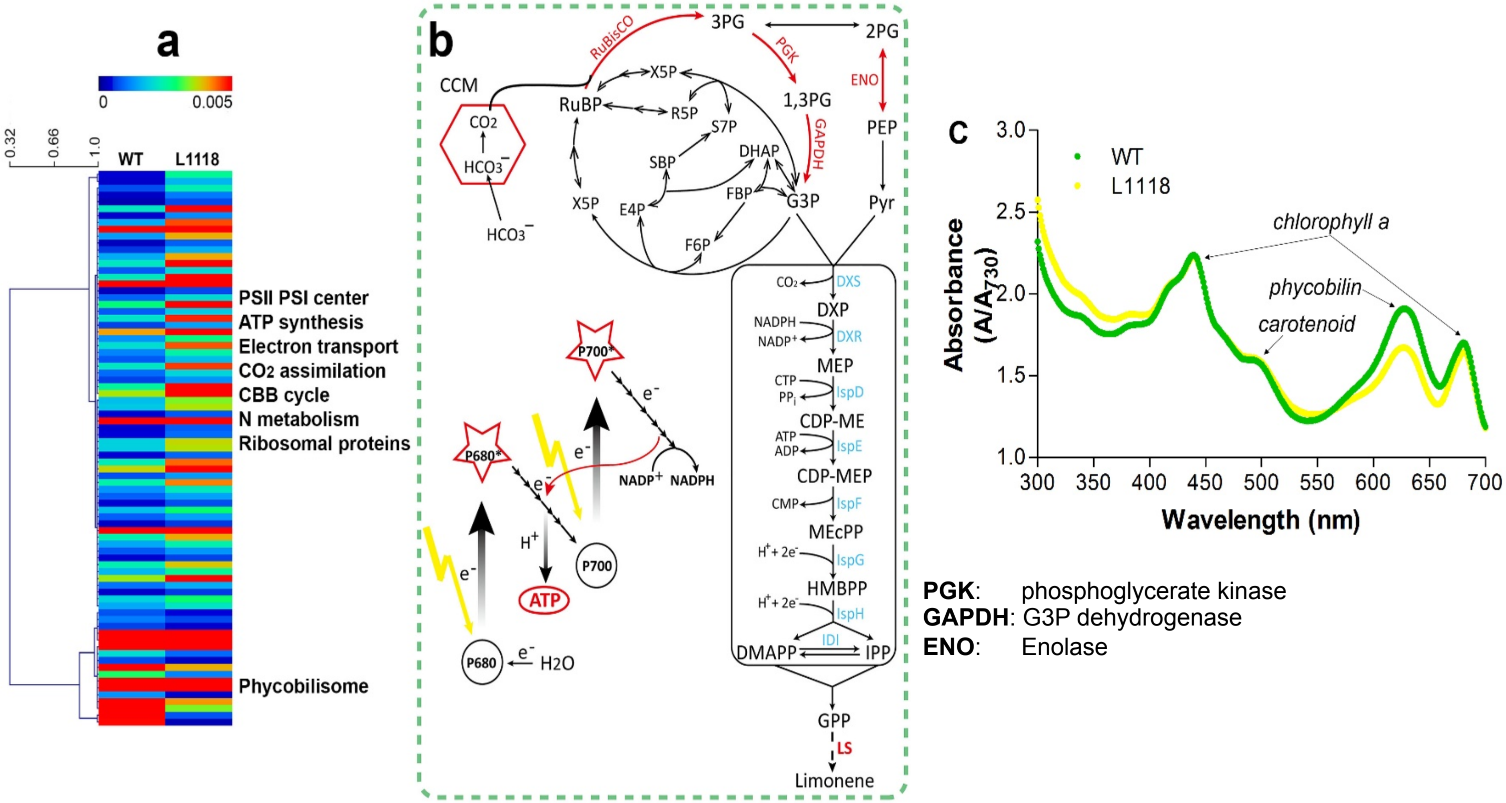
Tackling limonene synthase limitation increases productivity significantly



- Overcoming the pathway bottleneck lead to significantly enhanced limonene productivity
- 100 fold productivity increase compared to L111; >10 fold productivity increase compared to stepwise metabolic engineering
- Proteomics analysis showed >13-fold increase for LS abundance in L1118 vs. L1115
- Growth is not affected when comparing with wildtype

Wang X, et al. (2016) Proc Natl Acad Sci U S A. 113(50): 14225.

Metabolic regulation revealed by proteomics study



PGK: phosphoglycerate kinase
GAPDH: G3P dehydrogenase
ENO: Enolase

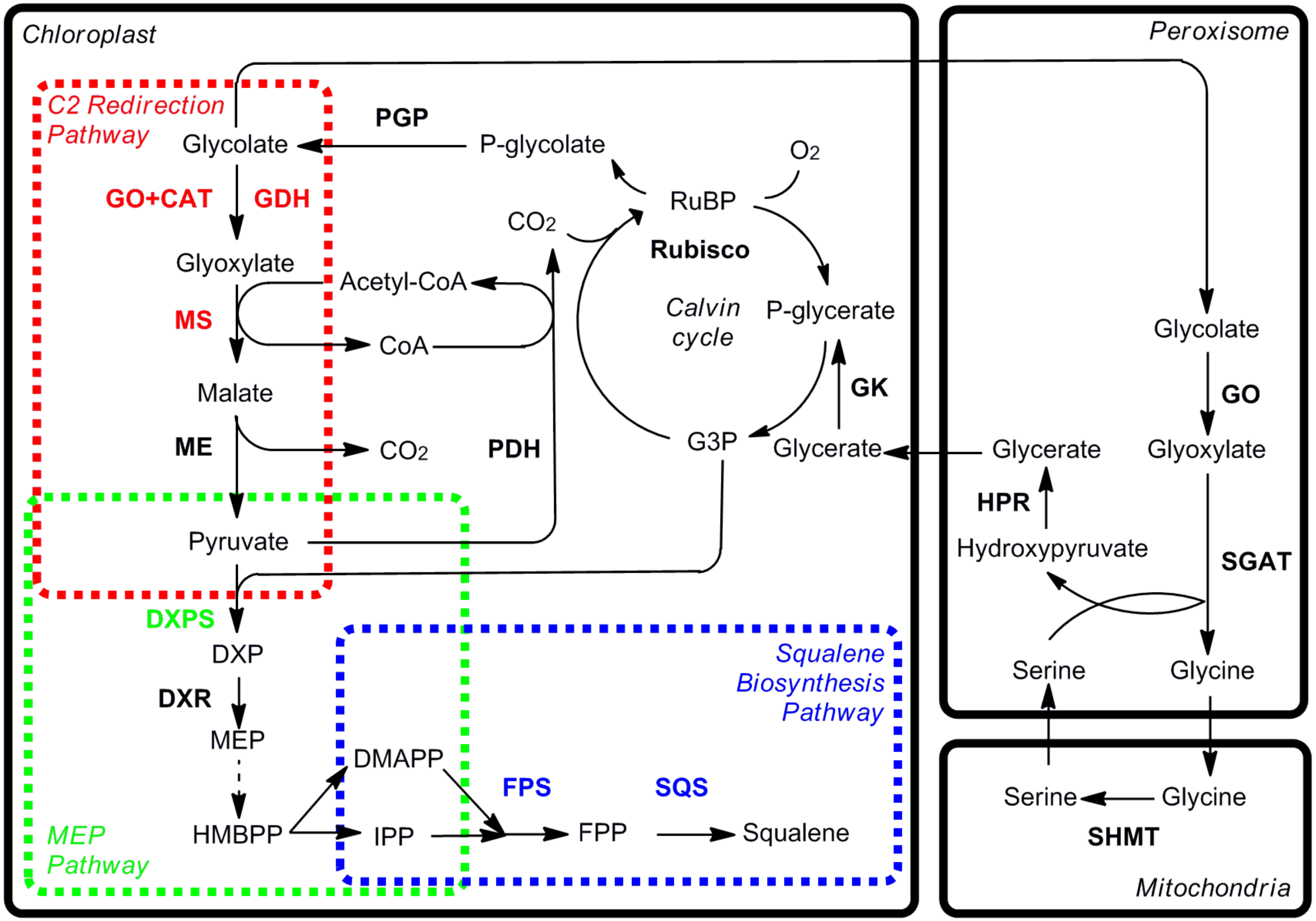
- Multidimensional Protein Identification Technology (MudPIT) proteomic analysis
- Potential synergy between limonene carbon sink and upstream photosynthesis
- Cell absorbance suggested enhanced MEP flux rather than terpene precursor redirection

Wang X, et al. (2016) Proc Natl Acad Sci U S A. 113(50): 14225.

Summary

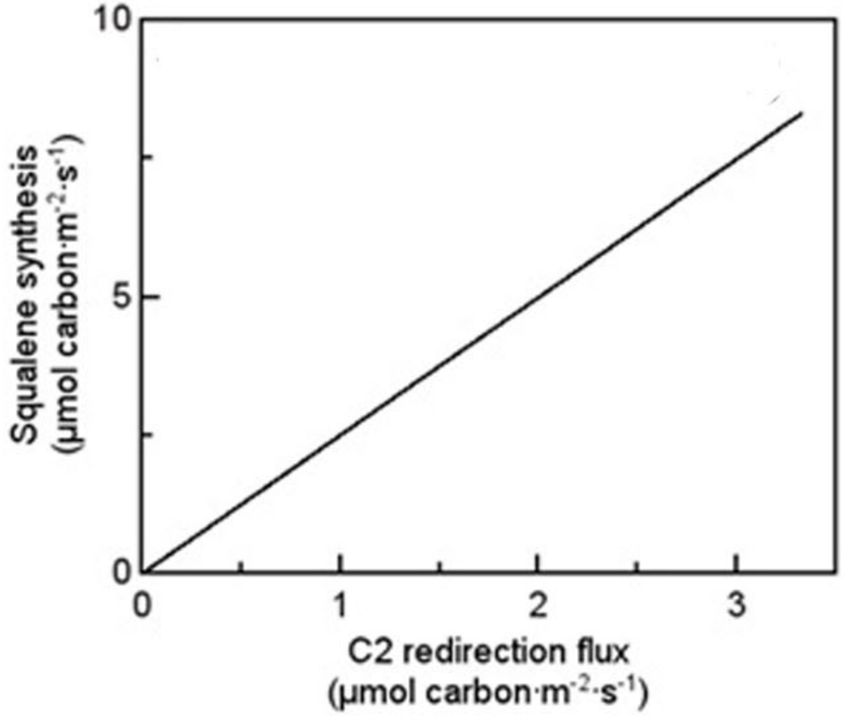
- Mathematical modeling revealed a metabolic bottleneck for photosynthetic terpene production.
- Overcoming the bottleneck with synthetic biology design has led to 100 times increase of limonene productivity and achieved a record yield.
- The high limonene productivity stimulated the photosynthesis carbon fixation, yet the additional biochemical bottlenecks limited the further increase of terpene level.

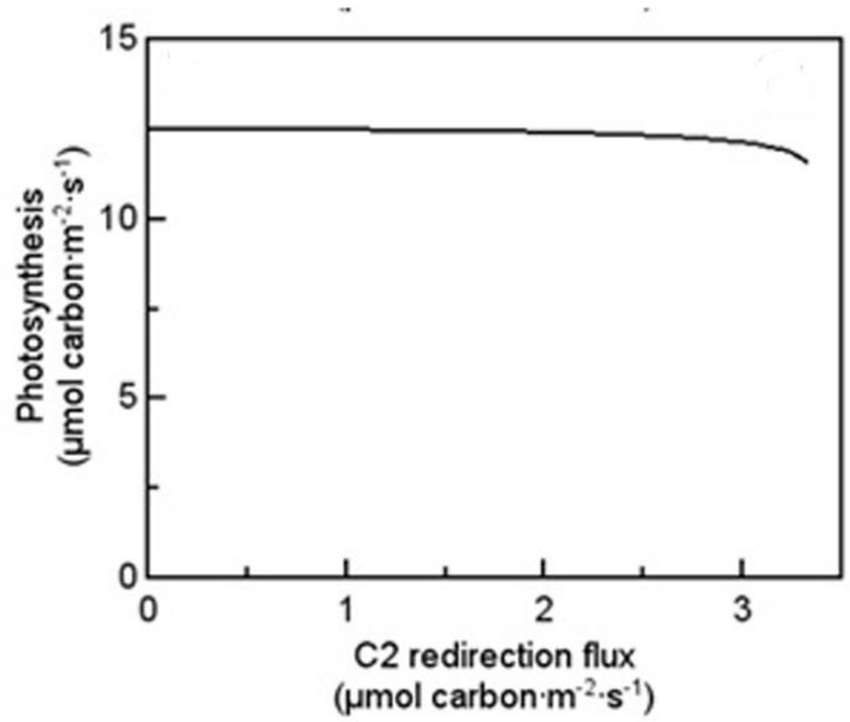
Rewiring Photorespiration Products



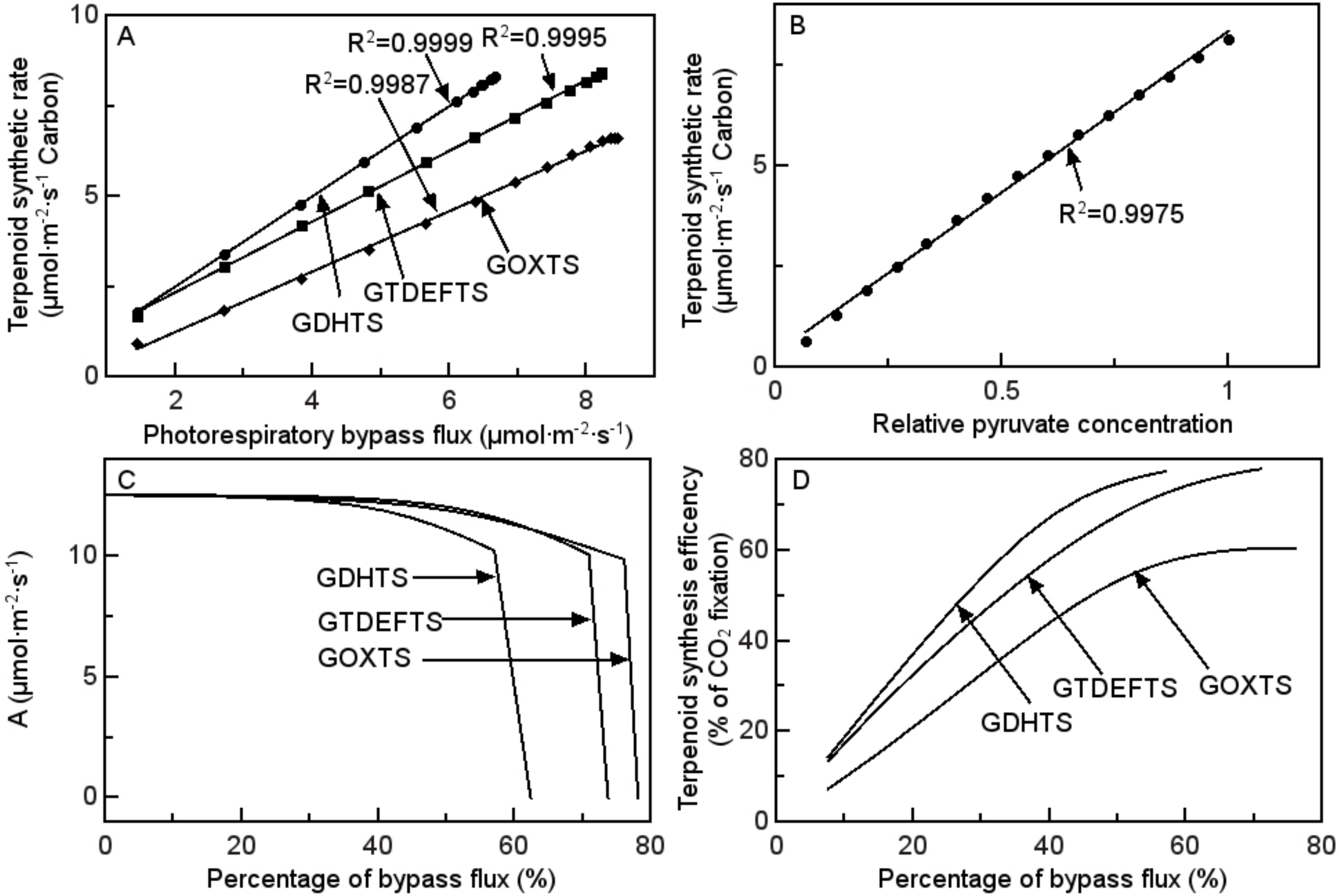
CAT, catalase; **DXPS**, 1-deoxy-D-xylulose 5-phosphate synthase; **DXR**, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; **FPS**, farnesyl diphosphate synthase; **GDH**, glycolate dehydrogenase; **GK** glycerate kinase; **GO**, glycolate oxidase; **HPR**, hydroxypyruvate; **ME**, malic enzyme; **MS**, malate synthase; **PGP**, phosphoglycolate phosphatase; **SGAT**, serine-glutamate aminotransferase; **SHMT**, serine hydroxymethyl transferase; **SQS**, squalene synthase; DMAPP, dimethylallyl diphosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; FPP, farnesyl diphosphate; IPP, isopentenyl diphosphate; MEP, 2-C-methyl-D-erythritol 4-phosphate.

Computational Modeling Indicated that C2 Redirection Could Increase Terpene Yield by Providing More Pyruvate



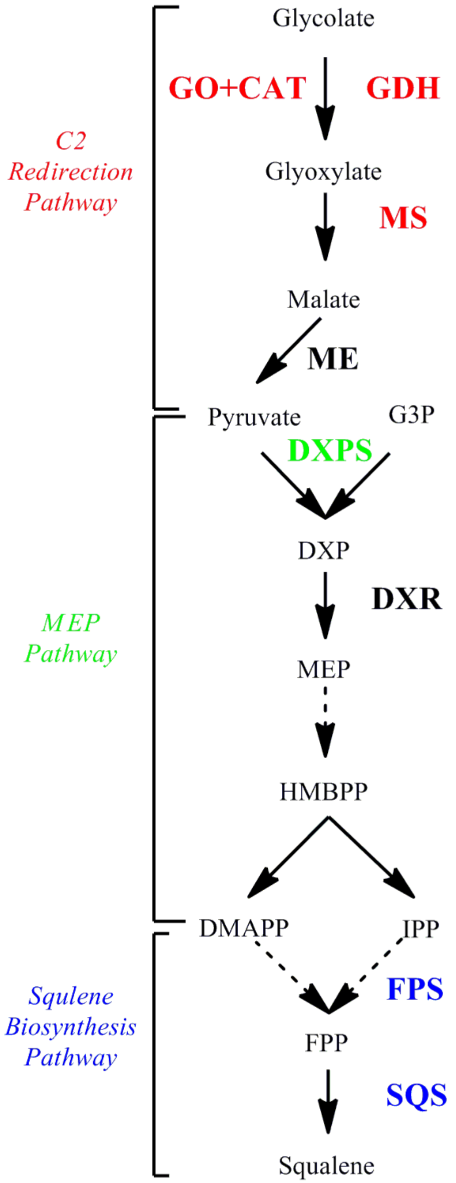


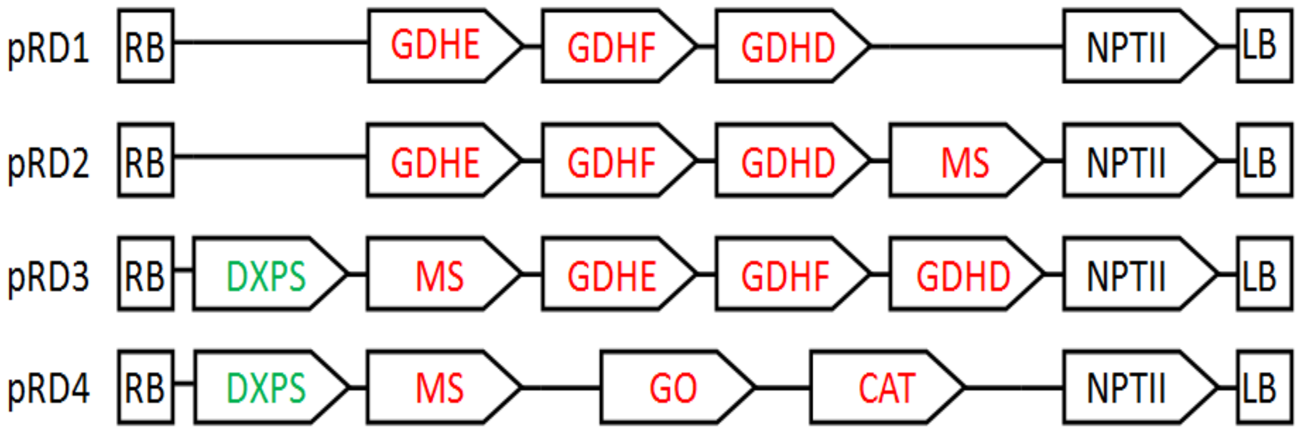
Photosynthesis rate will not be significantly impacted with our design to channel carbon to high value products



The effect of photorespiratory flux through the bypass on terpene synthesis (A), the effect of available pyruvate concentration on terpene synthesis (B), the effect of the percentage of photorespiratory flux through the bypass on photosynthesis (C), and the effect of the percentage of photorespiratory flux through the bypass on terpene synthesis efficiency (D) of different engineered terpene synthesis strategies.

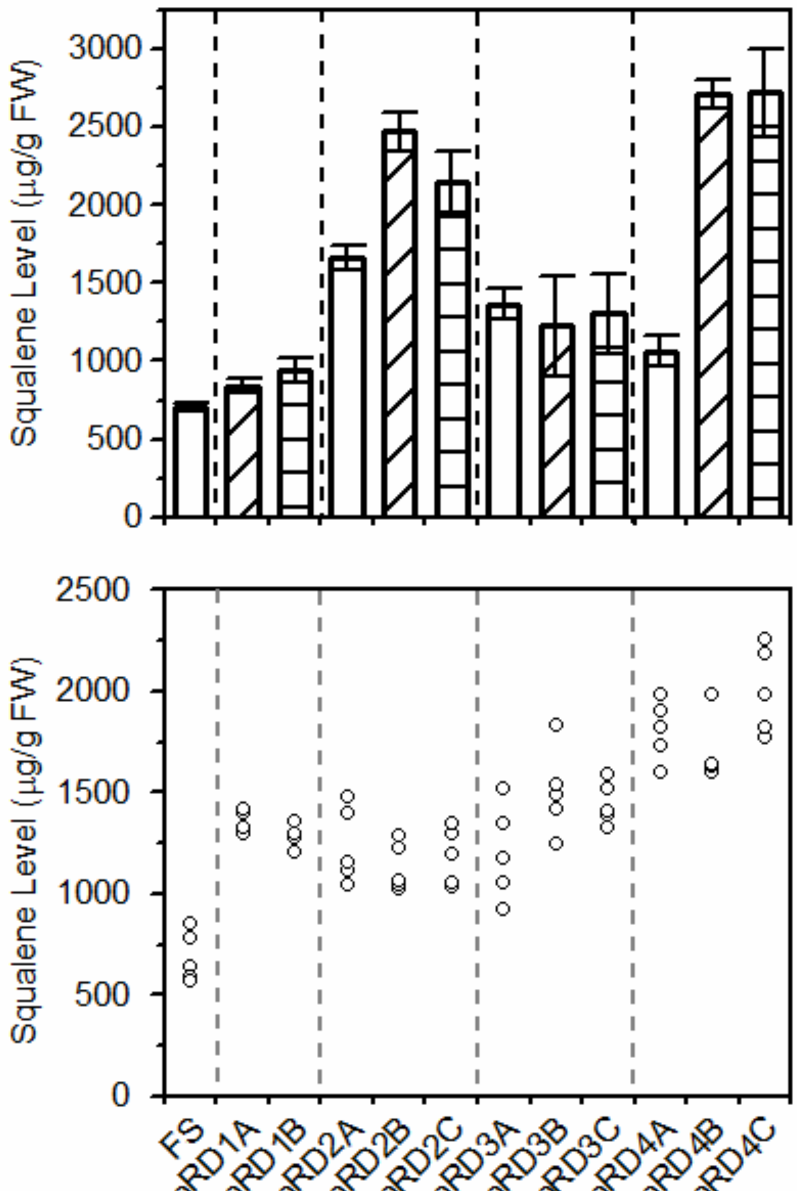
Pathway Design and Vectors





- pRD1=GDH only (partial by-pass)
- pRD2=GDH+MS (complete by-pass)
- pRD3=GDH+MS+DXP
- pRD4=GO+CAT+MS+DXP
- FS parental line= FPS+SQS

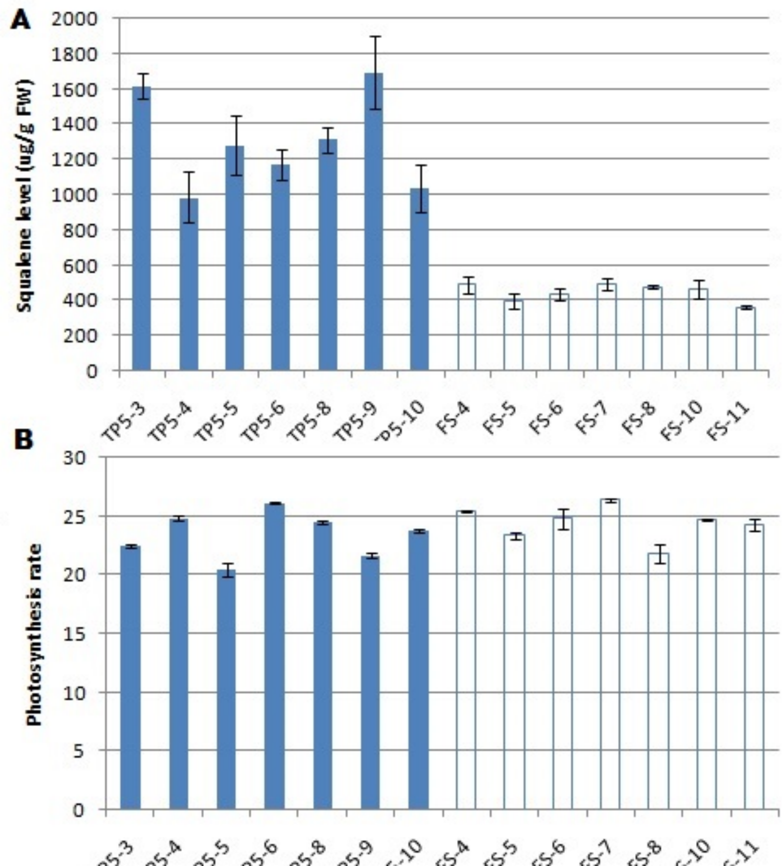
C2 Redirection Increases Squalene Production by 2 to 4 Folds



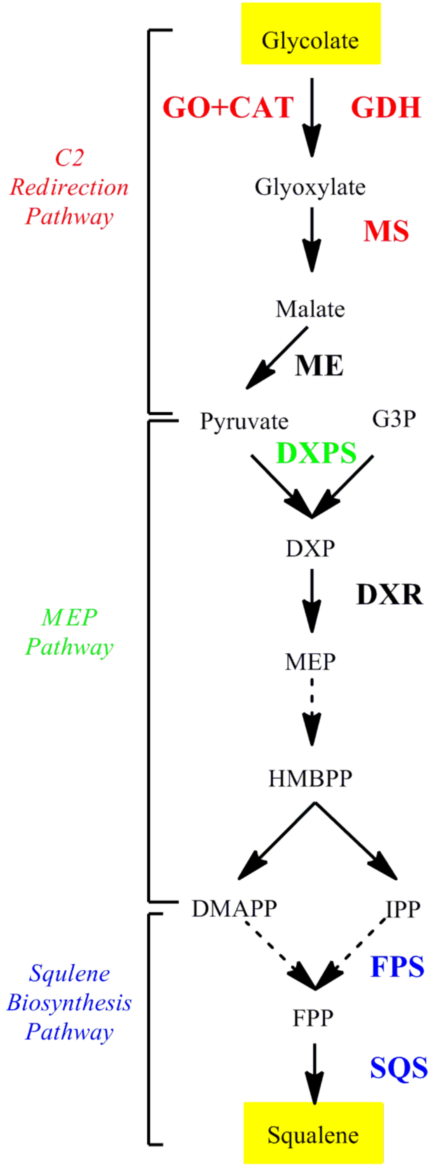
- pRD1=GDH
- pRD2=GDH+MS
- pRD3=GDH+MS+DXP
- pRD4=GO+CAT+MS+DXP
- FS parental line= FPS+SQS

Initial Field Trial Confirmed Squalene Increases

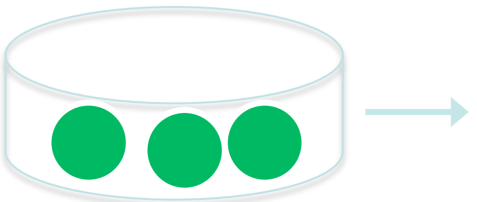
	Plant Height	Leaf No.	Leaf Length	Leaf width	Stem girth	Average internode length
1068	76.78±19.06	22.73±4.18	70.33±6.03	29.42±4.75	4.32±1.92	3.3±0.55
pRD4	50.55±9.03**	19.33±2.69*	57.5±8.08**	31.77±3.39	2.51±0.67**	2.58±0.31**
HG2	60.01±11.29**	20.13±2.67*	63.09±5.17**	33.41±3.37	3.69±0.8**	2.92±0.41*
HG4	55.32±9.35**	20.93±2.43*	58.3±8.41**	31.87±3.71	2.6±0.56**	2.59±0.34**



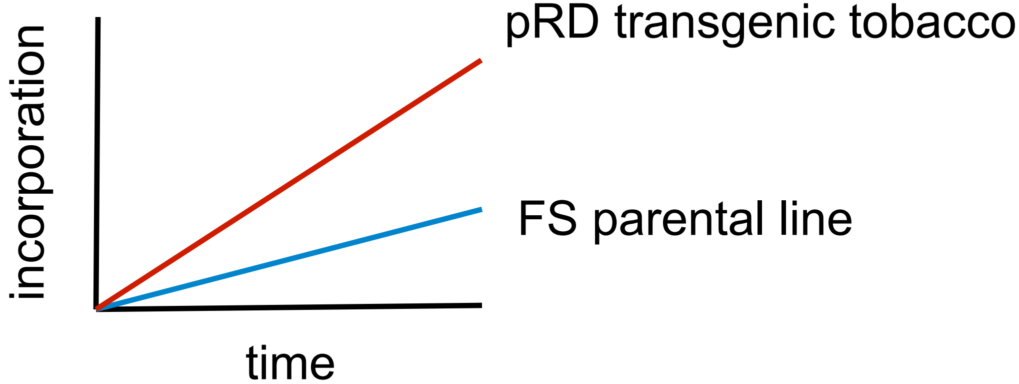
C14-Glycolate Feeding Assay to Evaluate Carbon Flux



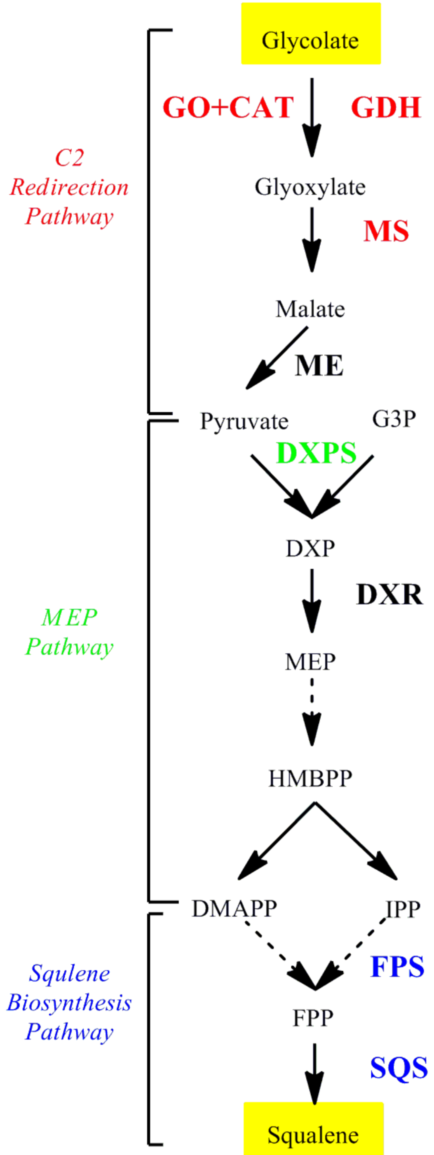
Pulse-label with radiolabeled glycolate

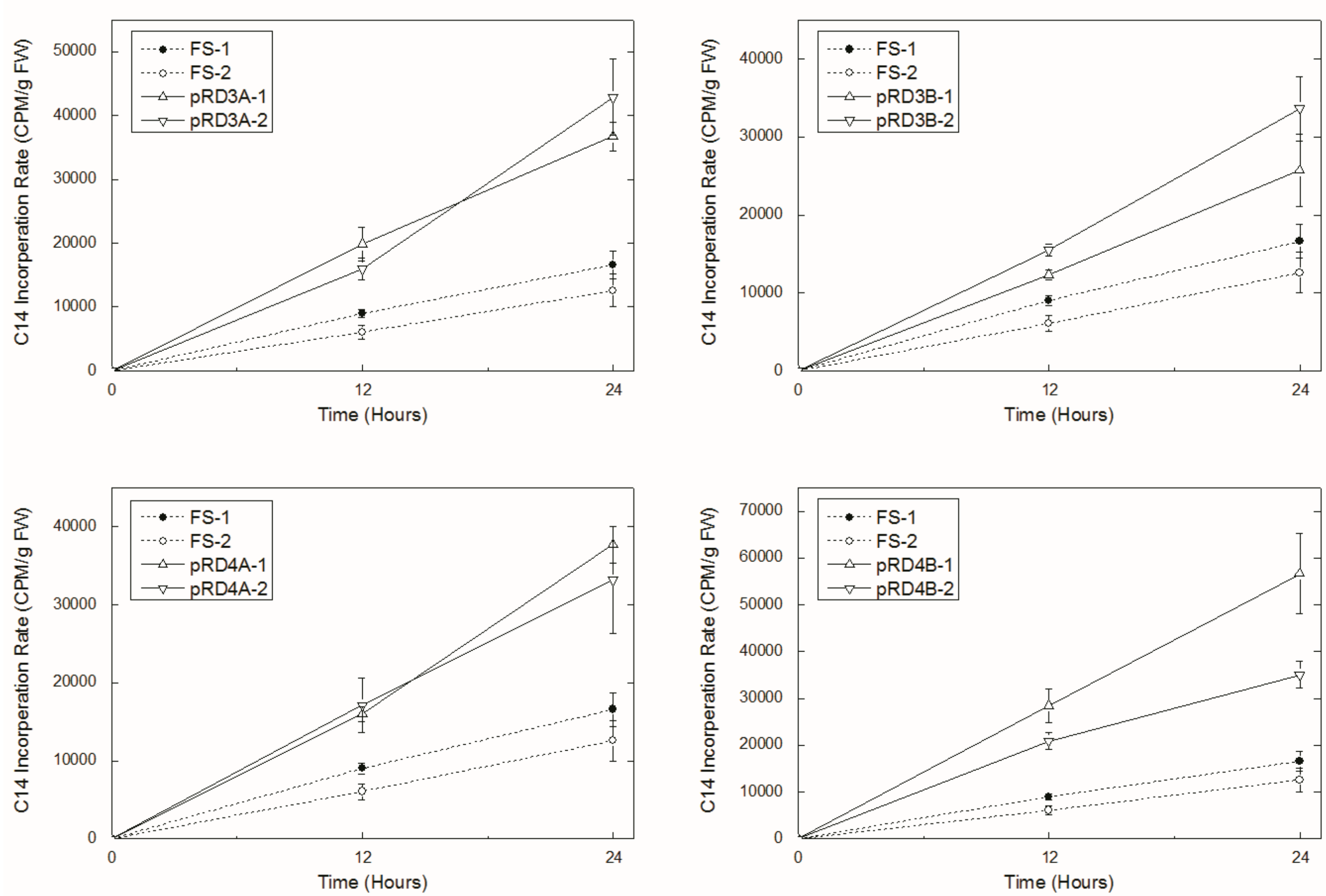


Collect samples at specific time points (0, 12, 24 hrs) and determine the amount of label incorporated into squalene

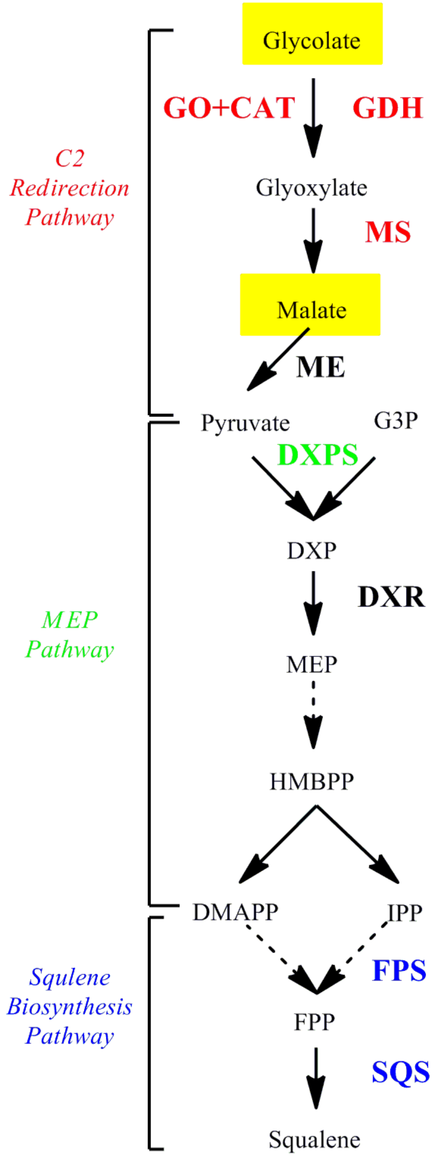


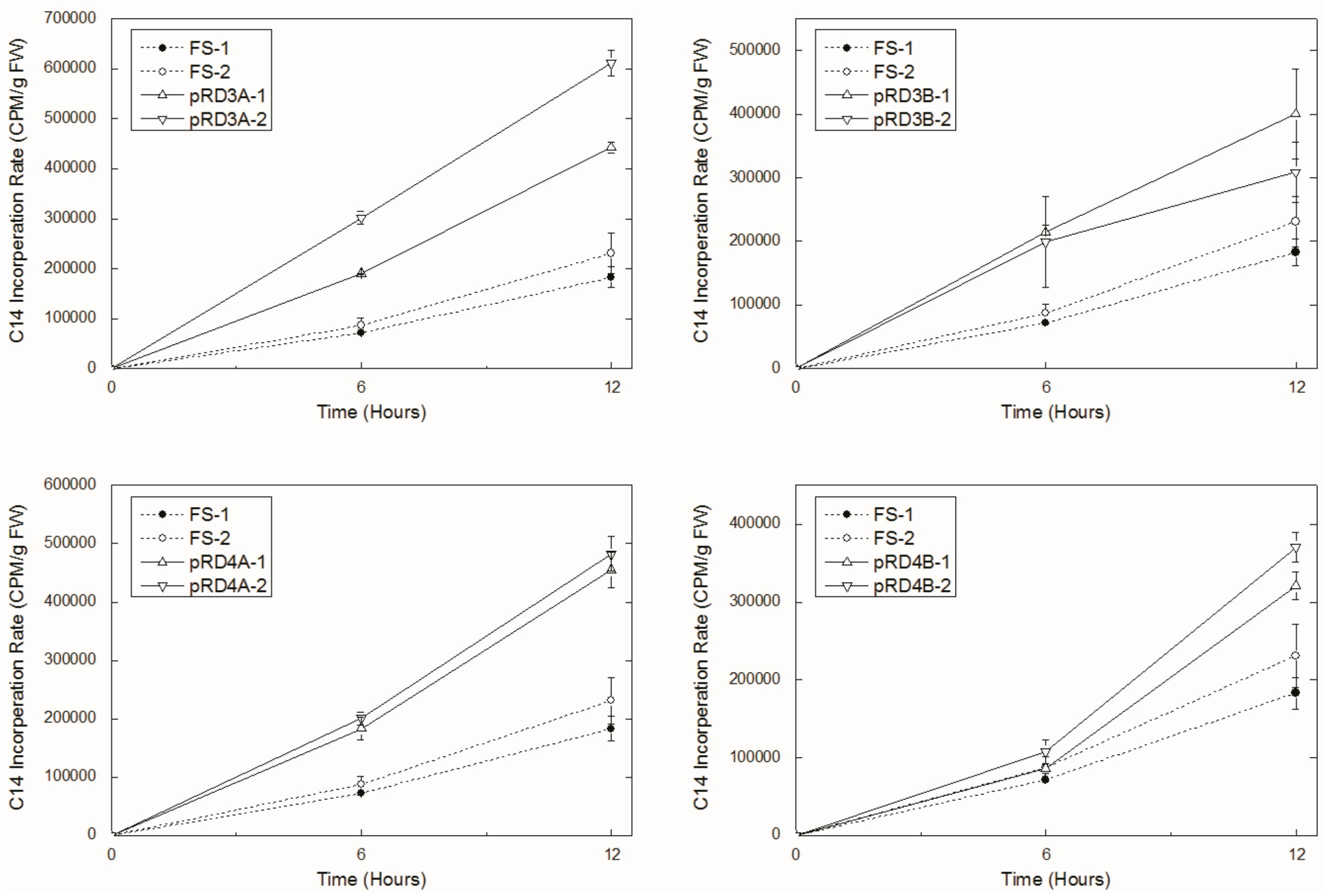
C2 Redirection Enhances Incorporation of Glycolate into Squalene



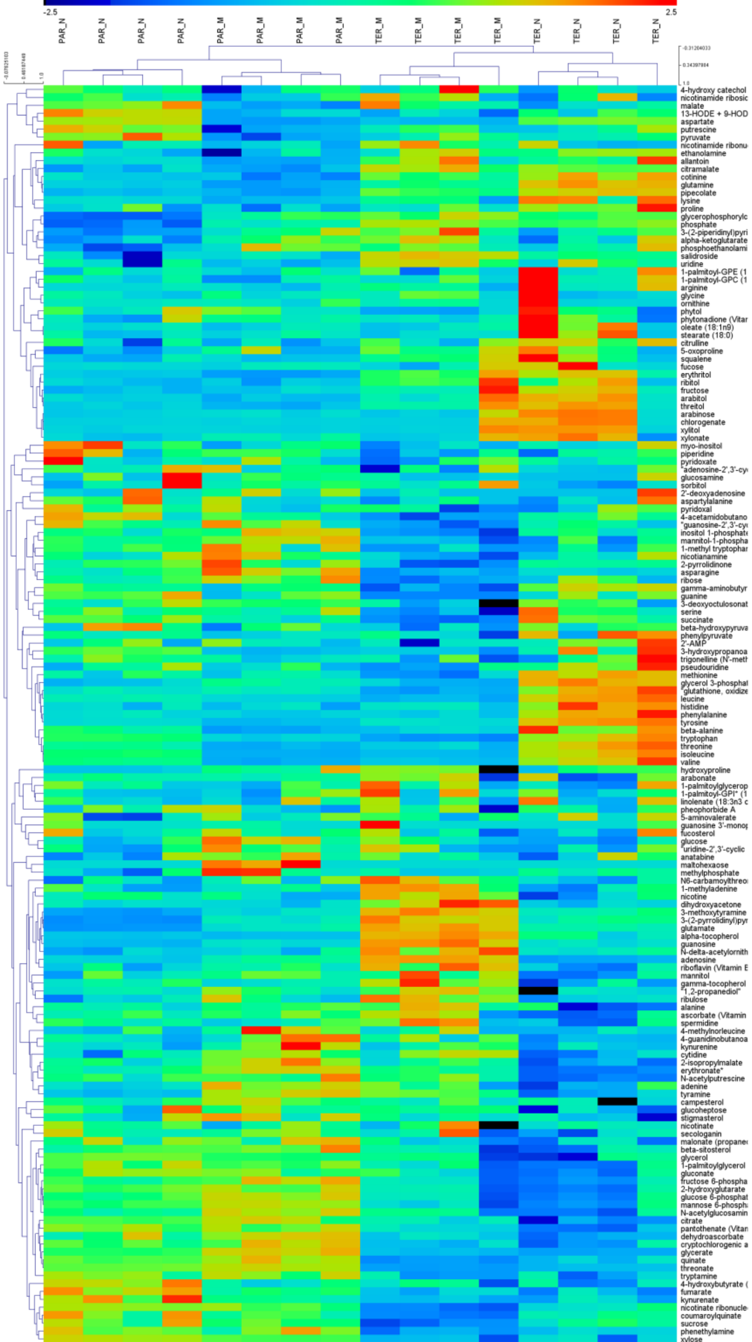


C2 Redirection Enhanced Incorporation of Glycolate into Malate





Global Metabolite Profiling to Evaluate Carbon Output

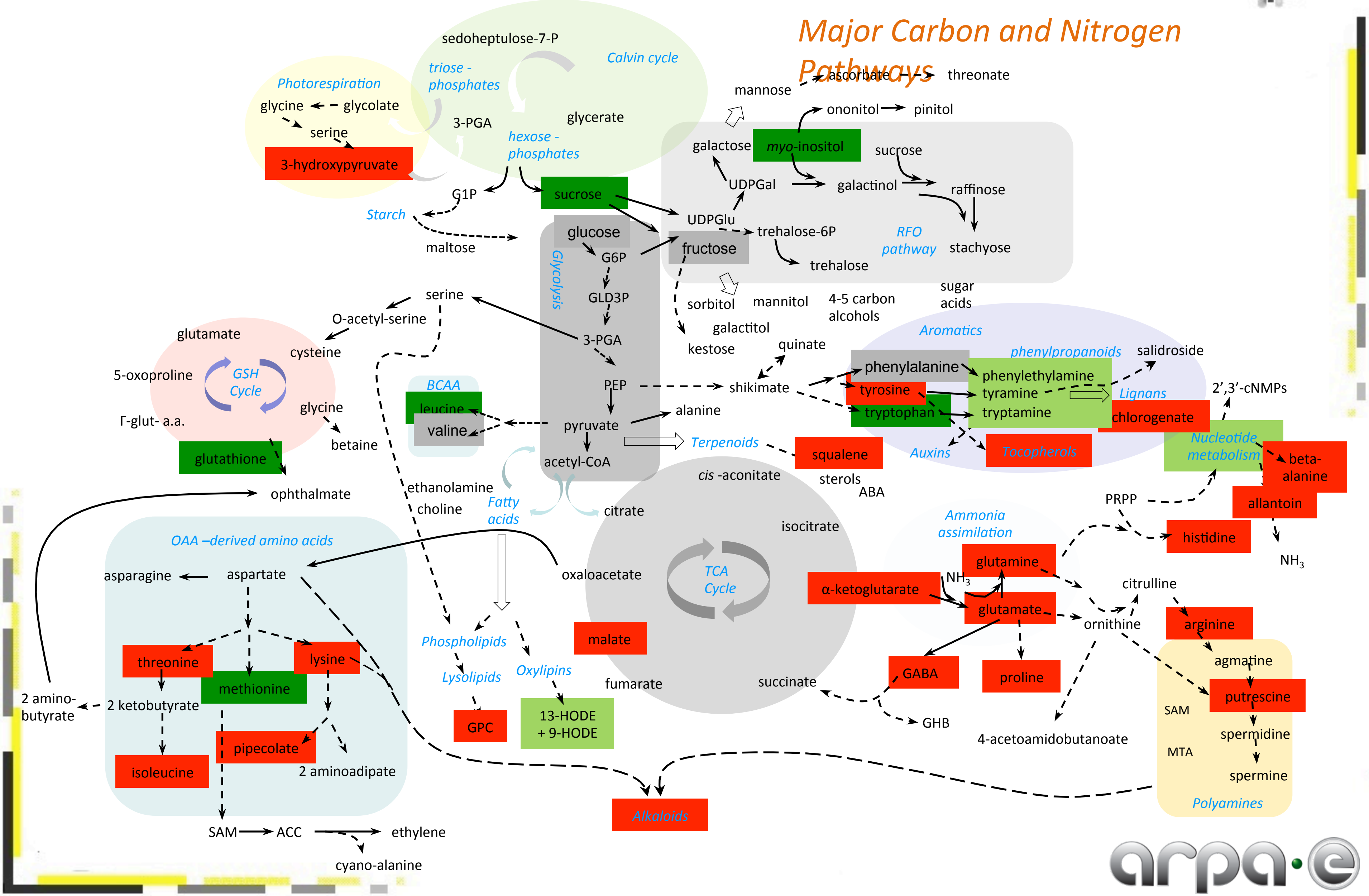


FS-N FS-M RD4-M RD4-N

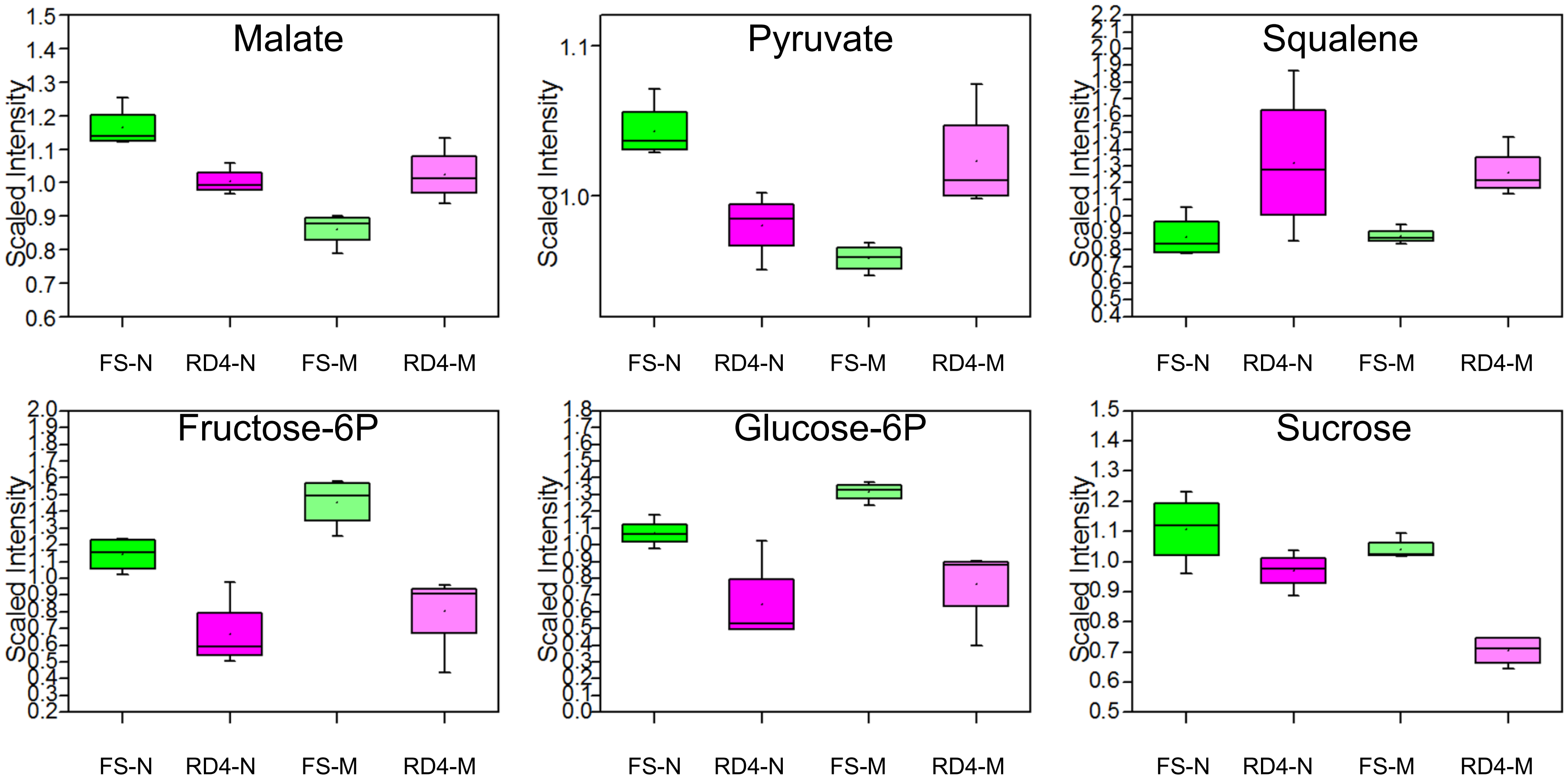
- GC-MS and UPLC-MS/MS platforms
- 162 metabolites identified
- 4 repeats of each sample clustered together

FS-N: FS plant samples collected during night time
FS-M: FS plants samples collected during daytime
RD4-N: RD4 plant samples collected during night time
RD4-M: RD4 plant samples collected during the daytime

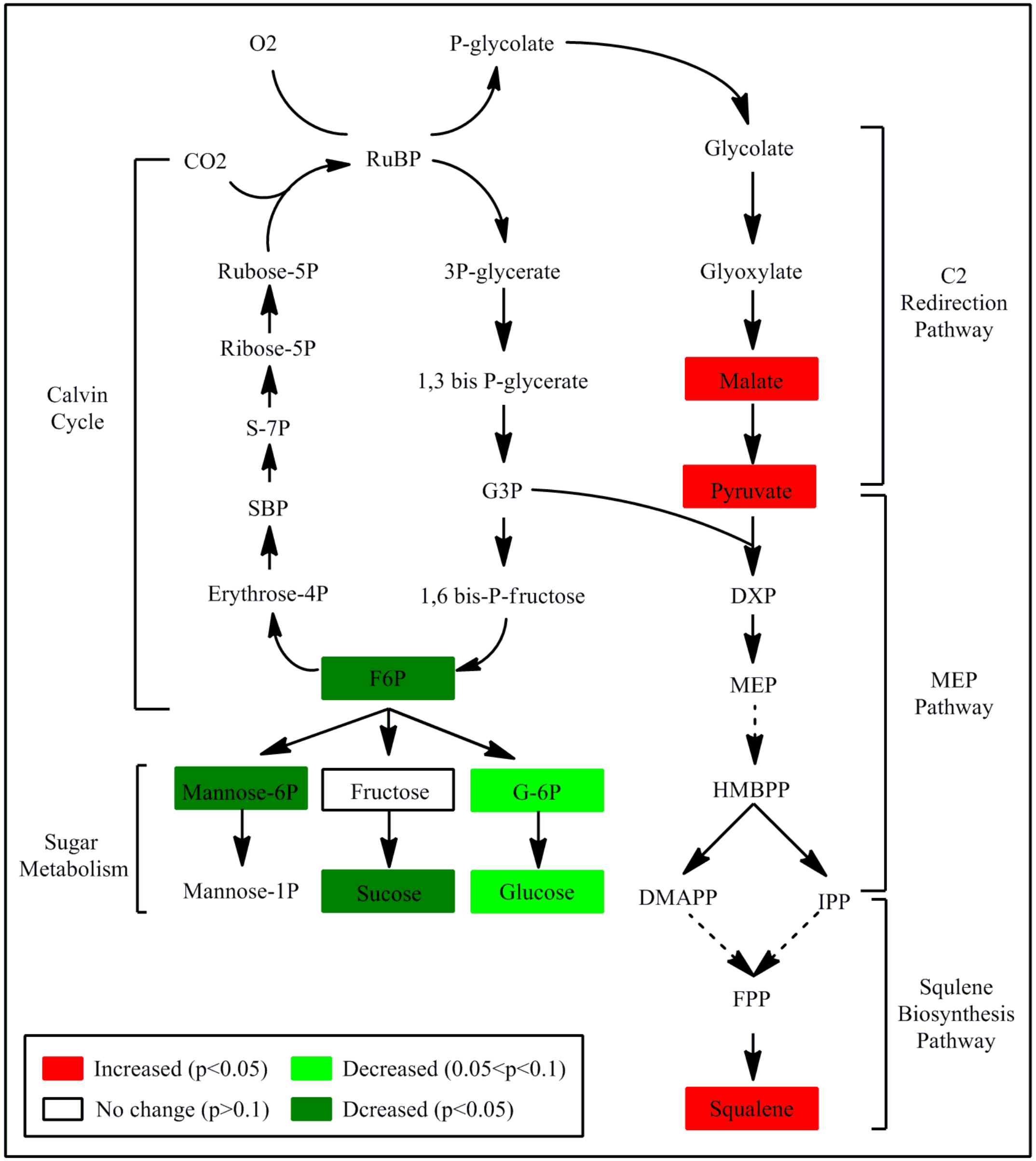
The Overview of Metabolite Changes in C2 Redirection Plants



Relative Abundance of Key Metabolites



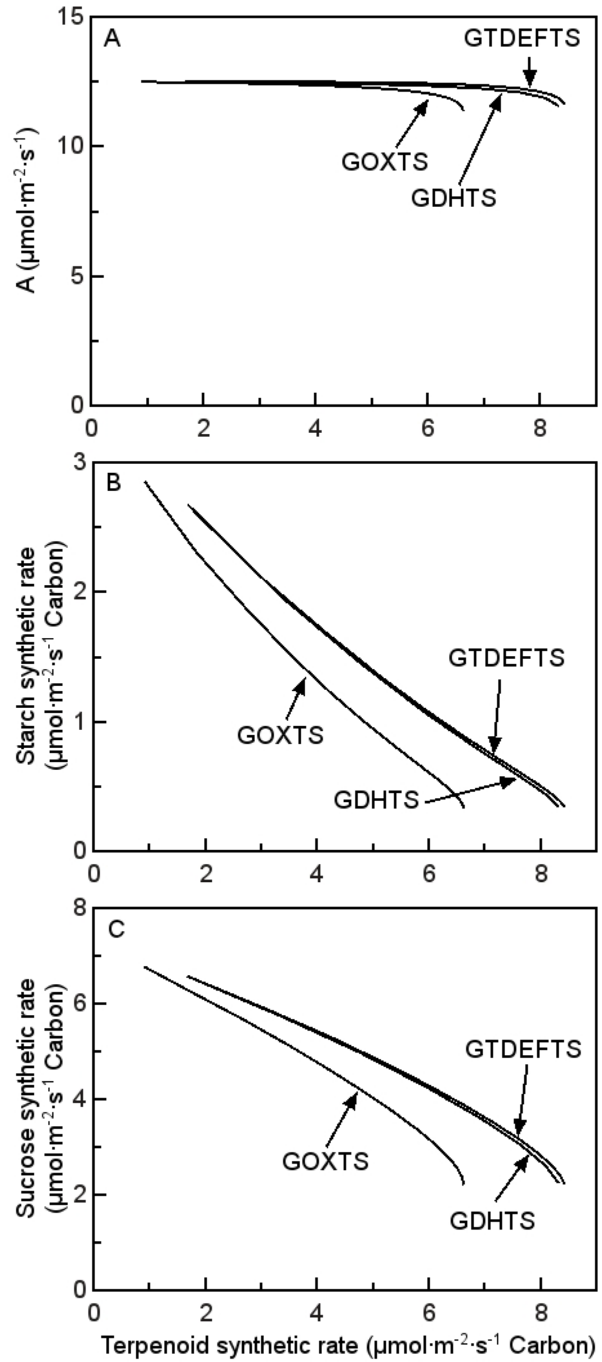
C2 Redirection Leads to a Significant Carbon Repartition



The mathematical model predicts an unchanged photosynthesis, but significantly decreased sucrose and starch output.

The modeling data fits very well into experimental data in three aspects:

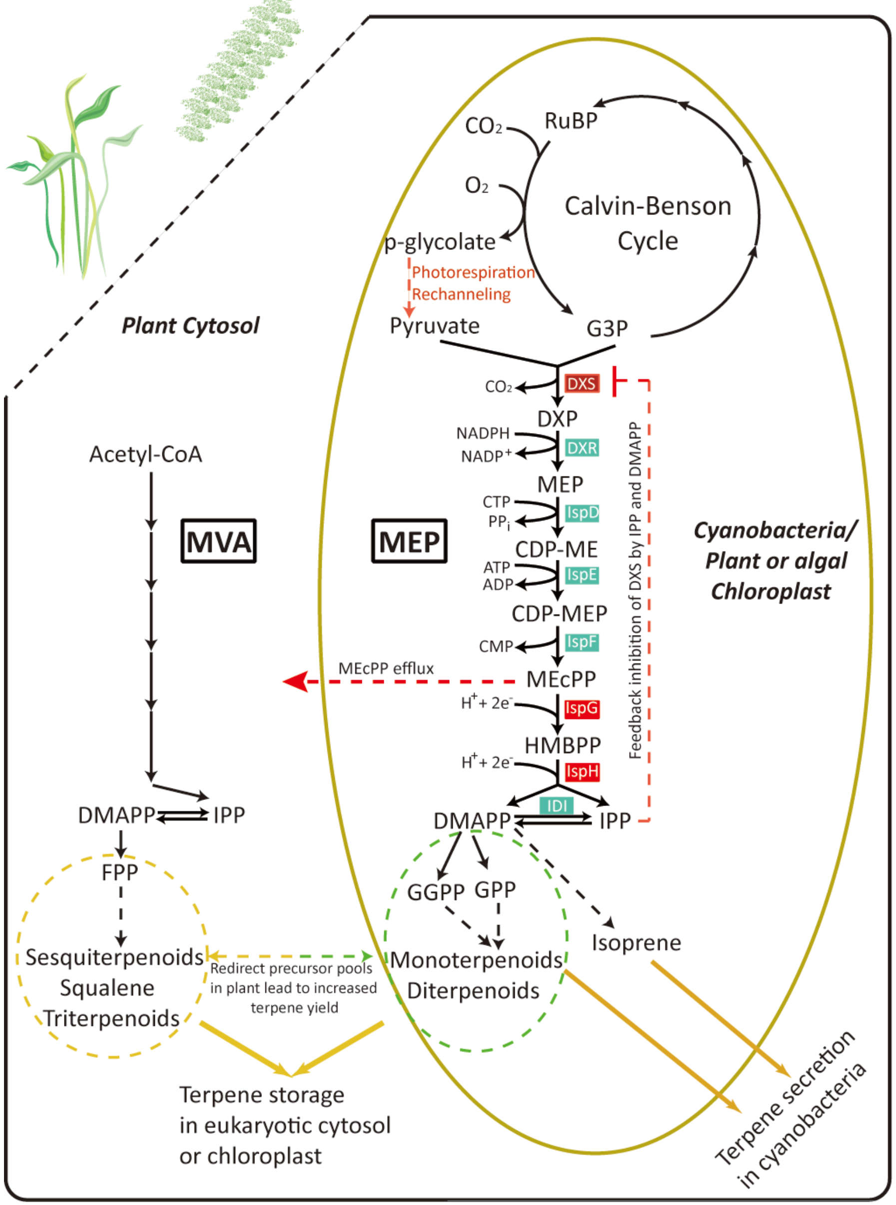
- Increased Terpene
- Decreased Sucrose and Starch Biosynthesis
- No Significant Changes in Photosynthesis



Summary

- C2 redirection was designed to enhance terpene yield and to achieve 2.7 mg/g FW of squalene, which is about 2-4 fold increases from terpene engineering only.
- A C14 labelling assay verified that a functional C2 redirection pathway increases glycolate flux malate and squalene.
- Metabolomics data also indicated a significant carbon repartition from sugar metabolism to terpene biosynthesis resulted from the C2 redirection.
- The combination of C2 redirection with photosynthesis acceleration could further increase terpene yield.
- This research established a novel approach to produce high level of terpenes toward fuels, chemicals and pharmaceuticals in plants. More importantly, it indicated that synergy between photosynthesis and downstream engineering is crucial for photosynthetic production of terpene and other products.
- Other strategies including C5 redirection, C3/C6 redistribution, synthetic droplet to enhance terpene storage are on-going.

Scientific Challenges



- **Scientifically, perfect unconventional sink!**
- **Low carbon partition**
- **Limited by carbon fixation in photosynthesis**
- **Limited by extensive feedback and downstream consumption of the compounds.**

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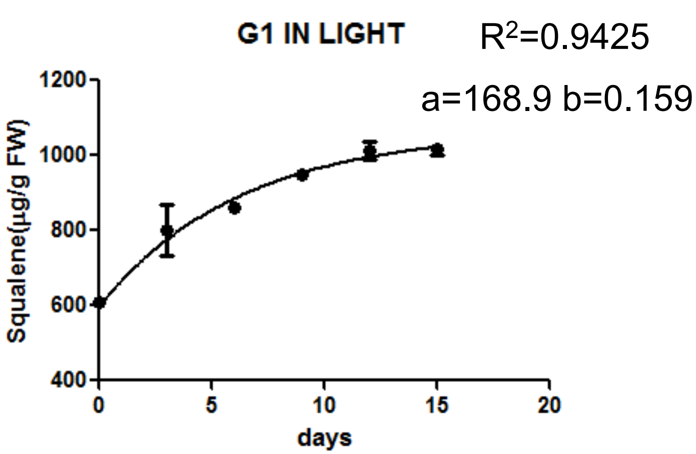
Mechanisms for Increased Squalene Accumulation

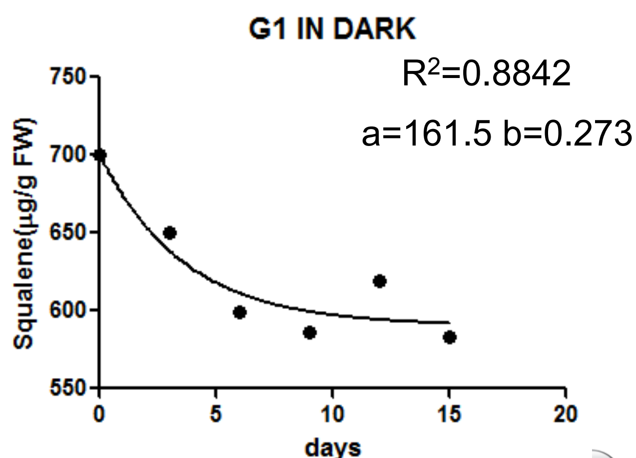
The model includes three parameters: 1.Squalene synthesis rate (a) is not changed during 15 days; 2. Squalene loss rate (*b*) is relevant to its concentration (*v*) with a constent (k): *b*= kv;3. all squalene leaking to cytosol is degraded. As squalene yield =total synthesis(∫at)-total loss(∫bt). We can come to a equation that: $V=at-\int_0^t kv\,dt$

(t)means time. And the equation can also be presented as:

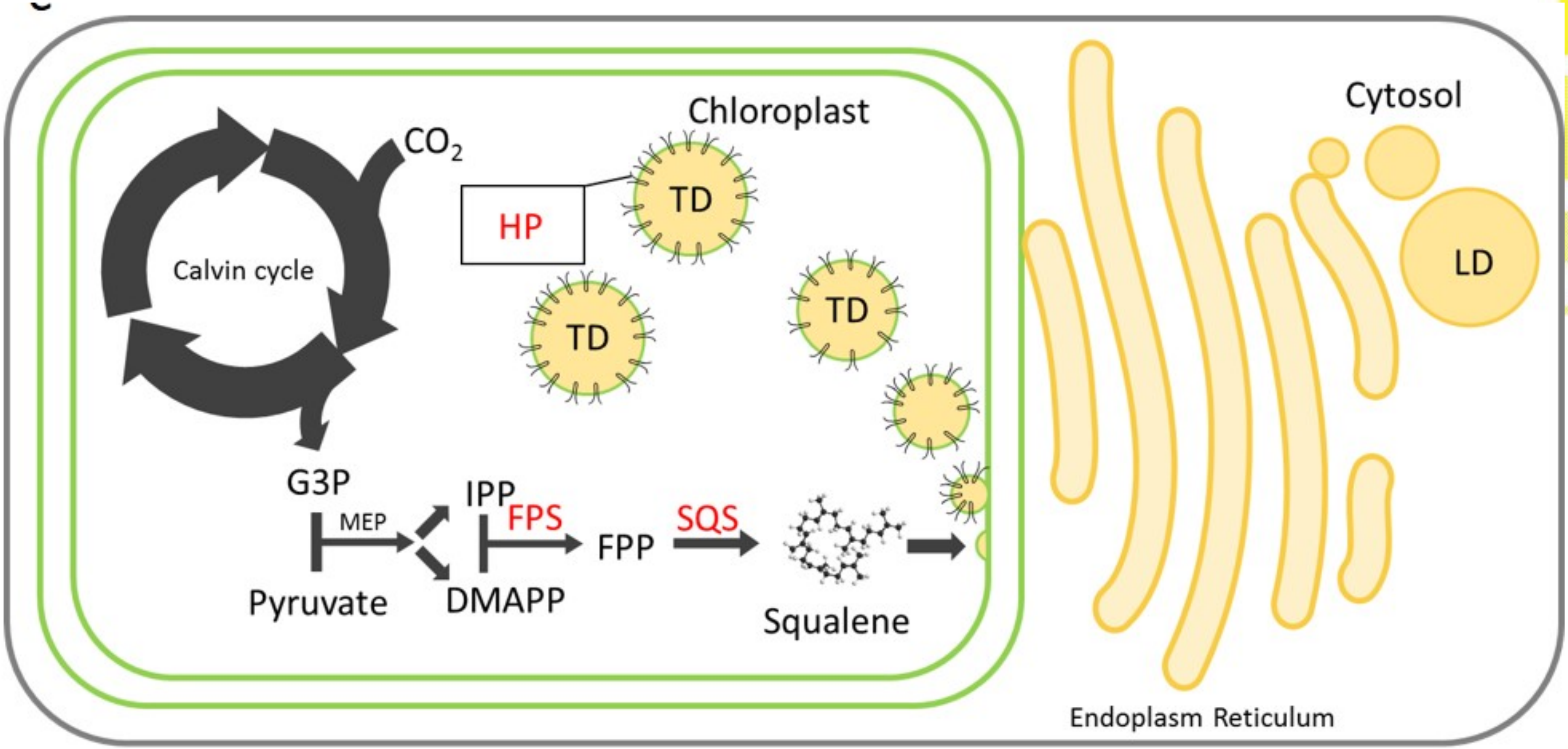
$$V=a/k-(a/k-V_0)e^{-kt}$$

(V_0) means squalene concentration at 0 day. We validated the model in two condition, light and dark.

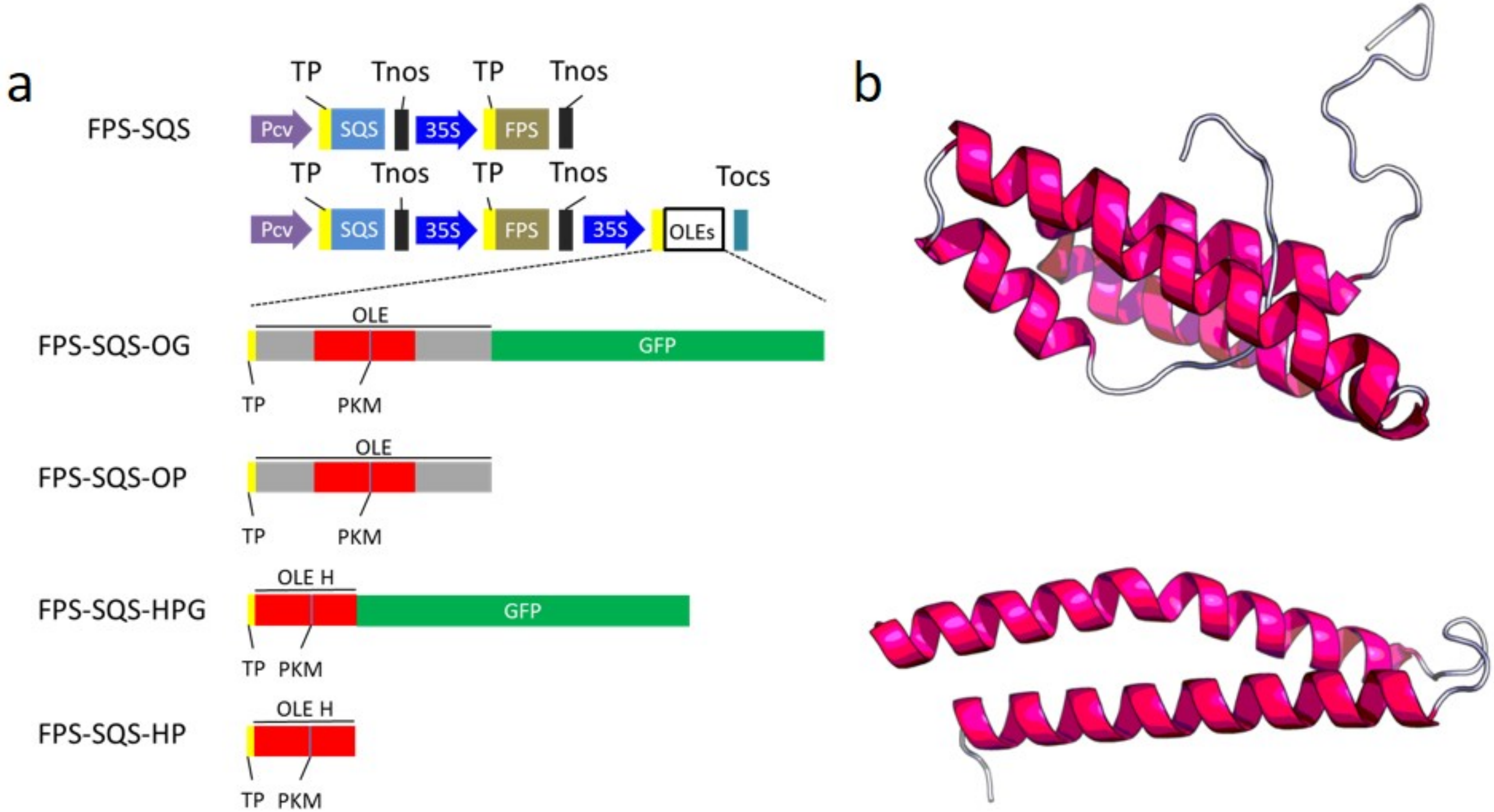




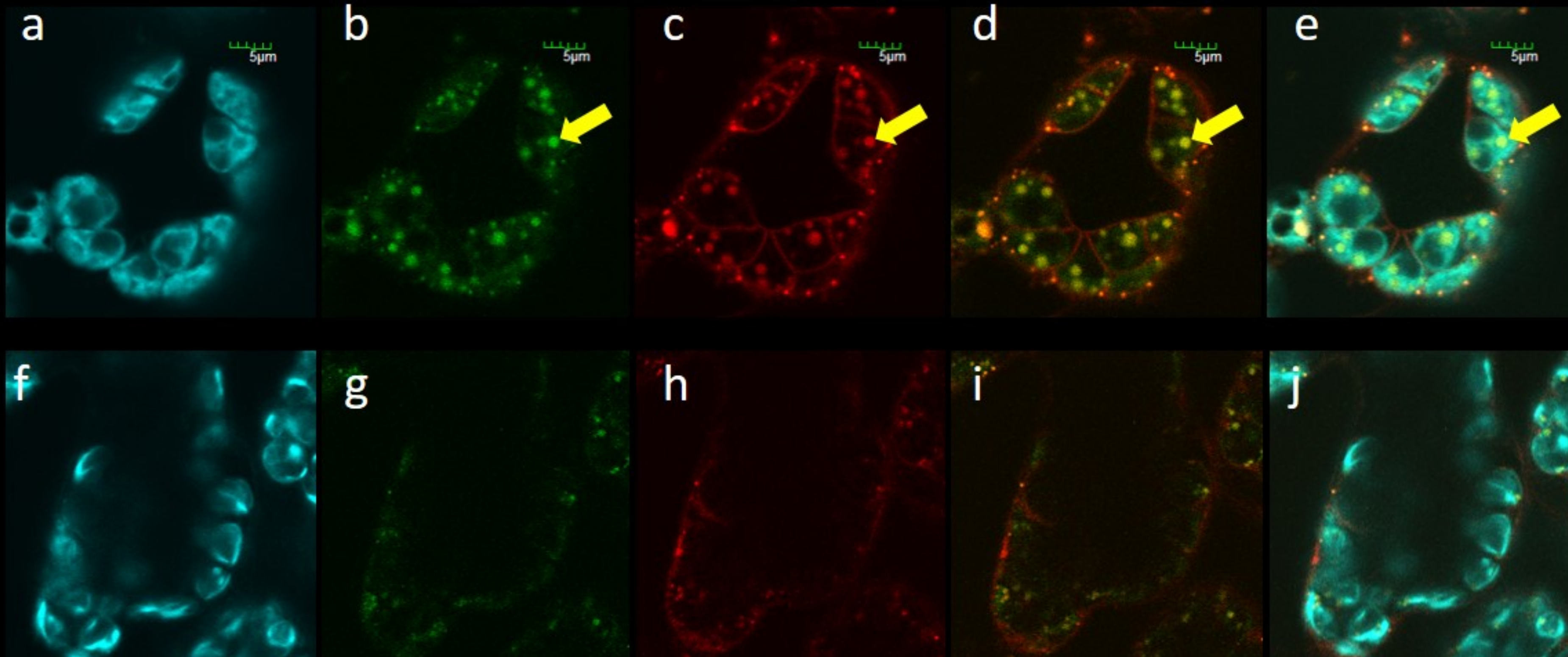
Design of a Synthetic Droplet



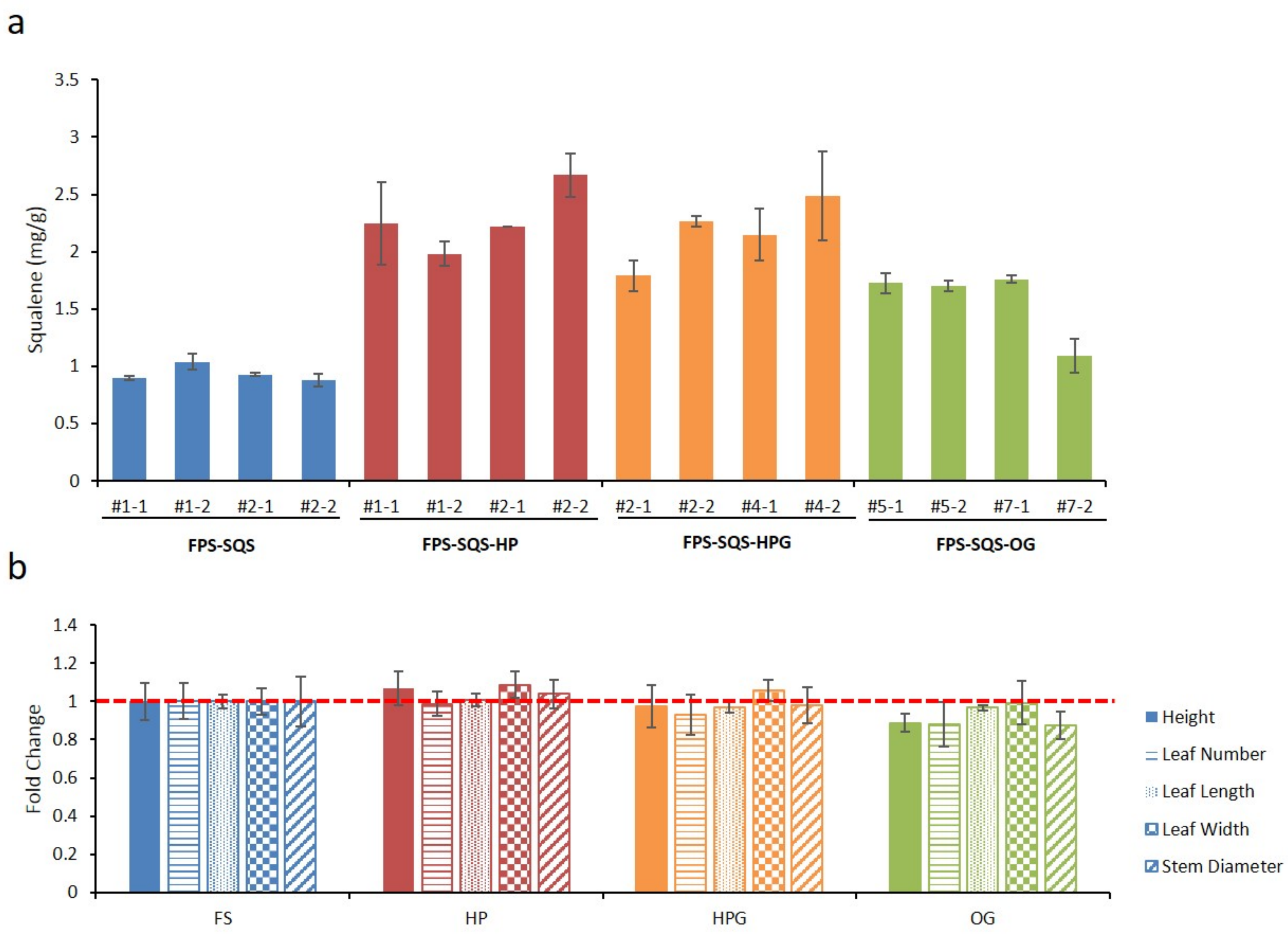
Design of a Synthetic Droplet

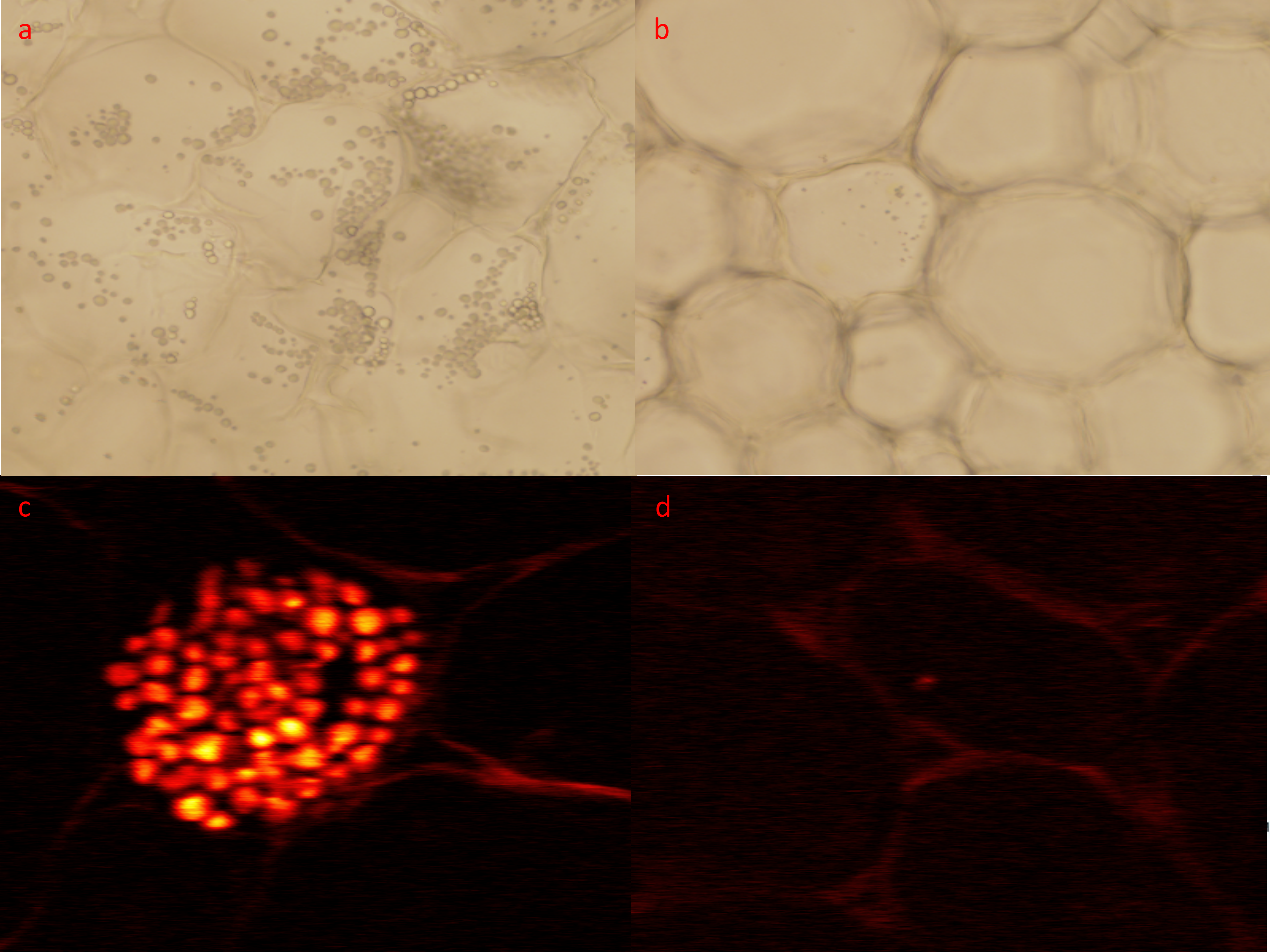


The Designed Droplet Locates in Chloroplast – The Same Location as Engineered Biosynthesis



Enhanced Squalene Production by Co-compartmentation of Synthesis and Storage




a
b
c
d

Summary

- The synthetic storage organelle can be formed in target subcellular location by proper design of hydrophobic protein.
- The co-compartmentation of storage with synthesis can lead to squalene accumulation at 2.6mg/G FW.
- Synthetic organelle contains a high concentration of squalene, representing a unique way to enhance target bioproduct production.
- The modeling experiments indicated that the prevention of membrane permeability may be the mechanisms for enhanced productivity.

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