NREL
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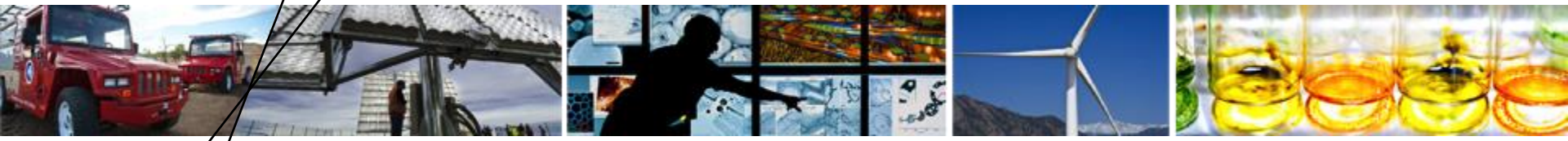
Biological Pyrolysis Oil Upgrading
WBS 2.3.2.301



2015 DOE BioEnergy Technologies Office (BETO) Project Peer Review

Date: March 24th, 2015

Technology Area Review: Thermochemical Conversion

Principal Investigator: Gregg T. Beckham

Organization: National Renewable Energy Laboratory

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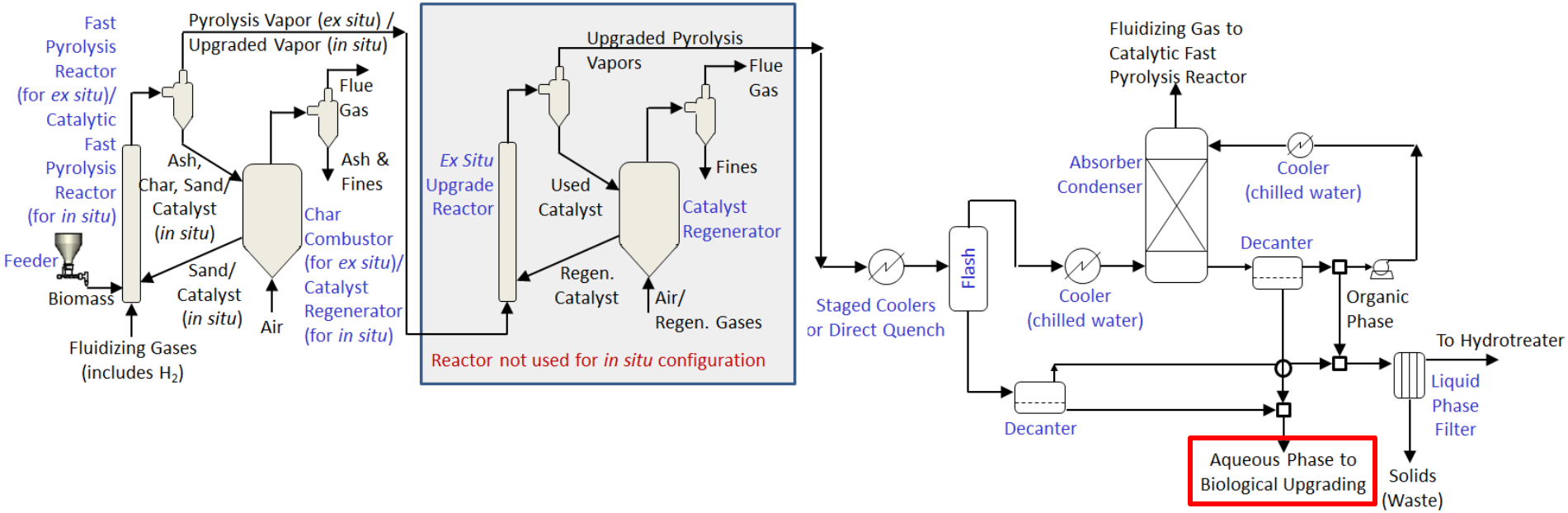
Goal Statement

Goal: develop approaches for waste valorization in pyrolysis processes

- Contribute to 2022 cost targets through valorization of waste streams to fuels or chemicals
- Focus on products with sufficient market size and growth potential to aid bioenergy industry



Waste valorization will be a major benefit to the US TC-based biorefinery infrastructure

- Conduct TEA/LCA to identify cost drivers and data gaps, and to refine process options
- Collaborate with industry and academic groups for development of tangible upgrading processes
- **Outcome:** demonstrated integrated approaches for converting TC waste streams to valuable compounds

Quad Chart

Timeline

- Start date: October 2014
- End date: September 2017
- Percent complete: 30%

Barriers

- **Tt-N Aqueous Phase Utilization and Wastewater Treatment**
- Tt-R Process Integration
- Tt-J Catalytic Upgrading of Bio-Oil Intermediates to Fuels and Chemicals

Budget

	FY14 Costs	Total Planned Funding (FY15-Project End Date)
DOE funded	$318,837	$934,163*

* This does not currently include a funding request for FY16 and FY17, but the project is slated to continue in both FY16 and FY17 with flat funding

Partners and Collaborators

- **Industry partners:** RTI International
- **NREL BETO Projects**: Thermochemical Platform Analysis – NREL, Catalytic Pyrolysis Science – NREL, Lignin Utilization, other NREL BETO-funded TC projects that produce aqueous waste streams
- **BETO-funded National Lab Projects:** Oak Ridge National Laboratory (A. Guss), PNNL (in discussions)
- **Office of Science funded efforts:** Environmental Molecular Sciences Laboratory, Pacific Northwest National Laboratory (through a competitively awarded proposal)
- **Academic collaborators:** Iowa State University, University of Georgia, University of Portsmouth, University of Tennessee Knoxville

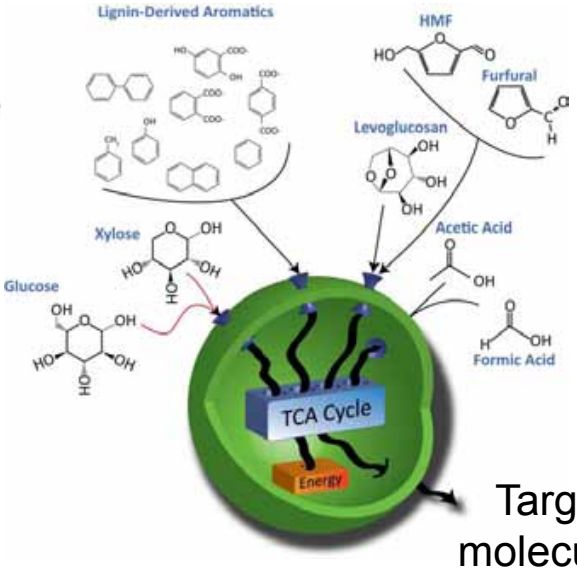
Project Overview

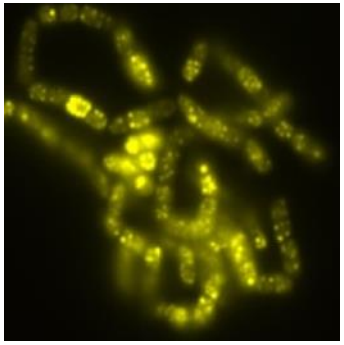
History: Valorization of waste streams from TC processes identified as a key MYPP technical barrier that currently places a large cost burden on wastewater treatment

- Project started as a BETO seed project in FY14, met major Go/No-Go decision in Sept 2014
- Leverage significant work in BC Platform Lignin Utilization project

Context: Nearly all TC processes produce aqueous waste streams at various points

- Recapture and valorize lost carbon
- Reduce burden on wastewater treatment
- Enable a value-added co-product stream
- Approach adaptable to most TC processes



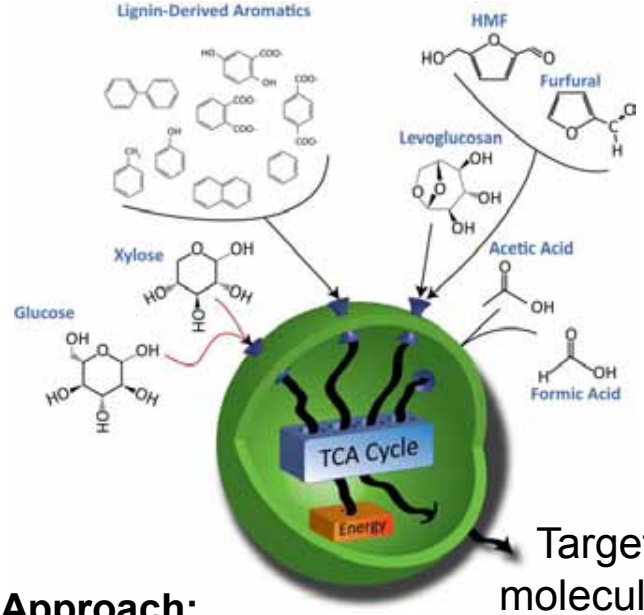


Project Objectives:

- *Develop biological strategies for valorization of TC waste streams*
- Conduct process development with “upstream” thermochemical conversion projects
- Employ TEA/LCA to define process targets and choose co-products of interest for fuels or chemical applications

Technical Approach

Aim 1: Develop biological catalysts that are able to metabolize a wide range of substrates

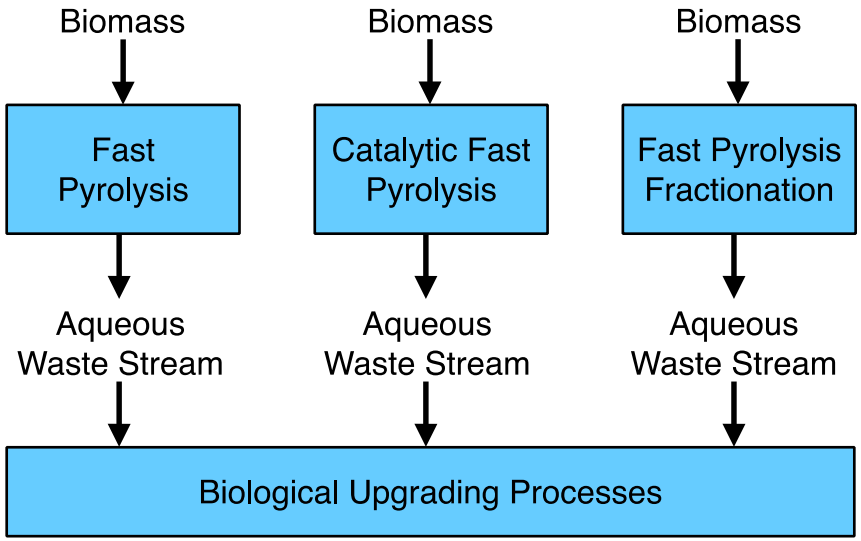


Approach:

- Engineer microbes to catabolize broad substrate ranges
- Evolve strains for higher tolerance

Challenges: Substrate specificity, yields, toxicity

Aim 2: Obtain and characterize streams from TC processes and tailor organisms to these streams



Approach:

- Characterize TC waste streams
- Tailor organism to process-relevant TC streams
- Conduct TEA to understand cost drivers

Challenges: Sufficient/consistent substrate

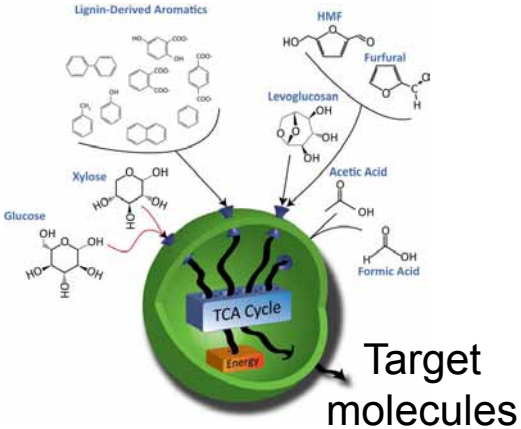
Critical Success Factors:

- Develop organism and process to achieve yields of co-products to achieve economic viability
- Incorporation into industrial processes for wastewater valorization from TC processes
- Discovery of novel biological transformations to build a “catabolic toolbox” for WW upgrading

Management Approach

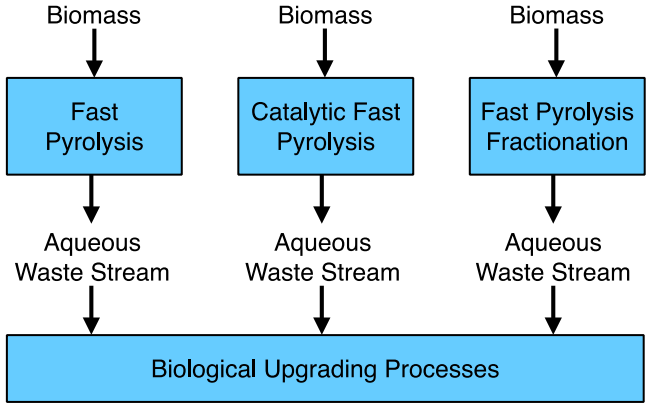
- *Develop simple, integrated approaches and use TEA/LCA and Go/No-Go's to refine options*
- *Employ fundamentals-driven science/engineering approach with an interdisciplinary approach*

Aim 1: Develop biological catalysts that are able to metabolize a wide range of substrates



Target molecules

Aim 2: Obtain and characterize streams from TC processes and tailor organisms to these streams



Assembled team of experts in metabolic engineering and organism development

- Milestones in this aim center on **substrate utilization** and **organism selection**
- Leverage biological work from **Lignin Utilization** (BC) project as a basis for this work
- Surpassed major "Go/No-Go" milestone at end of FY14, enabling further project work

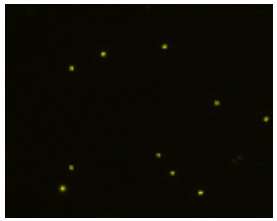
Collaborate with RTI, NREL, Iowa State University and other TC research groups to obtain process-relevant streams

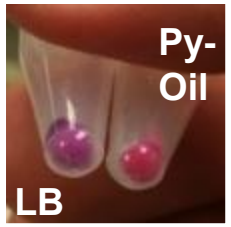
- Milestones in this aim center on **TEA modeling**, **substrate characterization**, and **tailoring/evolving organisms**
- Team includes TEA and fermentation expertise
- Leverage new techniques from our group to fingerprint molecules in waste streams

Technical Results – Outline

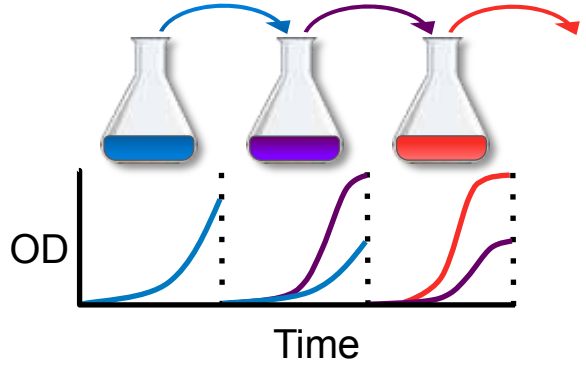
- Developed base model for FP aqueous streams as a “starting” point

- Expanding substrate utilization in a robust biocatalyst: phenol, guaiacol, levoglucosan, cellobiosan, furfural, HMF, and beyond





- Initial tests on mock aqueous pyrolysis oil



- Streams in hand from FP, CFP, fractionation of FP streams

- Initial strain evaluations and strain evolution going forward

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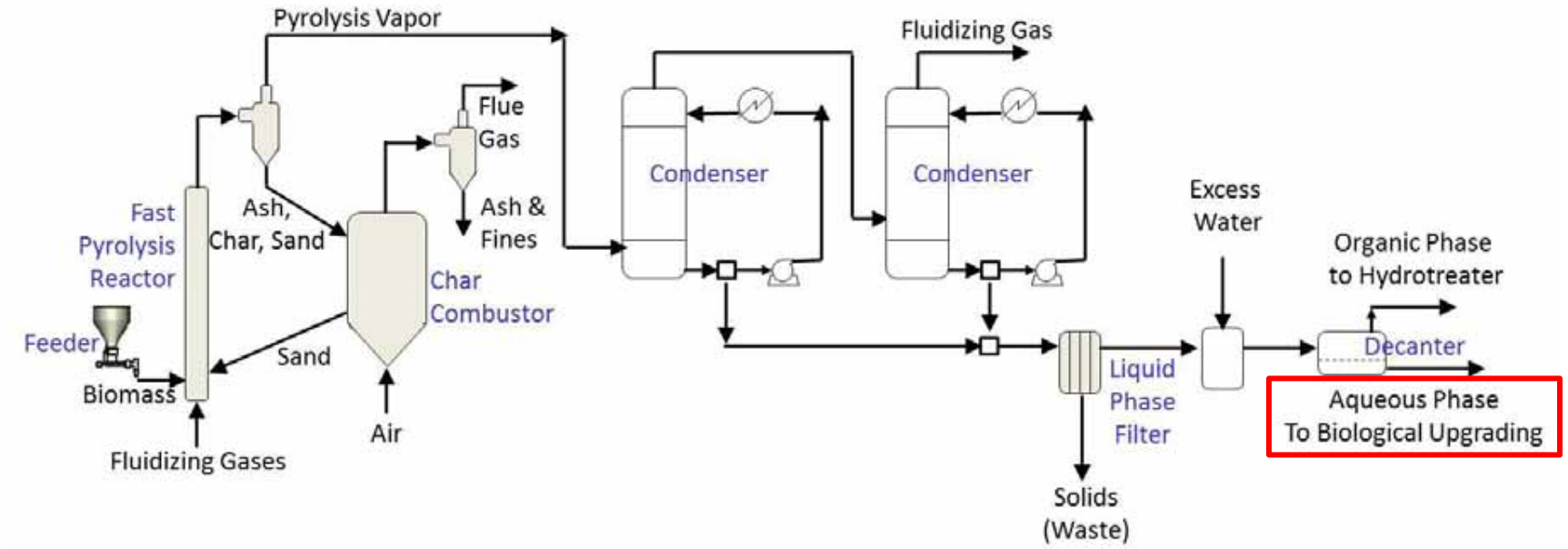
Base model development for Fast Pyrolysis



Leveraged extensive literature data for development of an initial composition model for base studies

- At elevated water content, bio-oils can be fractionated into an aqueous rich and organic rich phase
- An aqueous fraction model was constructed from experimental studies that produced aqueous fractions according to this protocol
- Compositions from GC-FID, HPLC, GC-MS, etc.
- Mass/carbon closures from many of these studies are ~60% or less
- Work ongoing to confirm composition from NREL experimental data
- **Highlighted initial slate of compounds for metabolic engineering**

functionality	g/L
aldehydes	43.6
sugars	42.2
ketones	27.6
acids	24.0
aromatics/phenols	13.2
alcohols	3.3

Studies considered include: Vispute *et al. Green Chem* **2009;** Vispute *et al. Science* **2010;** Tessini *et al. J Chromatogr A* **2011;** Valle *et al. Int J Hydrogen Energy* **2013;** Sukhbaatar *et al. Bioresour Technol* **2014;** Remon *et al. Int J Hydrogen Energy* **2014**

Toxic concentrations of pure compounds for *P. putida* KT2440

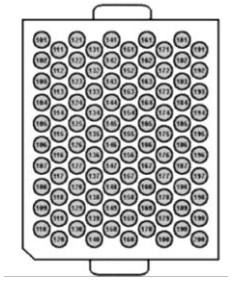
Results of Bioscreen C growth assays:

- In general, aldehydes (4-hydroxy-benzaldehyde, hydroxyacetaldehyde, vanillin) are more toxic than organic acids
- At concentrations found in aqueous pyrolysis oil, catechol and hydroxyacetaldehyde will be particularly challenging due to their high toxicities
- ***P. putida* KT2440 is capable of using the compounds highlighted in green as carbon sources**
- Further toxicity studies are being conducted with additional compounds as they are identified and quantified

Species	Conc where Growth Observed (g/L)	Conc where No Growth Observed (g/L)	Conc present in Pyrolysis Oils (g/L)
4-Hydroxybenzaldehyde	1.2	1.5	
4-hydroxybenzoic acid	22.5	45.0	
Acetic acid	6.0	12.0	14.7
Benzoic acid	1.2	6.0	
Catechol	2.0	5.0	27.5
p-Coumaric acid	15.0	30.0	
Ferulic acid	28.0	28.0	
Furfural	2.9	3.8	2.0
Guaiacol	1.2	6.0	1.2-3.6
HMF	4.0	20.0	8.1
Hydroxyacetaldehyde	0.3	0.5	3-52
Hydroxyacetone	12.5	25.0	14.7
Malonic acid			
Phenol	1.0	1.9	
Syringaldehyde	2.9	-	
Syringol	0.5	1.0	
Vanillic acid	20.0	40.0	

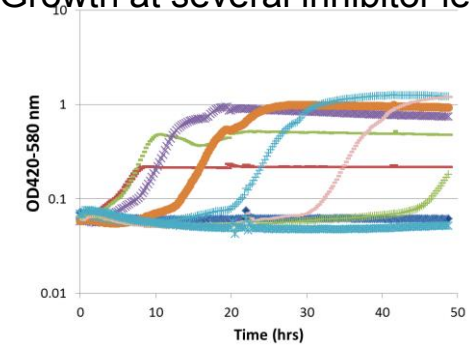
Culture of *P. putida* KT2440

Inoculation in minimal medium with and without glucose

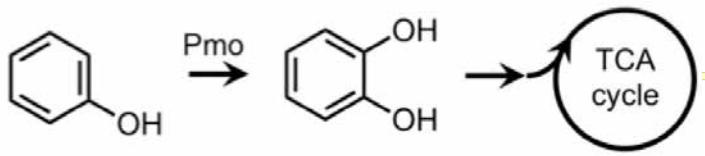


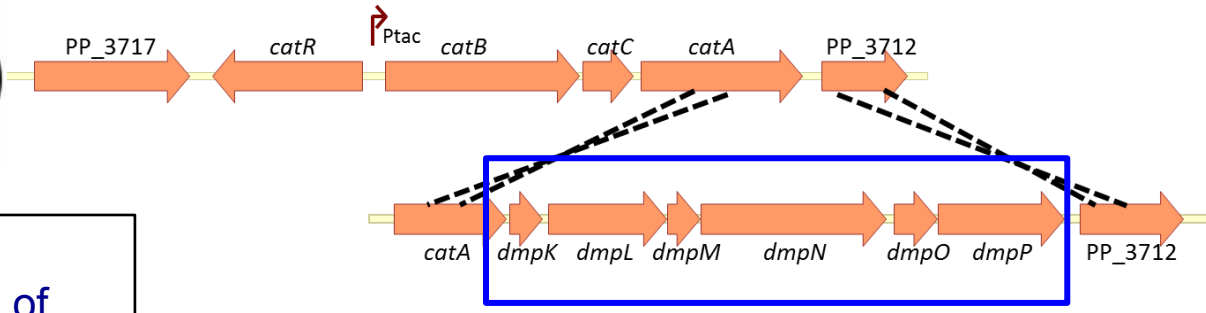
Incubation at 30°C

Growth at several inhibitor levels



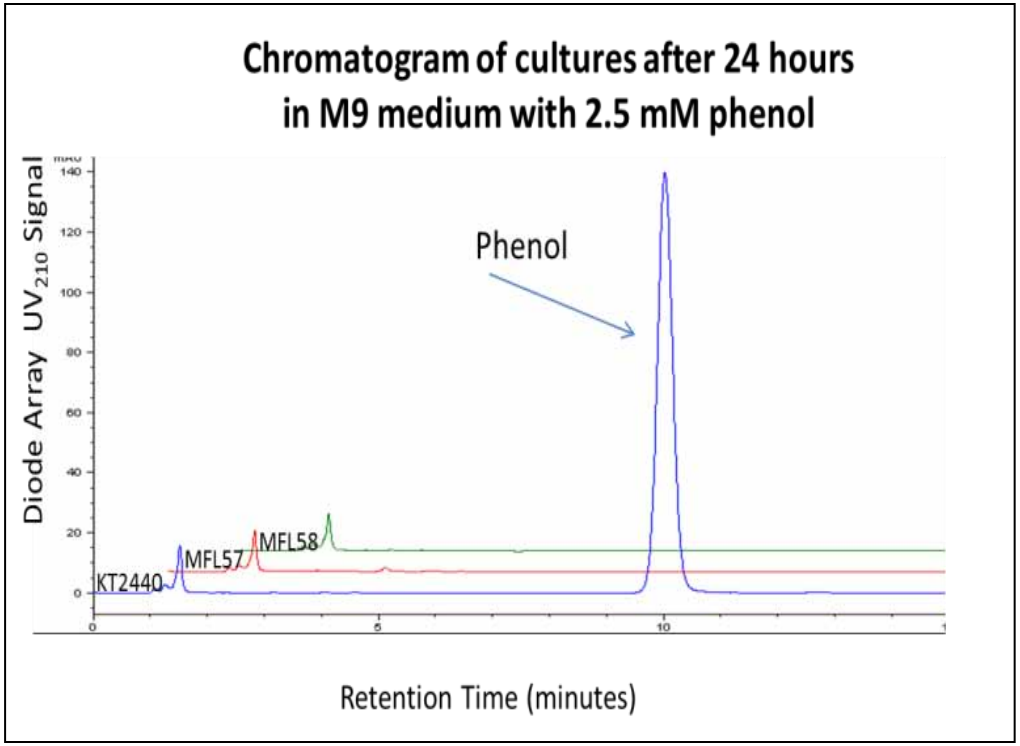
Phenol utilization by *P. putida* KT2440





Incorporation of the phenol monooxygenase enables utilization of phenol as a sole carbon source

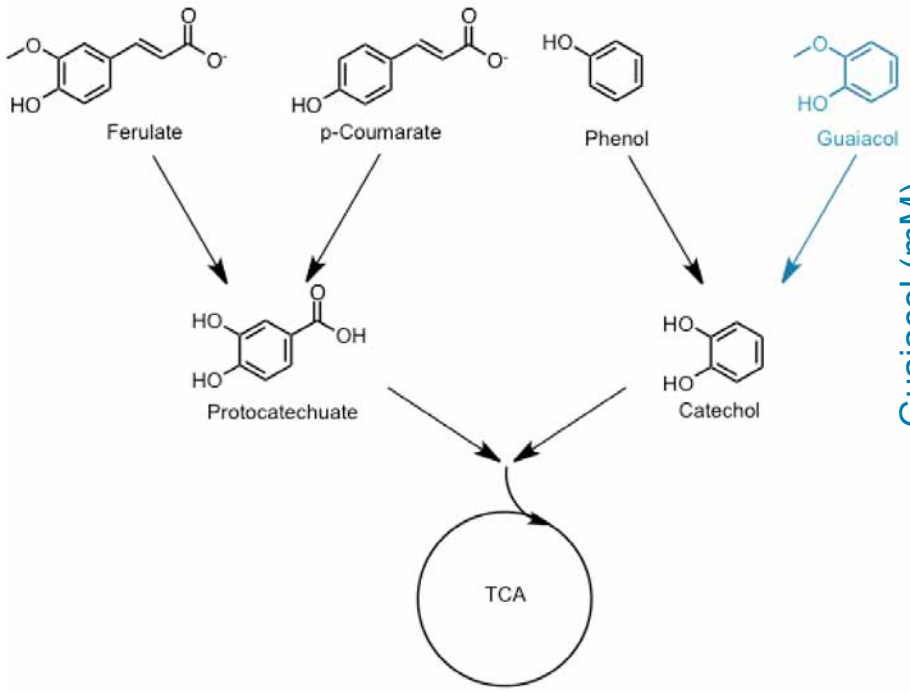
- Six subunit components (top figure) of phenol monooxygenase from *Pseudomonas sp.* CF600 were incorporated behind catA using an integration vector via homologous recombination
- Two strains were designed: MFL58 has the operon driven by the tac promoter and the cat operon in MFL57 is driven by the native cat promoter
- Growth was observed on phenol
- HPLC analysis (bottom figure) shows the utilization of phenol by MFL57 & MFL58 and not by wild-type KT2440

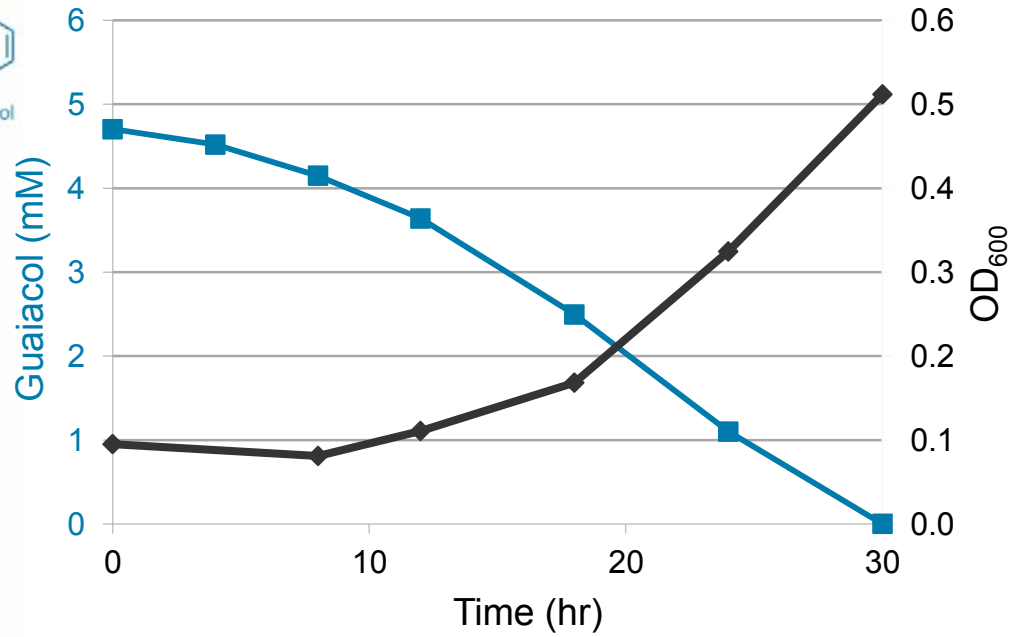


Guaiacol utilization by *P. putida* KT2440

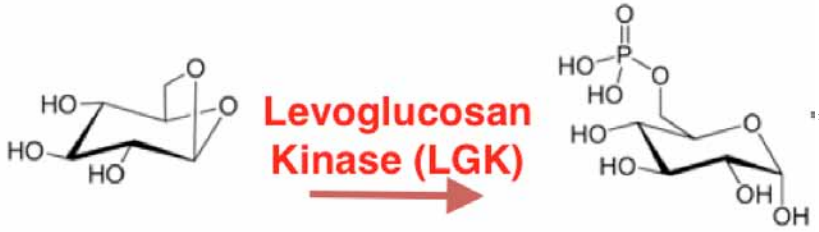
Introduction of a guaiacol O-demethylase and a co-transcribed reductase into *P. putida* enabled growth on guaiacol as a sole carbon source

- Guaiacol is a common pyrolysis product that some bacteria are able to metabolize through catechol
- Previous work has partially described O-demethylases that convert guaiacol to catechol, but the genes encoding these enzymes were not identified
- We discovered a gene responsible for this transformation





Levoglucosan utilization by *P. putida* KT2440

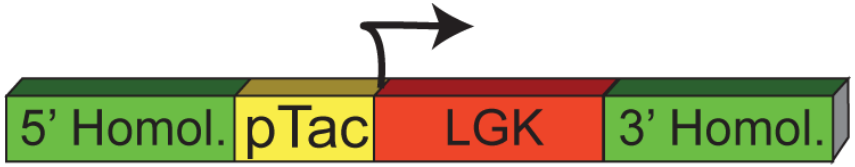


Levoglucosan

Glucose-6-Phosphate

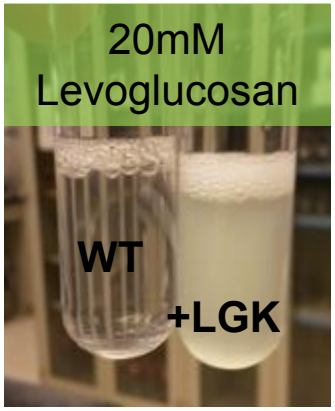
Pentose Phosphate Pathway

Glycolysis



Generated Integrative DNA Expression Construct

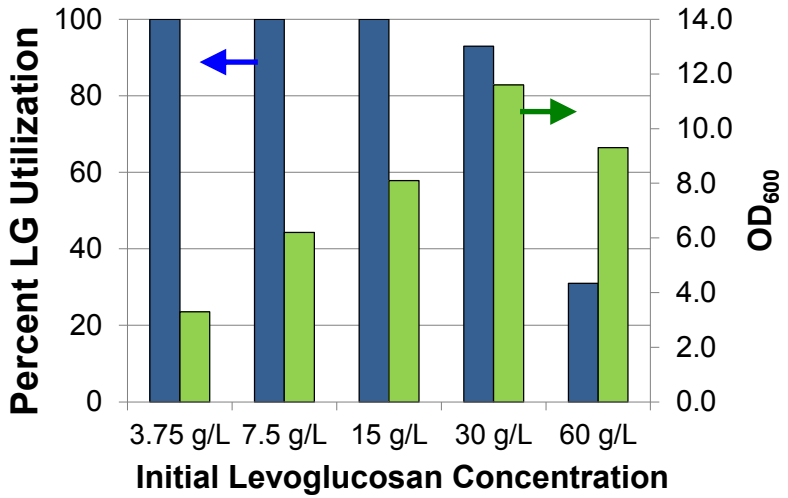
Transformed *P. putida*



Growth in Levoglucosan

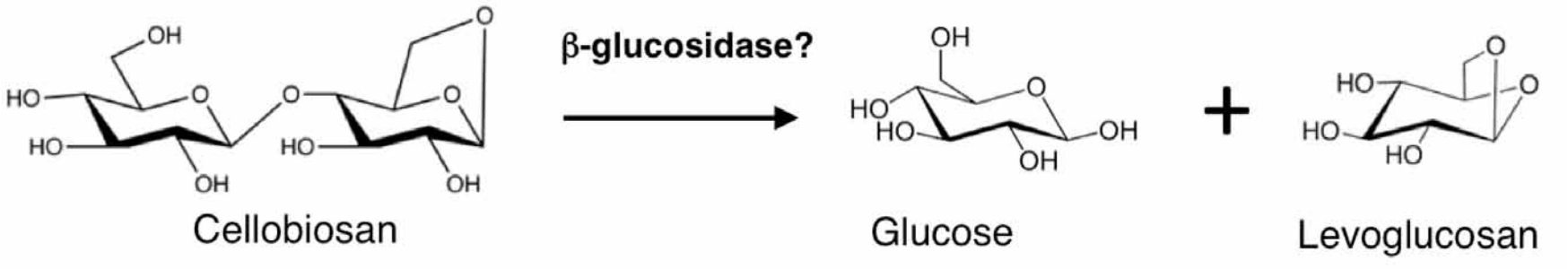
LGK integrated into *P. putida* enables growth on levoglucosan as a sole carbon source

- Levoglucosan is often found in FP aqueous streams at high concentration
- Levoglucosan kinase can convert LG to G-6-P, which enters PPP or glycolysis
- Integrated the LGK into *P. putida*

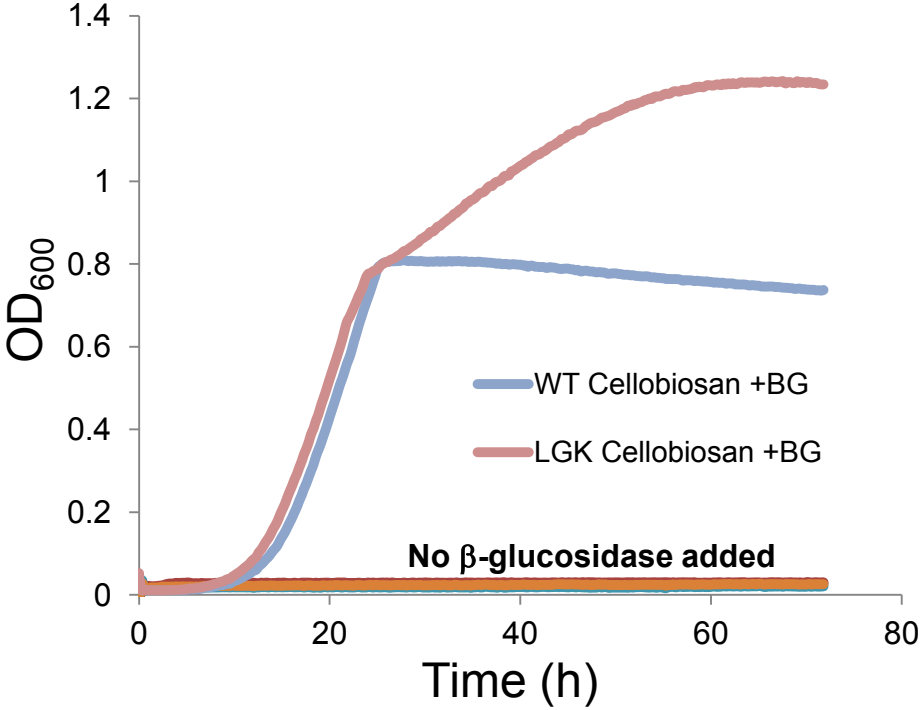


LGK from work of Layton *et al., Bioresource Tech.* 2011

Cellobiosan utilization by *P. putida* KT2440



Levoglucosan utilizing *P. putida* strain can fully utilize cellobiosan with β-glucosidase addition



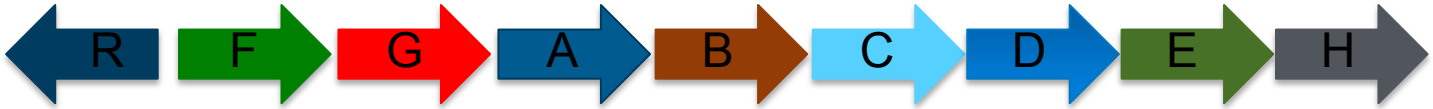
Exogenous β-glucosidase with LGK enables complete utilization of cellobiosan

- Cellobiosan is also often found in FP aqueous streams at high concentration
- Hypothesized that β-glucosidases could convert cellobiosan to glucose and levoglucosan
- Demonstrated that 4 different β-glucosidases turnover cellobiosan

Furfural and HMF utilization by *P. putida* KT2440

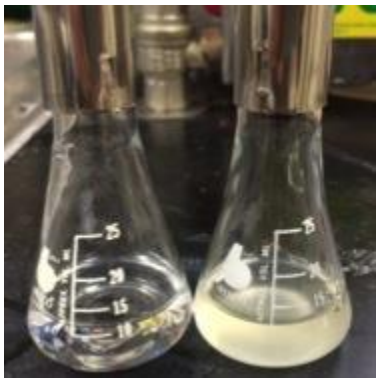
Integration of furfural and HMF genes into *P. putida* enables use of these species as sole carbon sources

- Operon conferring furfural/HMF utilization was recently identified (Koopman, *PNAS* 2010)
- Gibson assembly employed for reconstruction of an ~11kB *hmf* operon
- Incorporation of *hmf* operon into *P. putida* conferred growth on furfural and HMF as sole carbon sources
- >75% furfural utilization observed in engineered strains following 24 hr growth
- Efforts underway to integrate *hmf* operon into *P. putida*



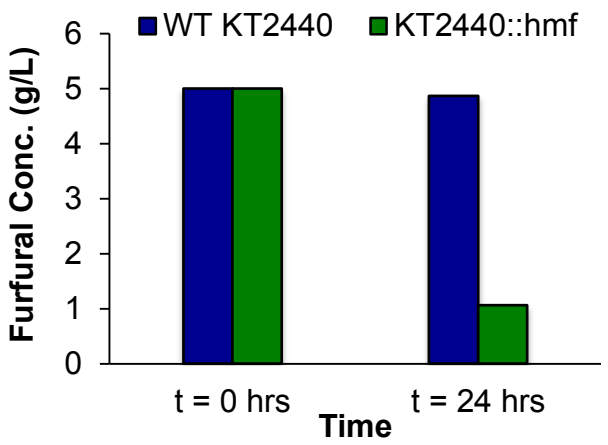
Selection on Furfural & HMF

Growth on HMF



KT2440 (wild type) ***hmf*:KT2440**

Enhanced Furfural Utilization

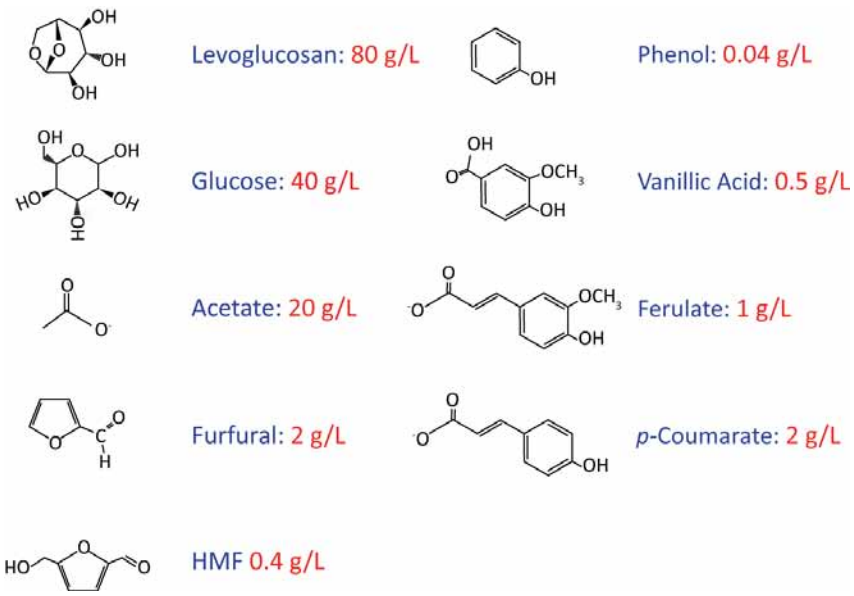


FY14 Go/No-Go Milestone Results

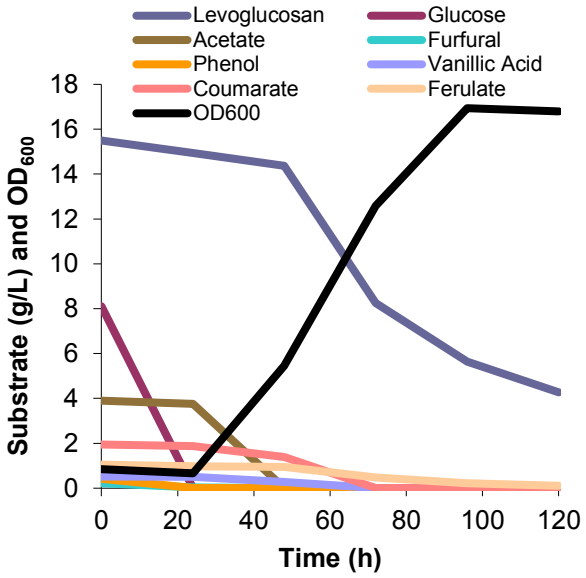
Used a mock FP aqueous stream to achieve a "Go" at the end of FY14 in an engineered strain of *P. putida* KT2440

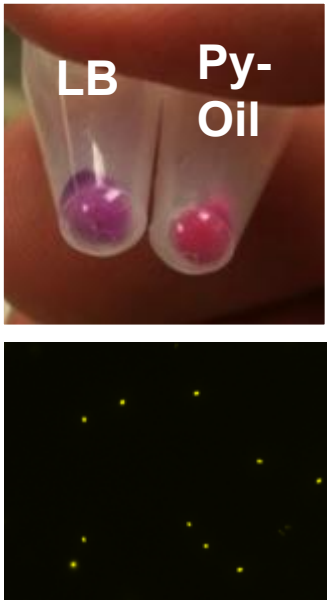
- Demonstrated biological utilization of furfural, levoglucosan, and phenol in *P. putida* KT2440
- Demonstrated ability of wild-type *P. putida* to grow in the aqueous fraction of pyrolysis oil
- Demonstrated ability of engineered *P. putida* to convert multiple py-oil components to intracellular polymers (*mcl*-PHAs), which can be converted to alkanes or hydroxy-acids (Linger *et al., PNAS* 2014)

Primary components in mock aqueous FP stream



1:5 Dilution





Finding genes for other substrates

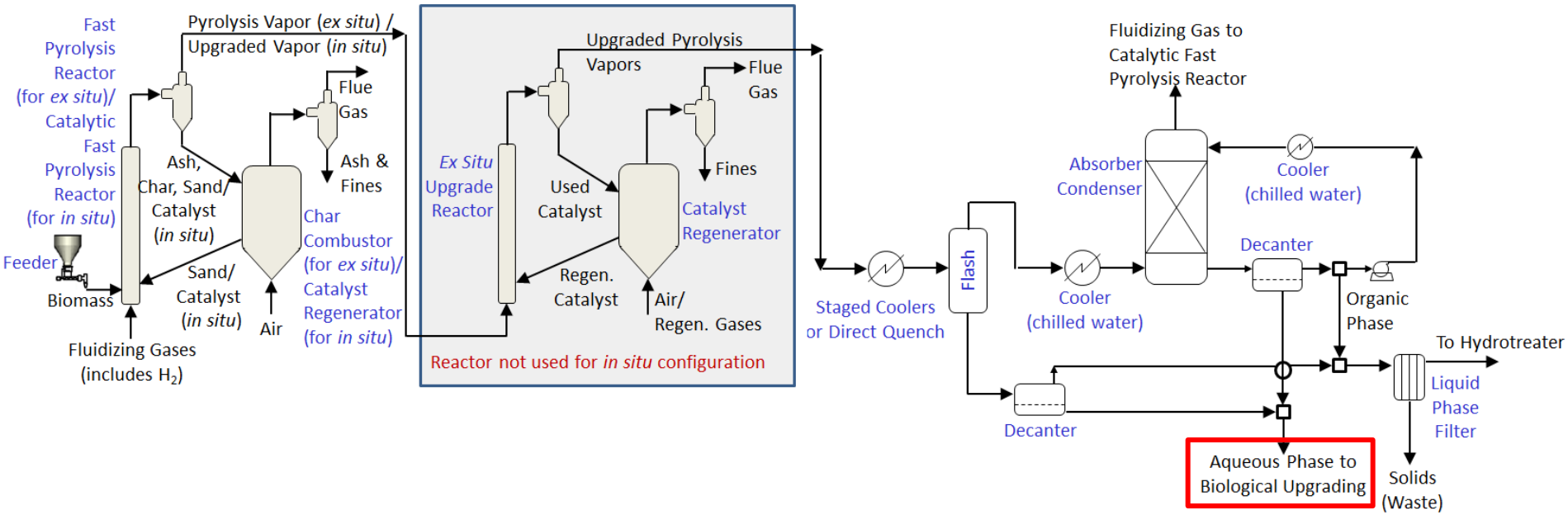
Metabolic pathways for several key species present in py-oil waste streams are unknown

- Tested numerous bacteria for their ability to grow in minimal medium containing as only carbon source pure molecules which are present in py-oil
- Positive results show bacteria that posses genes to metabolize molecules of interest
- Genes can be identified and used in *P. putida* KT2440 to expand substrate specificity

	Positive Control	Hydroxyacetaldehyde		Hydroxyacetone		2-furanone		3-methyl-1,2-cyclopentadione		Pyrogallol		Syringol	
		2 mM	10 mM	2 mM	10 mM	2 mM	10 mM	2 mM	10 mM	2 mM	10 mM	2 mM	10 mM
SOIL BACTERIA (15)													
Pseudomonas putida KT2440	++++	-	-	+/-	+/-	-	-	-	-	+	+	-	-
Pseudomonas putida mt-2	++++	-	-	-	-	-	-	-	-	+	+	-	-
Pseudomonas fluorescens Pf-5	++++	-	-	-	-	-	-	-	-	+	+++	-	-
Cupriavidus necator H16	- (Glu-)	-	+	-	-	-	-	-	-	+	+++	-	-
Azotobacter vinelandii Lipman, NRS 16	-	-	-	-	-	-	-	-	-	-	-	-	-
Acinetobacter sp. strain ADP1	-	-	-	-	-	-	-	-	-	+	+++	-	-
Citrobacter freundii	++++	-	-	-	-	-	-	-	-	+	+	-	-
Enterobacter lignolyticus SCF1	++++	-	-	-	+/-	-	-	-	-	++	++	-	-
Amycolatopsis sp. 75iv2 (ATCC 39116)	++++	-	-	-	-	-	-	-	-	+	+	-	-
Rhodococcus jostii RHA 1	++++	-	-	++	-	++	-	++	-	+	+	-	-
Rhodococcus erythropolis U23A	++++	-	-	-	-	-	-	-	-	-	+++	-	-
Bacillus subtilis	++++	-	-	-	-	-	-	-	-	-	-	-	-
Bacillus megaterium	-	-	-	-	-	-	-	-	-	-	-	-	-
Burkholderia phytofirmans	++++	-	-	-	-	-	-	-	-	++	+++	-	-
Pseudomonas putida S12	++++	-	-	-	-	-	-	-	-	+	+	-	-
MARINE BACTERIA (7)													
Sagitulla stellata E-37	+/-	-	-	-	+++	-	+	-	-	+	-	-	-
Citreicella sp SE45	+/-	-	-	-	-	-	-	-	-	-	-	-	-
Roseovarious nubinhibens ISM	-	-	-	-	-	-	-	-	-	+	-	-	-
Ruegeria pomeroyi DSS-3	+/-	-	-	-	-	-	-	-	-	-	-	-	-
Sulfitobacter sp. NAS-14.1	-	-	-	-	-	-	-	-	-	-	-	-	-
Sulfitobacter sp. EE-36	+/-	-	-	-	-	-	-	-	-	-	-	-	-
Halomonas sp.1	-	-	-	-	-	-	-	-	++	+	-	-	-

+ → Optical densities at least two-fold higher that the non-inoculated solution.
++ → Optical densities at least three-fold higher that the non-inoculated solution.
+++ → Optical densities at least four-fold higher that the non-inoculated solution.

Aqueous streams of interest: CFP



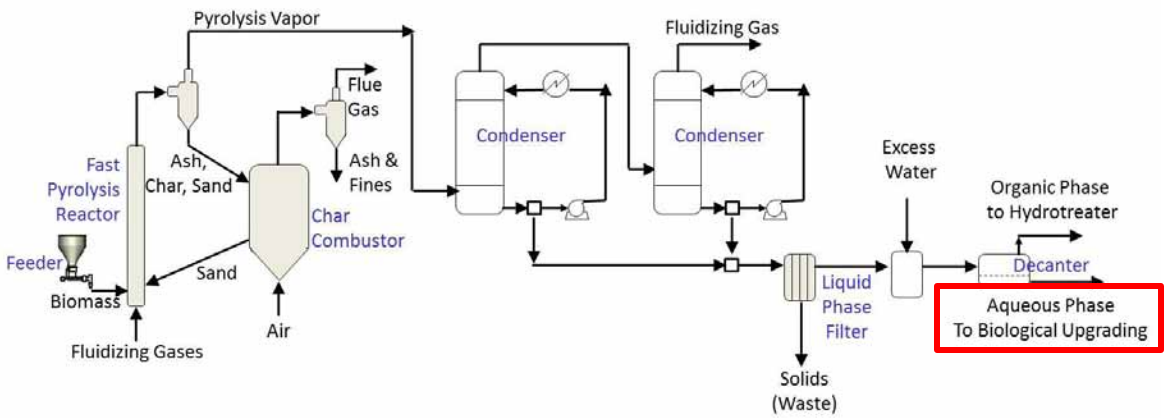
Collaborating with RTI to upgrade CFP aqueous waste streams

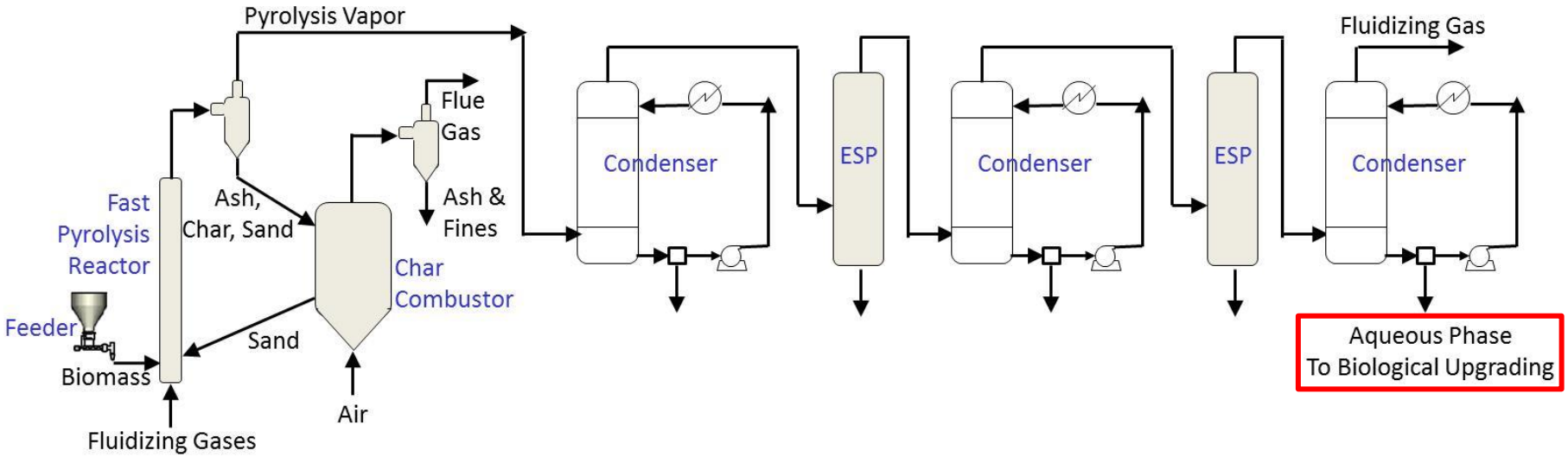
- Two waste streams from oak and pine
- Compositional analysis ongoing
- Initial growths of bacteria show some dilution is required, likely as a result of aldehyde content present in the waste stream
- Laboratory evolution ongoing for these waste streams

Aqueous streams of interest: FP

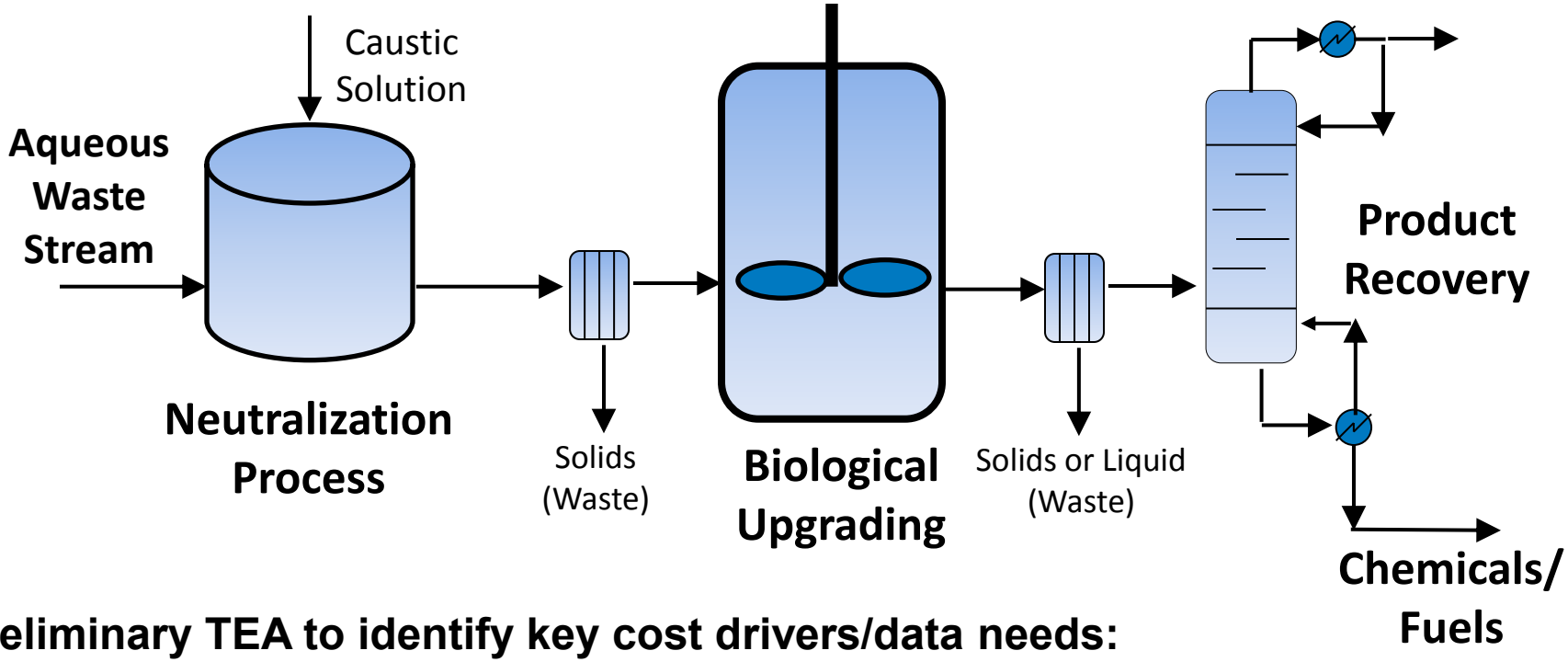
Collaborating with ISU and NREL to obtain FP waste streams

- Single waste streams for each approach
- Compositional analysis ongoing
- Initial growths of bacteria show partial dilution is required





Preliminary TEA



Preliminary TEA to identify key cost drivers/data needs:

Neutralization process:

- Required pH for upgrading, caustic required to neutralize, carbon losses

Biological Upgrading: ***intracellular storage products and gas-phase products***

- Titer, yield, productivity and carbon conversion to desired product

Product recovery

- Efficiency of recovery process and capital/operating costs for purification

Potential cost savings through process integration

- Reduce WWT requirements, lower hydrotreating severity, utilize off-gas/heat

Relevance

Valorization of aqueous waste streams will be a major contributor to 2022 HC cost targets

Highlighted in MYPP as a key barrier in TC Platform: **"Research is needed to characterize organics in the aqueous phase and to convert these organics to hydrogen, biochemicals, or hydrocarbon fuels."**

Key MYPP areas:

<u>Aqueous Phase Utilization and Wastewater Treatment</u>
- Enables selective, tunable route for upgrading "waste" carbon
- Potential for both fuels and chemicals production via a biological route
- Maximizing use of biomass carbon

<u>Process Integration</u>
- Working with process-relevant streams from 3 TC approaches

<u>Catalytic Upgrading of Bio-Oil Intermediates to Fuels/Chemicals</u>
- Enables tuning of upstream catalytic steps to reduce HT cost

Key Stakeholders and Impacts:
- **Economics and sustainability of TC processes could significantly benefit from co-product manufacturing**
- Work will enable production of fuels or chemicals from waste streams in TC biorefinery designs
- Impacts the "**Whole Barrel of Oil**" initiative
- **Portfolio of chemicals from waste carbon will diversify and accelerate development of the biomass value chain**
- Significant amounts of peer-reviewed science and IP will be generated from this work
- Methods to upgrade heterogeneous intermediates can be adapted by other platforms, e.g., Lignin Utilization

Future Work

Aim 1: Develop biological catalysts that are able to metabolize a range of substrates

- Finalize gene sets for primary aldehydes, acids, ketones, and organic acids present in 3 TC process streams for "plug-and-play" organism engineering

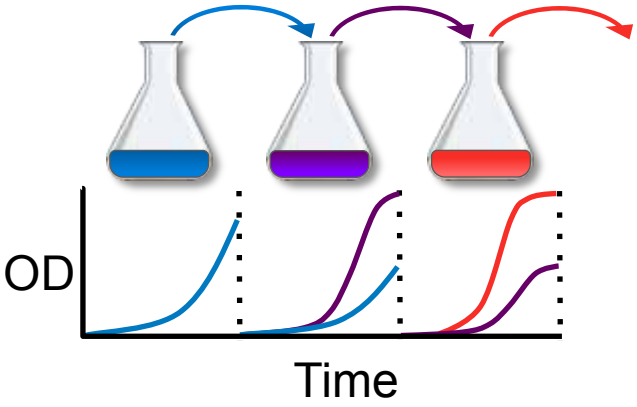
Aim 2: Obtain and characterize streams from TC processes and tailor organisms to these streams

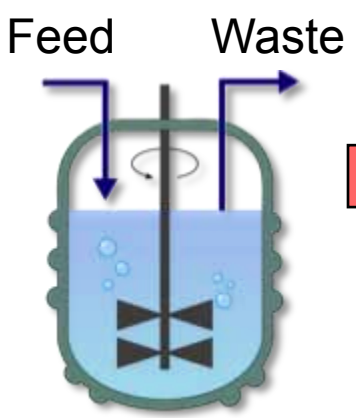
- Finalize chemical analysis of each stream
- Select an optimal co-product based on TEA modeling of waste valorization processes

Tailored genes

Specific process streams

Laboratory strain evolution





***Tailored* organisms for each process stream**

Goal: deliver tailored organisms for initial bench-scale integrated process evaluations in FY16/17, priority dictated by BETO TC Platform cost targets

Summary

1) Approach:
   - develop biological catalysts that can metabolize a broad range of waste carbon and are tolerant to TC-derived aqueous process streams
   - collaborate widely with academic, national lab, and industrial partners including TC Platform tasks

2) Technical accomplishments
   - demonstrated incorporation of multiple genes into a host organism, *P. putida* KT2440 for catabolism of multiple, process-relevant organic species such as phenolics and furans
   - identified multiple organisms with pathways for additional major aldehydes and ketones
   - applying an adapted laboratory evolution approach to increase strain tolerance to toxic streams
   - demonstrated PHA production in mock pyrolysis oil stream with minor dilution only

3) Relevance
   - reduce economic and sustainability burden on wastewater treatment in TC process configurations
   - co-products essential to meet DOE hydrocarbon cost targets
   - addresses Whole Barrel of Oil Initiative and bolsters the biomass value chain

4) Critical success factors and challenges
   - stream toxicity, **economic** *and* **sustainable** production of co-products, high yields of products needed

5) Future work:
   - complete comprehensive set of catabolic genes for “plug-and-play” organism engineering
   - ramp up efforts on adaptive laboratory evolution for tailoring organisms to specific process streams

6) Technology transfer:
   - working with **industry** to build commercialization path to wastewater valorization in TC processes

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Additional slides

- Publications
- Acronyms
- Literature data for FP oil model development
- Bioscreen activity assay

Publications

Publications in preparation:

1. J.G. Linger *et al.*, Levoglucosan and cellobiosan utilization in *Pseudomonas putida*
2. C.W. Johnson *et al.*, Characterization of a guaiacol O-demethylase
3. M.T. Guarnieri *et al.*, Furfural and 5-hydroxymethylfurfural utilization in *Pseudomonas putida*

Acronyms

- CFP: Catalytic Fast Pyrolysis
- FP: Fast Pyrolysis
- LCA: Life-Cycle Analysis
- LGK: Levoglucosan Kinase
- TEA: Techno-Economic Analysis

Vispute *et al. Green Chem* **2009** data

- Fast pyrolysis of oak
- They prepare their aqueous fraction by mixing 80 g water with 9 g bio-oil, centrifuge, then decant
- Components identified via GC-FID, HPLC, and GC-MS
- Carbon closure: 60%

Table 1 Identification of major components of aqueous fraction of bio-oil. The aqueous fraction of bio-oil was made by mixing 80 g of water with 9 g of bio-oil

Quantification method	Species	Concentration (mmole carbon L^{-1})	% of total carbon
GC-FID	Hydroxyacetone	135.5	6.5
GC-FID	Hydroxyacetaldehyde	28.1	1.4
GC-FID	Guaiacols and derivatives	30.8	1.5
HPLC	Sugars	377.4	18.2
HPLC	Levoglucosan	390.6	18.8
GC-FID	Acetic acid	182.2	8.8
GC-MS	Furfural and 2-furanone	100.0	4.8
	Total carbon content identified by GC & HPLC	1244.6	60.0
	Total carbon content measured by TOC	2075.9	100

Vispute *et al. Science* **2010** data

- Fast pyrolysis of pine
- They prepare their aqueous fraction by mixing 28g water with 7 g bio-oil, centrifuge, then decant
- Components identified via GC-FID, HPLC, and GC-MS
- Carbon closure: 57%

Chemical analysis of water soluble fraction of pine wood bio-oil (WSBO) and single & two-stage hydrogenation product of WSBO

Following table depicts the detailed composition of WSBO feed.

Table S3 Composition of WSBO feed*

Compound	mmol carbon L^{-1}	Classification
Hydroxyacetaldehyde	427.6	Aldehyde
Acetic acid	244.1	Acid
Hydroxyacetone	199.3	Ketone
2-Furanone	37.6	Ketone
Phenol	2.5	Phenolic
3-Methyl-1,2-cyclopentadione	45.7	Ketone
Guaiacol	10.3	Phenolic
Catechol	249.8	Phenolic
1-Hydroxy-2-butanone	20.2	Ketone
Furfural	20.9	Aldehyde
2-Cyclopenten-1-one	21.9	Ketone
5-Hydroxymethylfurfural	63.9	Aldehyde
4-Methyl catechol	47.5	Phenolic
Levoglucosan	652.5	Sugar
Sugars	124.4	Sugar
Methanol	24.4	Alcohol
Total carbon Identified	2192.6	
Total carbon as measured by TOC	3879.4	

*made by mixing 7 gm pine wood bio-oil with 28 gm water. The WSBO has 3879.4 mmol carbon L^{-1}, hence the carbon concentration of each component is given in mmol carbon L^{-1} from than compound in WSBO. Fraction carbon contribution of each compound can be found by dividing mmol carbon L^{-1} for that compound by 3879.4 mmol carbon L^{-1}.

Tessini *et al. J Chromatogr A* **2011** data

- Fast pyrolysis of "sawdust"
- Their method for preparing the aqueous fraction was not transparent
- Components identified via HPTLC

Table 2
Analytical parameters for HPTLC-sugar determination in bio-oil.

Sugars	Linear range (ng)	Detection limit (ng)	Quantification limit (ng)	Intermediate precision[a] (RSD (
Levoglucosan	100–800	60	180	11%
Cellobiosan	100–700	80	240	20%
Xylose	50–400	16	48	–
Arabinose	50–300	14	42	–
Cellobiose	50–300	18	59	–

[a] The intermediate precision was determined using three different bio-oil samples in triplicate, at different days and with different HPTLC plates.

Table 3
Sugar concentrations in fresh bio-oil samples and extracts.

Samples	Glucose	Levoglucosan (wt%)	Cellobiosan (wt%)	Xylose (wt%)	Arabinose
Bio-oil 1	ND[a]	1.27	1.46	ND	ND
Bio-oil 2	ND	1.90	1.99	ND	ND
Bio-oil 3	ND	1.68	0.98	ND	ND
Bio-oil 4	ND	2.26	1.40	ND	ND
Bio-oil 4 aqueous phase	ND	1.81	0.93	ND	ND
Bio-oil 4 n-butanol/phase	ND	0.78	0.82	ND	ND
Bio-oil 4 pyrolytic lignin	ND	0.75	0.88	ND	ND

[a] ND, not detected; wt%, weight/weight percent.

Valle *et al. Int J Hydrogen Energy* **2013** data

- Fast pyrolysis of pine
- They prepare their aqueous fraction by adding water to bio-oil in the mass ratio 2:1
- Components identified via GC-MS
- Mass closure: 57 wt % (dry basis)

Table 1 – Mass composition and molecular formula of the aqueous fraction of the bio-oil used.

Compound	wt%
Acetic acid	19.1
Acetone	1.0
Formic acid	2.7
Methanol	1.0
1-Hydroxy-2-propanone	8.7
Hydroxyacetaldehyde	1.8
1-Hydroxy-2-butanone	2.0
Levoglucosane	19.6
Hexose	2.7
Other ketones	6.1
Other acids	4.7
Esters	3.1
Other aldehydes	5.5
Phenols	13.4
Ethers	0.3
Alcohols	3.6
Others	1.1
Unidentified	3.8
Molecular formula	$C_{4.1}H_{7.4}O_{2.7}$

Sukhbaatar *et al. Bioresour Technol* **2014** data

- Fast pyrolysis of pine
- They prepare their aqueous fraction by adding 2 L water to 2 L bio-oil, shaking, and decanting
- Components identified via HPLC

Table 3
Composition of bio-oil water fraction resulting from different detoxification steps.

g/L	Raw bio-oil	After water extraction BWF	After n-butanol extraction EBWF	After hydrolysis and n-butanol evaporation DBWF	After combination treatment step CAP
Levoglucosan	113 ± 0.4	77.02 ± 1.57	76.60 ± 0.31	ND	ND
Glucose	ND	ND		114.19 ± 1.12	248.62 ± 0.88
Xylose	ND	ND	ND	4.91 ± 1.29	18.64 ± 0.71
Galactose	ND	ND	ND	10.05 ± 0.21	18.98 ± 0.79
Arabinose	ND	ND	ND	ND	ND
Mannose			8.11	31.49 ± 1.05	53.51 ± 0.69
5-HMF	4.52 ± 0.17	3.03 ± 0.69	ND	ND	ND
Furfural	5.14 ± 0.03	2.28 ± 0.18	ND	ND	ND
Acetic acid	15.01 ± 0.10	10.15 ± 1.78	ND	ND	ND
n-Butanol	ND	ND	38.43 ± 2.11	19.33 ± 0.06	ND

ND – not detected.

Remon *et al. Int J Hydrogen Energy* **2014** data

- Fast pyrolysis of pine
- They prepare their aqueous fraction by adding bio-oil to water until water to carbon molar ratio of 5.5 is reached (calculated to correspond to a 3.44:1 water:oil mass ratio), then separate precipitate by filtration
- Components identified via GC-MS
- Mass closure: 58 wt % (dry basis)

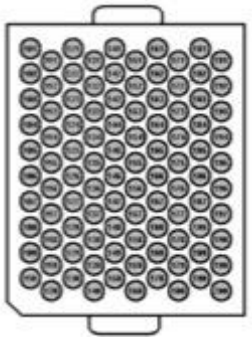
Table 5 – Comparison between the chemical compositions (in dry basis) of the bio-oils and the aqueous fractions prepared. Results are expressed in mg/g as mean ± standard deviation.

	Fluidized bed		Spouted bed		*p*-Value
	Bio-oil	A. Fraction	Bio-oil	A. Fraction	
Carboxylic acids	**49.6 ± 8.4^{A}**	**77.7 ± 35.9^{A}**	**61.0 ± 17.0^{A}**	**65.7 ± 24.0^{A}**	**0.7090**
Acetic acid	33.2 ± 3.3^{A}	44.1 ± 19.8^{A}	43.0 ± 9.8^{A}	37.0 ± 0.0^{A}	0.7506
Formic acid	13.9 ± 4.2^{A}	30.5 ± 16.6^{A}	12.81 ± 6.6^{A}	23.4 ± 23.4^{a}	0.6247
Propionic acid	2.4 ± 0.9^{B}	3.1 ± 0.5^{B}	5.2 ± 0.6^{A}	5.3 ± 0.9^{A}	0.037
Alcohols	**5.8 ± 0.5^{A}**	**17.6 ± 6.9^{A}**	**7.9 ± 0.2^{A}**	**18.0 ± 6.4^{A}**	**0.1209**
Methanol					
Aldehydes	**155.7 ± 99.5^{A}**	**303.3 ± 236.0^{A}**	**240.7 ± 145.5^{A}**	**350.6 ± 219.7^{A}**	**0.7485**
Hydroxyacetaldehyde	144.8 ± 99.6^{A}	277.3 ± 234.8^{A}	225.0 ± 151.2^{A}	315.9 ± 219.8^{A}	0.8107
Acetaldehyde	1.0 ± 1.0^{A}	3.7 ± 0.9^{A}	4.0 ± 3.9^{A}	5.2 ± 3.0^{A}	0.4884
Formaldehyde	9.9 ± 1.0^{C}	22.2 ± 2.1^{B}	11.8 ± 1.7^{C}	29.4 ± 2.8^{A}	0.0018
Ketones	**33.4 ± 11.1^{A}**	**44.2 ± 20.8^{A}**	**59.0 ± 19.8^{A}**	**82.2 ± 37.5^{A}**	**0.3386**
2-Propanone,1-hydroxy-					
Furans	**5.1 ± 0.2^{A}**	**4.0 ± 0.1^{B}**	**1.8 ± 0.2^{C}**	**1.8 ± 0.0^{C}**	**0.001**
Furfural					
Sugars	**110.3 ± 10.8^{A}**	**126.2 ± 2.2^{A}**	**71.7 ± 3.6^{B}**	**87.4 ± 24.2^{B}**	**0.050**
Levoglucosan					
Aromatics	**34.2 ± 10.6^{A}**	**11.2 ± 0.4^{B}**	**10.7 ± 2.7^{B}**	**9.4 ± 0.6^{B}**	**0.027**
Phenols	3.5 ± 0.2^{B}	3.1 ± 0.1^{B}	3.7 ± 0.4^{B}	4.8 ± 0.5^{A}	0.002
Guaiacols, syringols	30.7 ± 10.8^{A}	8.1 ± 0.3^{B}	7.1 ± 3.0^{B}	4.6 ± 0.1^{B}	0.028

A, B and C in each row represent statistically different homogeneous groups for bio-oils and aqueous fractions with 95% confidence.

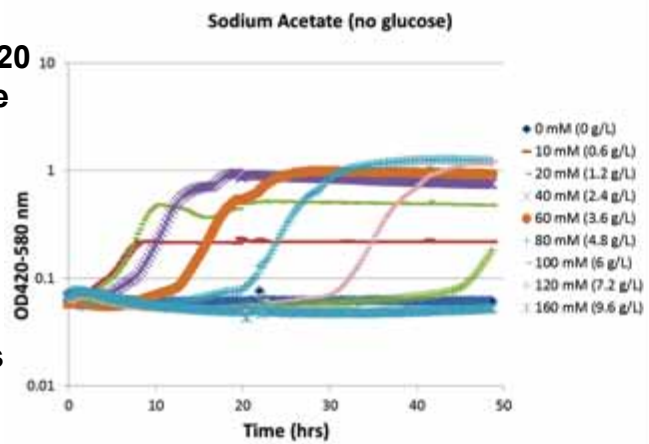
Bioscreen C toxicity assay screen

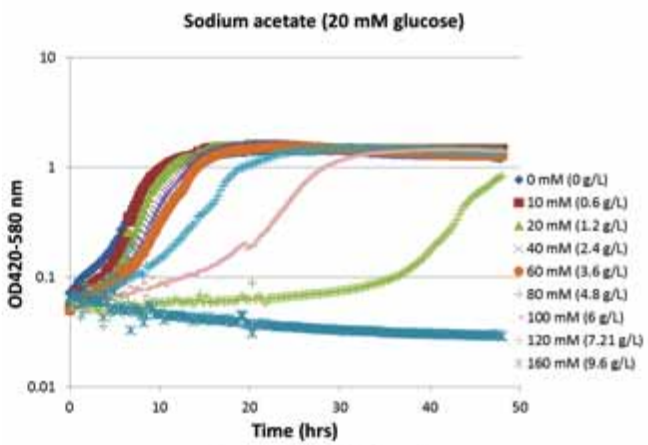
Culture of *P. putida* KT2440 in minimal medium containing 20 mM glucose

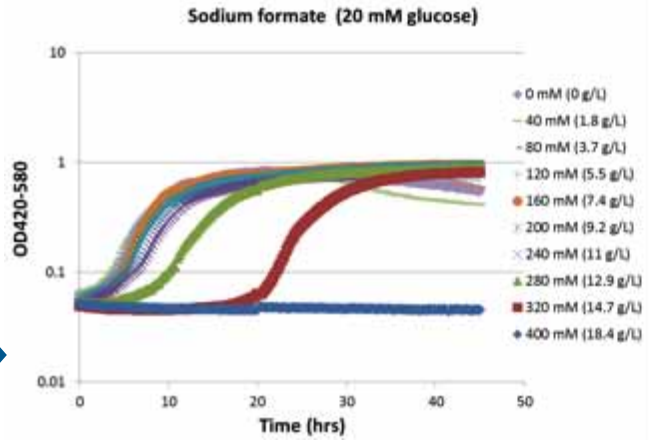


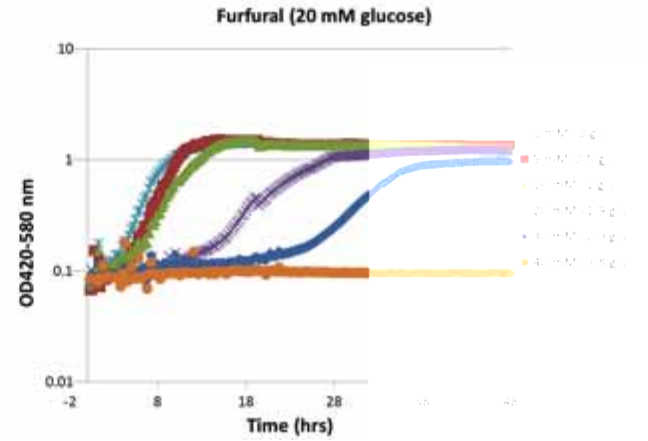
Inoculation into microplates

Incubation in Bioscreen C (automated turbidimeter) at 30°C with shaking



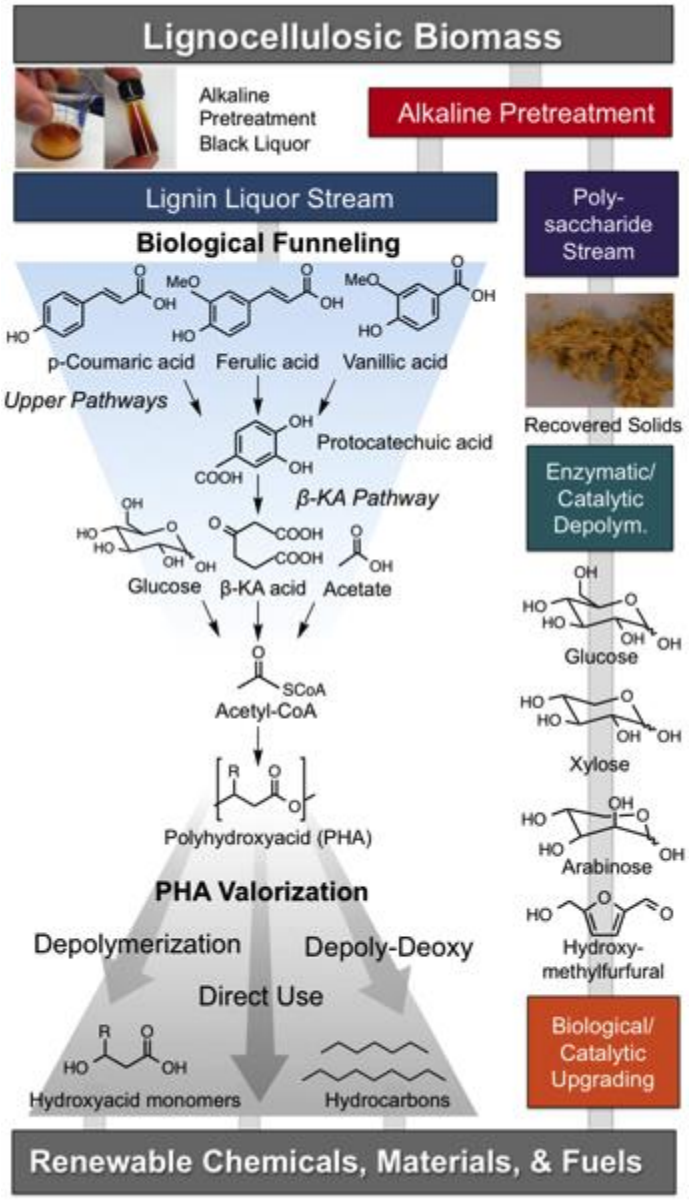




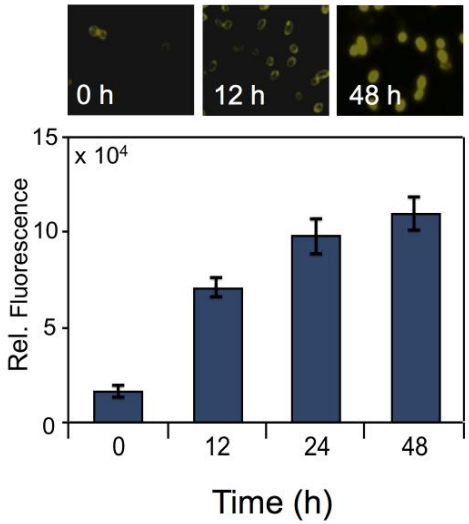


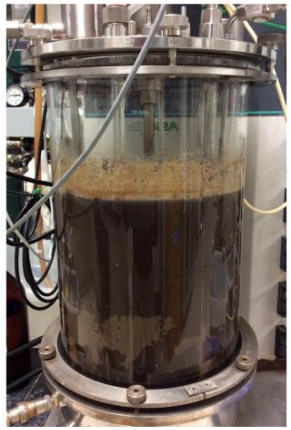
Growth curves at several inhibitor concentrations containing minimal medium with or without glucose. *P. putida* can not use formate and furfural as a carbon source.

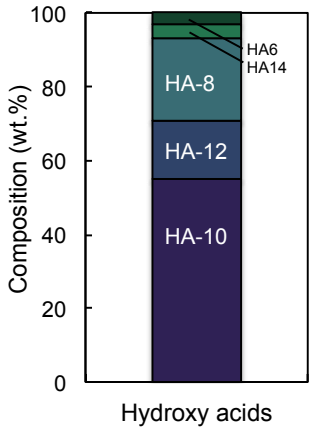
Basis for this project: Biological Funneling



Biological Funneling Cultivations on APL from Lignin Utilization Project in BC Platform







Biological Funneling enables conversion of lignin-derived aromatics into value-added compounds

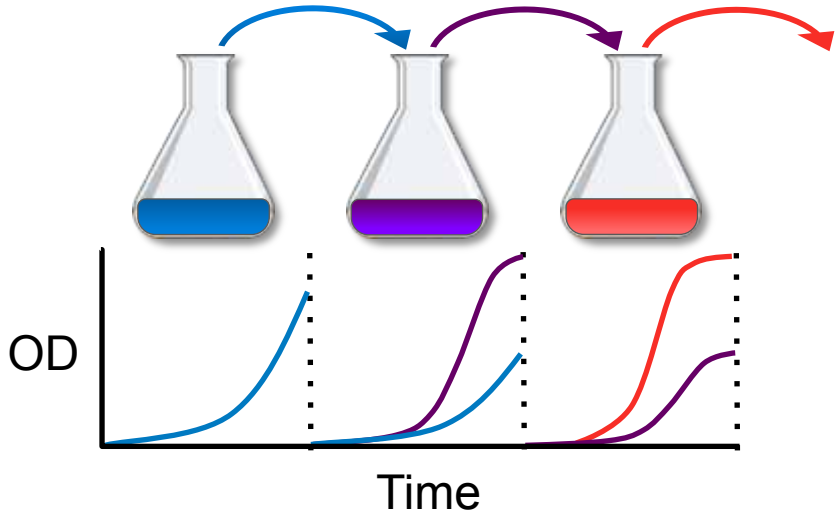
- Demonstrated *mcl*-PHA production in *Pseudomonas putida* KT2440 on alkaline pretreated liquor
- **Leveraging this work from another BETO-funded project as the basis for Biological Pyrolysis Oil Upgrading including the model organism and initial target product (*mcl*-PHAs)**

Linger, Vardon, Guarnieri, Karp *et al.*, *PNAS,* 2014

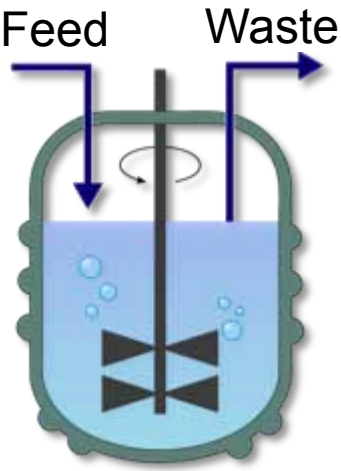
Adaptive Laboratory Evolution (ALE)

Currently ramping up efforts in adaptive laboratory evolution (ALE) with native and engineered strains of *P. putida* KT2440 for increased microbial resistance

Serial Transfer



Continuous Culture



Applikon MiniBio