DOE Bioenergy Technologies Office (BETO) 2015 Project Peer Review

Design, Construction, and Implementation of Novel Biofuel Production Capabilities in Filamentous Fungi

March 26, 2015
Technology Area Review

Kenneth S. Bruno
Pacific Northwest National Laboratory

On many slides, the slide notes section has important additional information

Goal Statement

- **Problem**: Lack of robust platforms for biochemical conversion of lignocellulosic feedstocks
  - Must have high levels of production
  - Must be able to utilize recalcitrant substrate
  - Must produce novel compounds (beyond ethanol, butanol…)
- **Goal**: Apply Synthetic Biology to filamentous fungi to meet needs of future biorefinery
  - Fungi are chiefly responsible for the degradation of plant material in the environment
  - Fungi have large capacity for secondary metabolite production
  - Fungi ***ARE*** genetically tractable
  - Fungi ***ARE*** scalable for industrial production
- Success of this project will be measured by increase in yield from lignocellulosic biomass that will expand the options for novel compounds for the biorefinery

Quad Chart Overview

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Timeline

- Project start date 6/1/2013
- Project end date 6/30/2015
- Percent complete 70%

Budget

	Total Costs FY 10 – FY 12	FY 13 Costs	FY 14 Costs	Total Planned Funding (FY 15-Project End Date
DOE Funded	0.00	360,366	1,452,661	589,584
University Cost Share	0.00	54,927	34,362	81,116
Industry Cost Share	0.00	0.00	24,276	71,524

Barriers

- Barriers addressed
  - **Biochemical Upgrading**
  - Bt-K: Product Acceptability and Performance

Partners

- **University Partners**
  - Berl R. Oakley , University of Kansas
  - Clay C. Wang University of Southern California
  - Blaine Pfeifer, University of Buffalo
- **Cost Share only Partners**
  - Novozymes of North America
  - Denmark Technical University
  - Bend Research

1 - Project Overview

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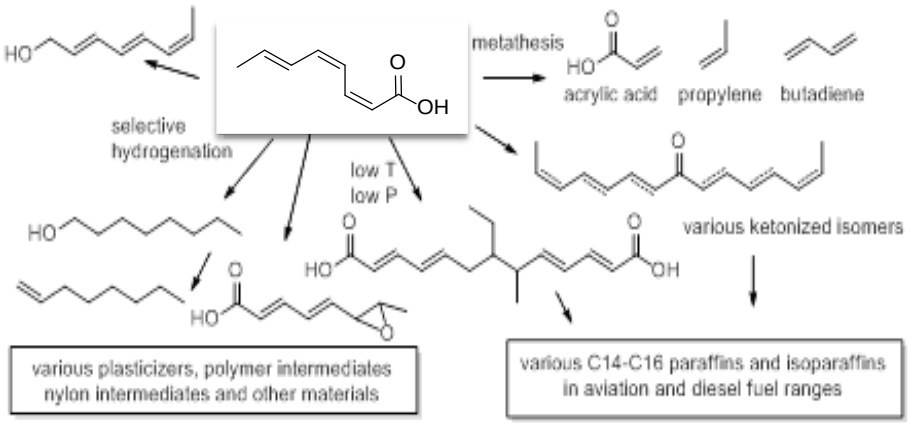
HISTORY

- Project participants have history of collaborative work
- Researchers at University of Kansas and University of Southern California have NIH funded project for novel compound discovery in fungi
- PNNL has Fungal Biotechnology Team grouped together with Process engineers and catalysis group
- SUNY-Buffalo group has experience with metabolic modeling for compound production

OBJECTIVES

- Develop a strain that can produce the target compound on pretreated Corn Stover (PCS)
- Utilize 30 liter bioreactors to analyze production and provide information to metabolic model
- Genetically manipulate organism to improve production
- Hit a target titer of 500 mg/liter on PCS within 2 years
- Discover additional novel compounds with desirable traits for downstream processing

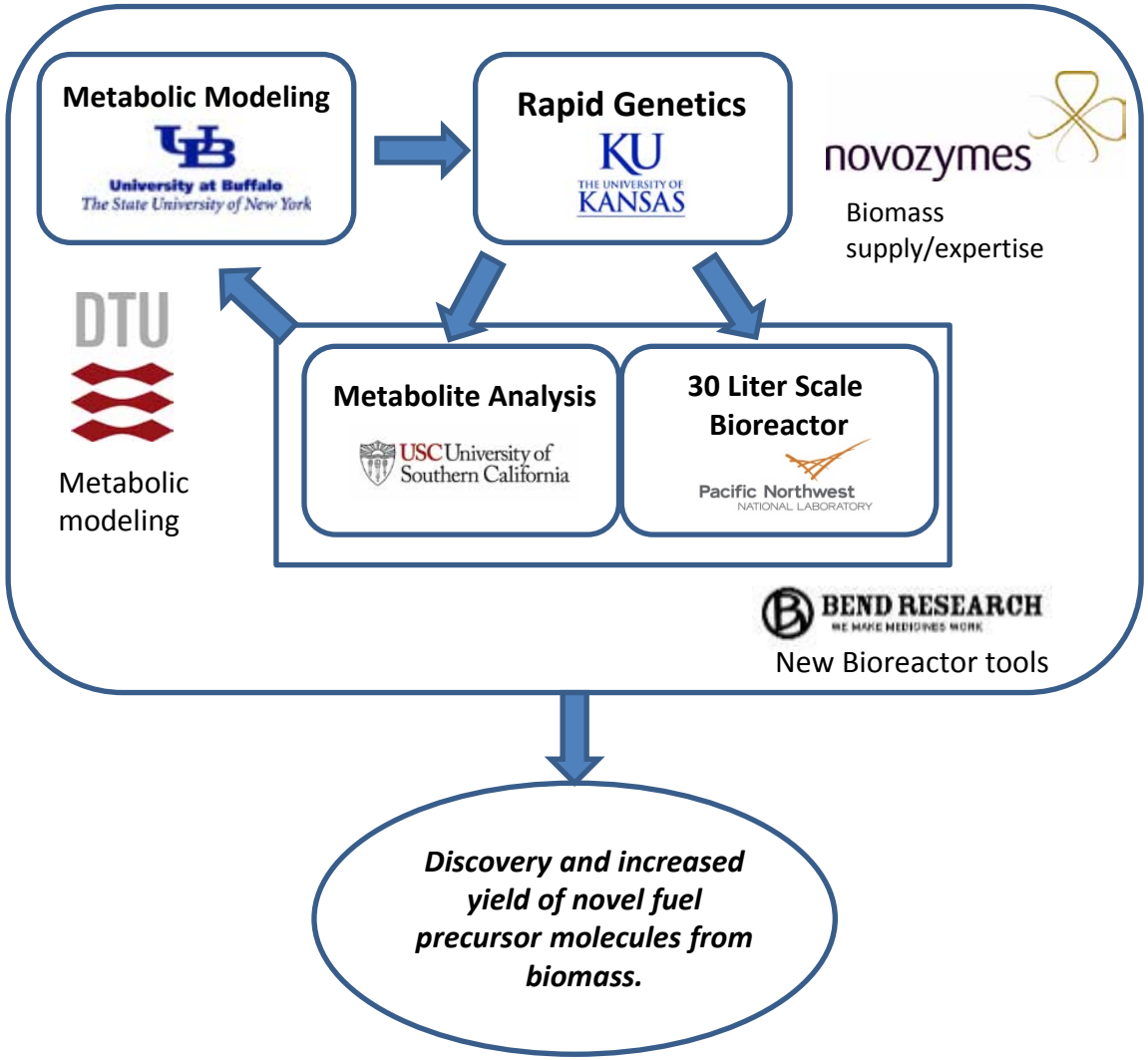


Predicted downstream treatment of precursor molecule

CONTEXT

- Bring together novel compound, process and analysis capabilities
- Combine rapid genetic manipulation with data analysis and metabolic modeling to bring Synthetic Biology to filamentous fungi
- Technical Financial plan developed to help guide progress/goals
- Downstream (post bioreactor) processing to real world products

2 – Approach (Technical)



- Project divided into four main tasks
  - Task A. Expand and Implement the Metabolic Model (SUNY)
  - Task B. Production on Hydrolysate (PNNL)
  - Task C. Iterative Genetic Improvements Made through Modeling (KU)
  - Task D. Metabolite analysis and discovery of more target molecules (USC)

2 – Approach (Management)

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- *Critical success factors to technical and commercial viability*
  - Develop a scalable process that includes a robust organism
  - Increase yield and lowering cost of production
    - Eliminate costly components
    - Overcome glucose inhibition
    - Reduce production time
- *Potential Challenges*
  - *Filamentous Fungi can be problematic in Bioreactors*
  - *Must develop method for high level induction in Corn stover*
  - *Metabolic model may provide large numbers of potential target genes*
  - *Final product stability in corn stover*
- *Management Approach*
  - *Milestones for each task*
  - *Final milestone for titer*
  - *Skype with partners often*
  - *Phone conference with Program Officer monthly*
  - *Cost share partners came to PNNL for visit in FY14*

3 – Technical Accomplishments/ Progress/Results

- *Key technical accomplishments*
  - *From 150-300 mg/l on lactose to 100-400 mg/L on pretreated corn stover*
  - *Developing a useable Genome Scale metabolic model that can be used for predictive analysis in both A. niger and A. nidulans*
  - *Determined key challenge in production (organism consumption)*
  - *Discovered other novel products of interest (one that is not consumed)*

(View the PowerPoint "Notes" page for additional information)

Manipulating Filamentous fungi

- Strains of *A. niger* and *A. nidulans* that have high level of homologous recombination used
  - Simplifies genetic targeting
  - Reduces time in screening
  - Gene deletion and promoter replacement is straightforward

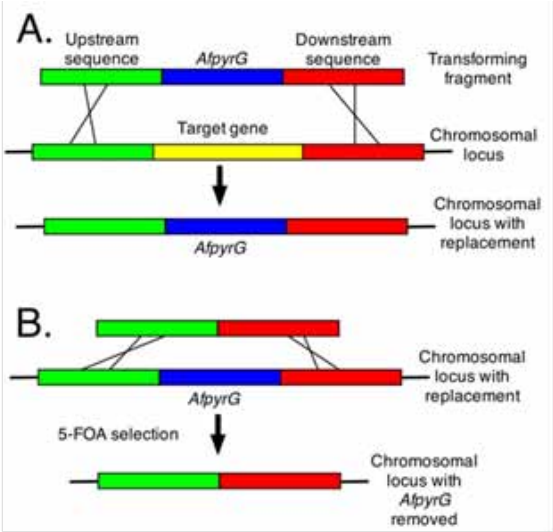


Figure 7. Gene replacement and marker recycling.

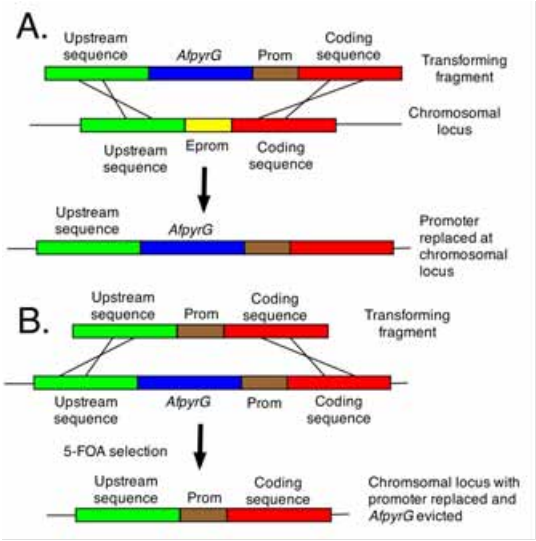
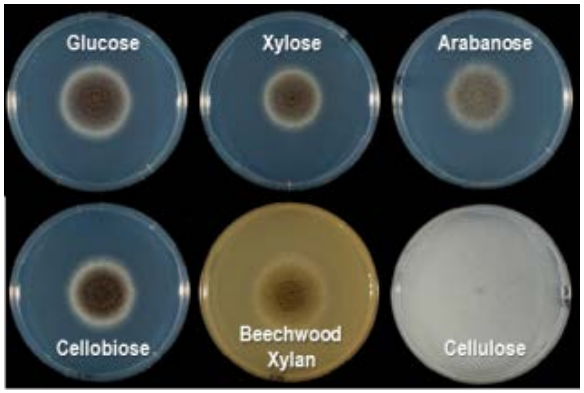


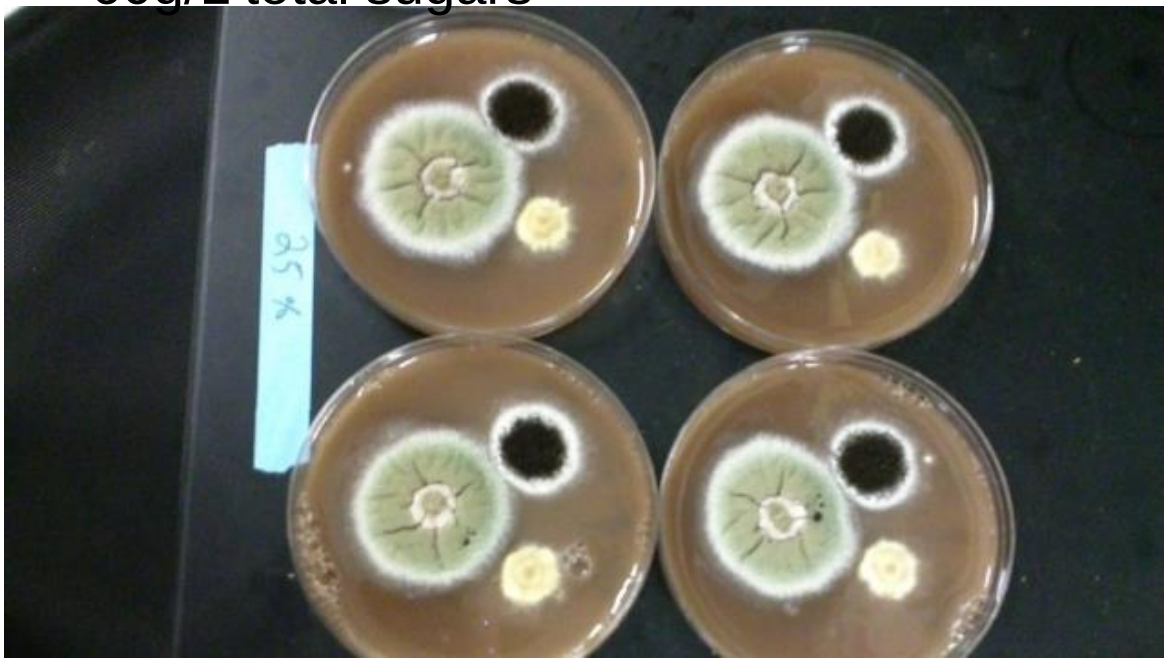
Figure 8. Promoter replacement and *AfpyrG* eviction.

Filamentous fungi grow well on plant material



Growth of ***A. niger*** 1015 on various sugars similar to what is present in lignocellulosic hydrolysate (www.fung-growth.org).

Aspergilli growing on Pretreated Corn Stover 60g/L total sugars



Compound Discovery: Lactose, 30ml

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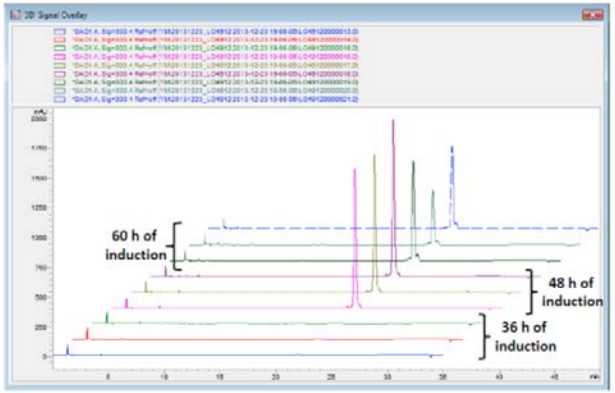
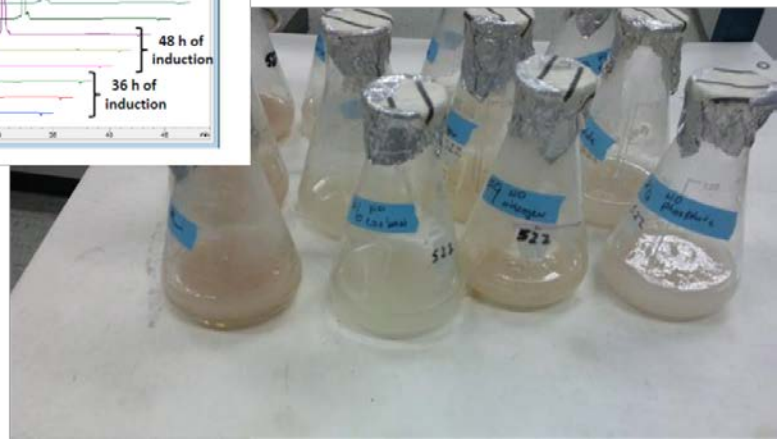
- The original compound discovery was performed in 30 ml shake flask studies where lactose was the sole carbon source
  - Lactose used because strongest promoters are glucose repressed
  - No larger scale prior to this funding
  - Yields ranged from (extrapolated) 1500-300 mg/liter from 15g/ L lactose
  - What is feasible yield??
  - ~ 5.2 g from 15 g glucose rough approximation





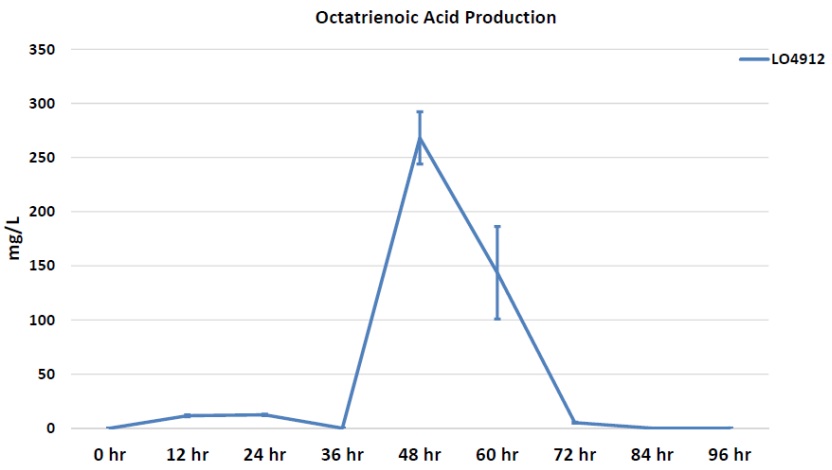
Per Ken Bruno										Per Jon Magnuson					
4 Malonyl-CoA + 3 NADPH + H2O --> 4 CO2 + 4CoA + 3 $NADP^+$ + 1 octatrienoic acid										Roughly	2	units glucose to get 4 units of malonyl-CoA			
											1/6	units of glucose to make 3 NADPH units			
and:	lactose + H2O -->		1 glucose + 1 galactose							Total	2.17	total units of glucose per unit of acid			
			1 glucose	--> 2 malonyl-CoA						or	1.08	moles lactose per mole acid			
net:	1 lactose + $2H_2O$ + 3 NADPH --> $4CO_2$ + 4 CoA + 3 $NADP^+$ + 1 octatrienoic acid														

MW glucose 180.1559
MW OTA 138.0681

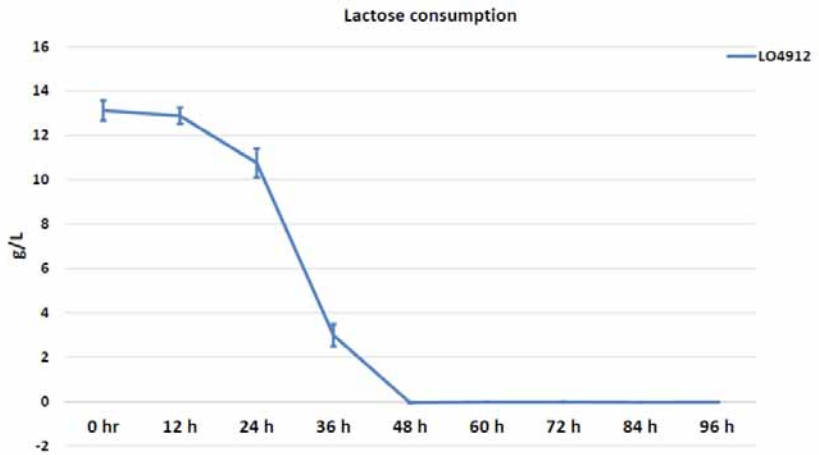
Production ranges determined during initial validation

-30 ml shake flasks with Lactose as carbon source
-Proposal titer hit but product is consumed by organism (experimentally verified)
-Huge variance between shake flasks

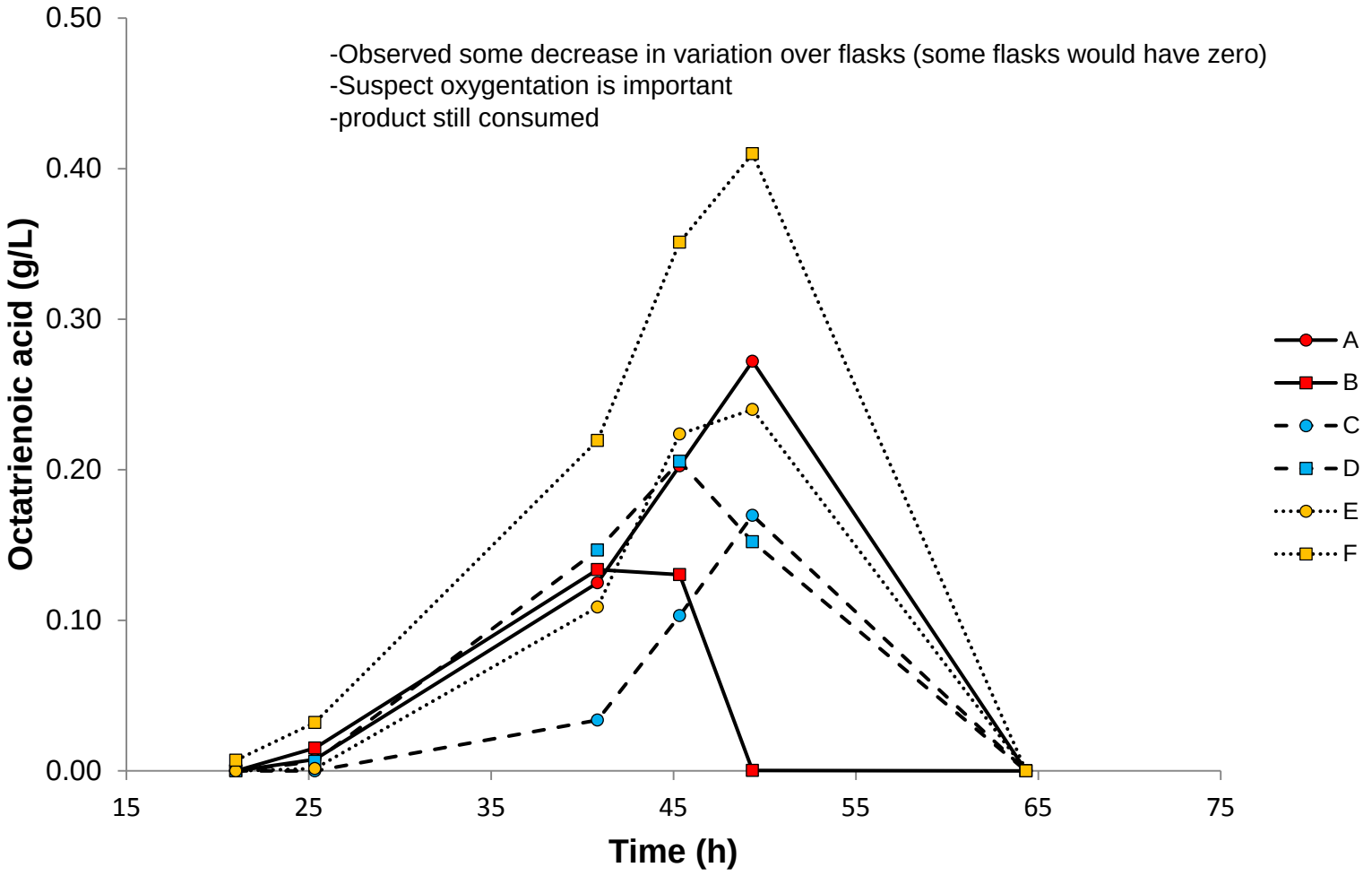
Kinetic of (2*E*, 4*E*, 6*Z*)-octatrienoic acid production



Kinetic of lactose consumption from LO4912

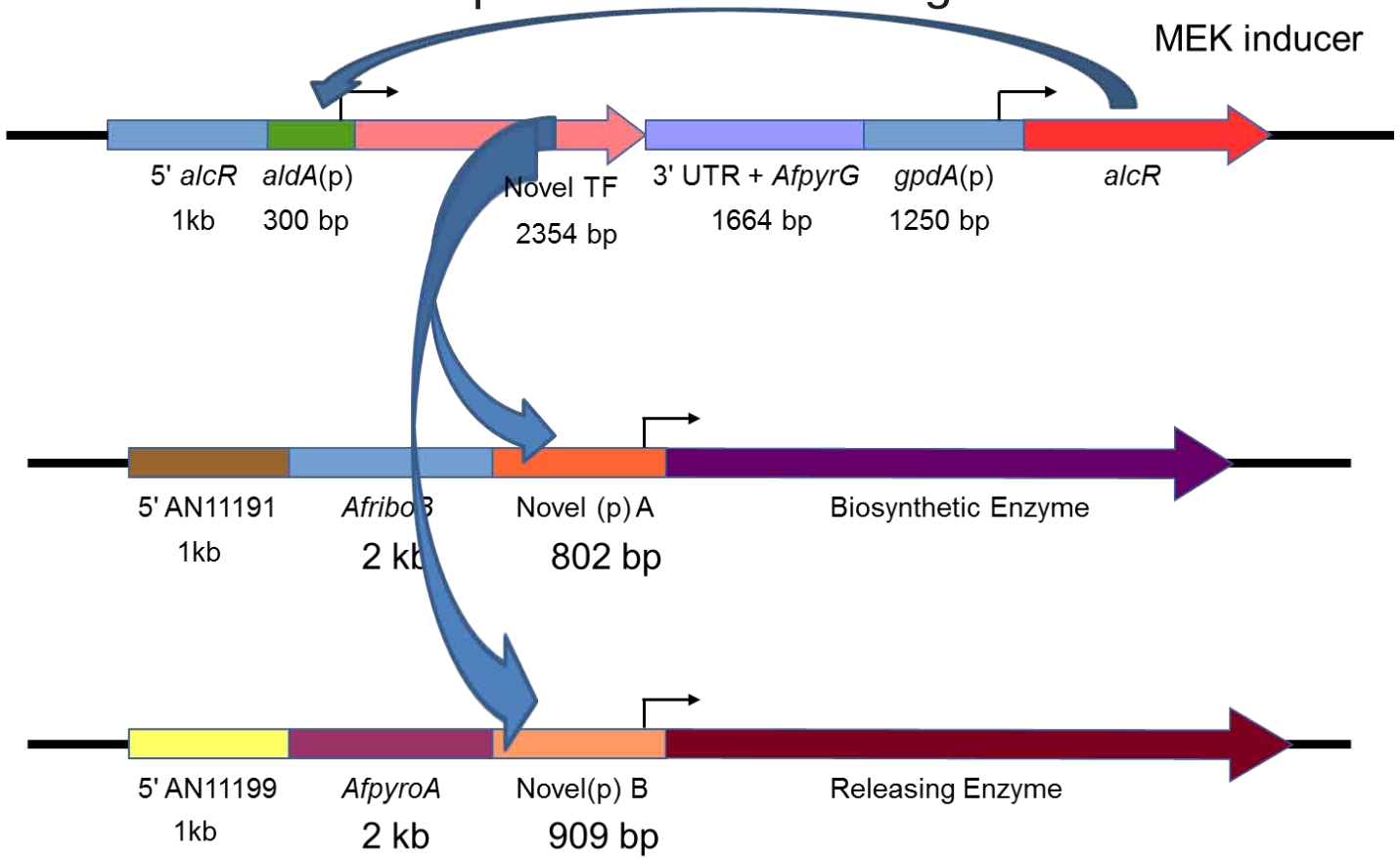


Octattrinoic acid production at 400ml scale in bioreactors



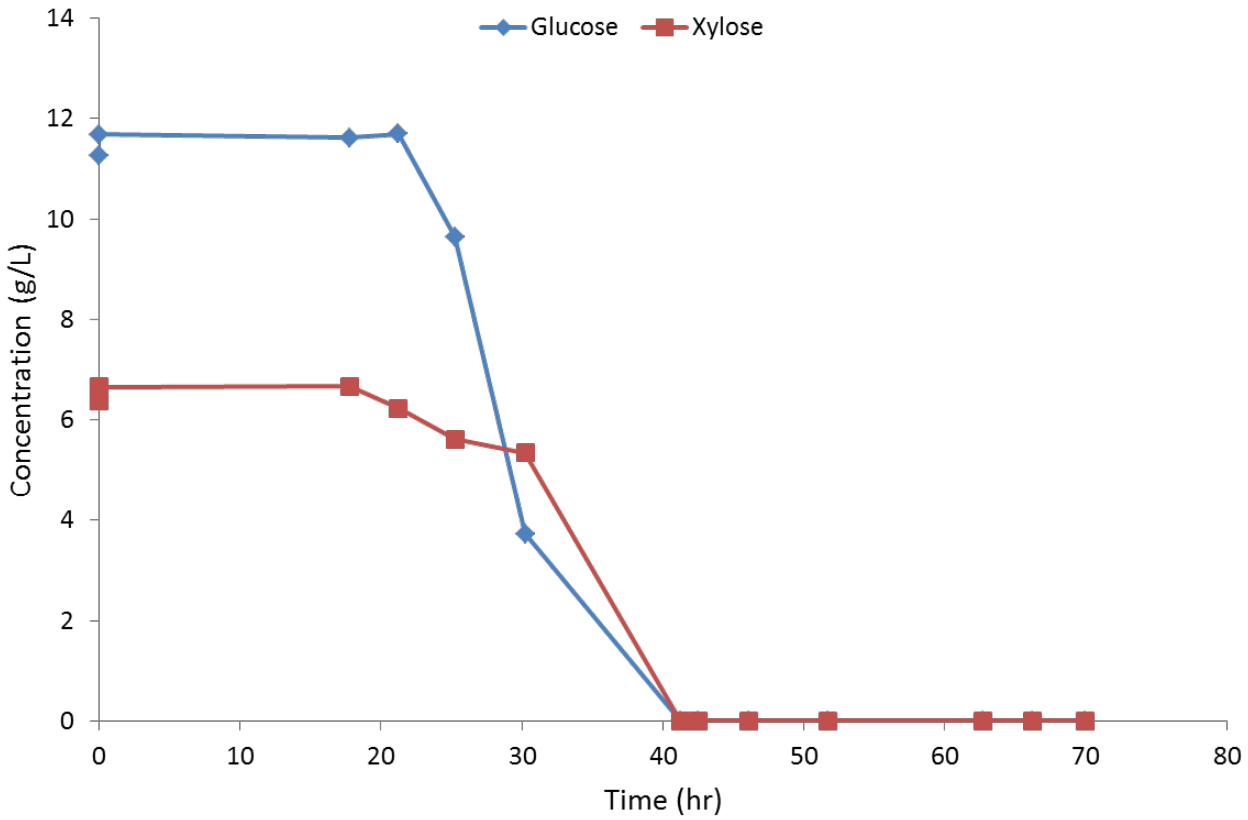
Engineering a promoter system to overcome glucose repression

- The *alcA* promoter system is strongly inducible yet repressed in presence of glucose
- A new combination of promoters was designed that overcomes this

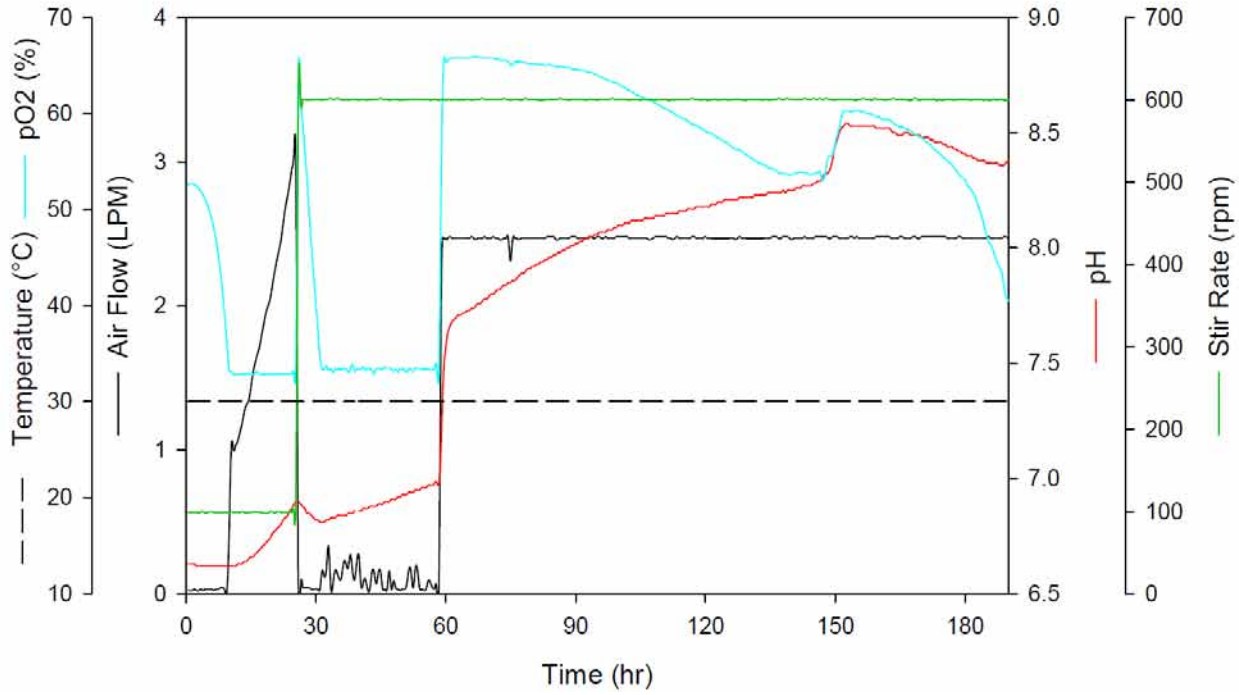


Production of compound, 30 L Bioreactor

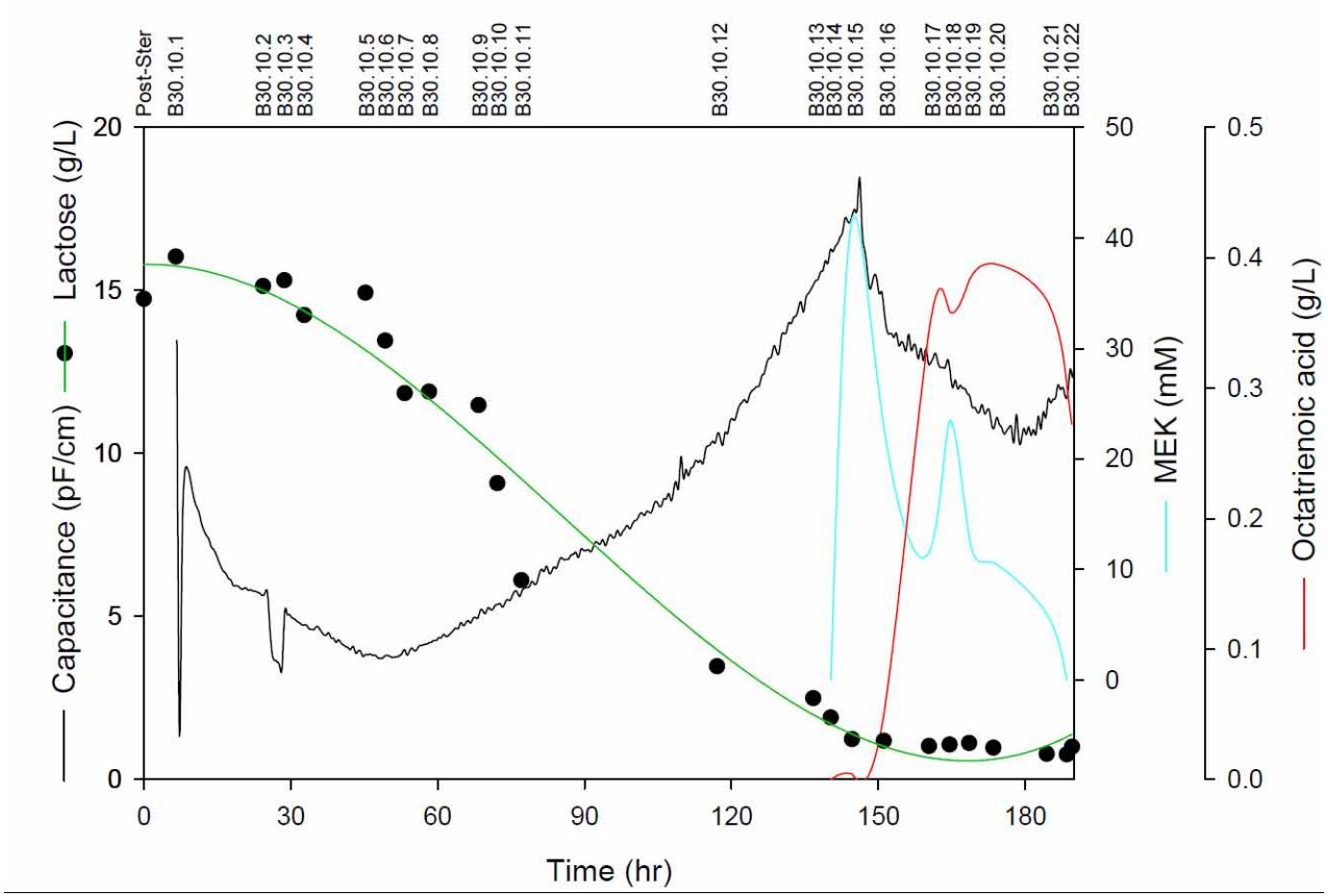
- Glucose repression diminished
- Strain grows rapidly on 2% total sugars from PCS
- Still losing compound to consumption



The 30 L bioreactor allows greater data collection for optimization



Dielectric spectroscopy probe correlates with growth, responds to induction



A computational metabolic model for *Aspergillus*

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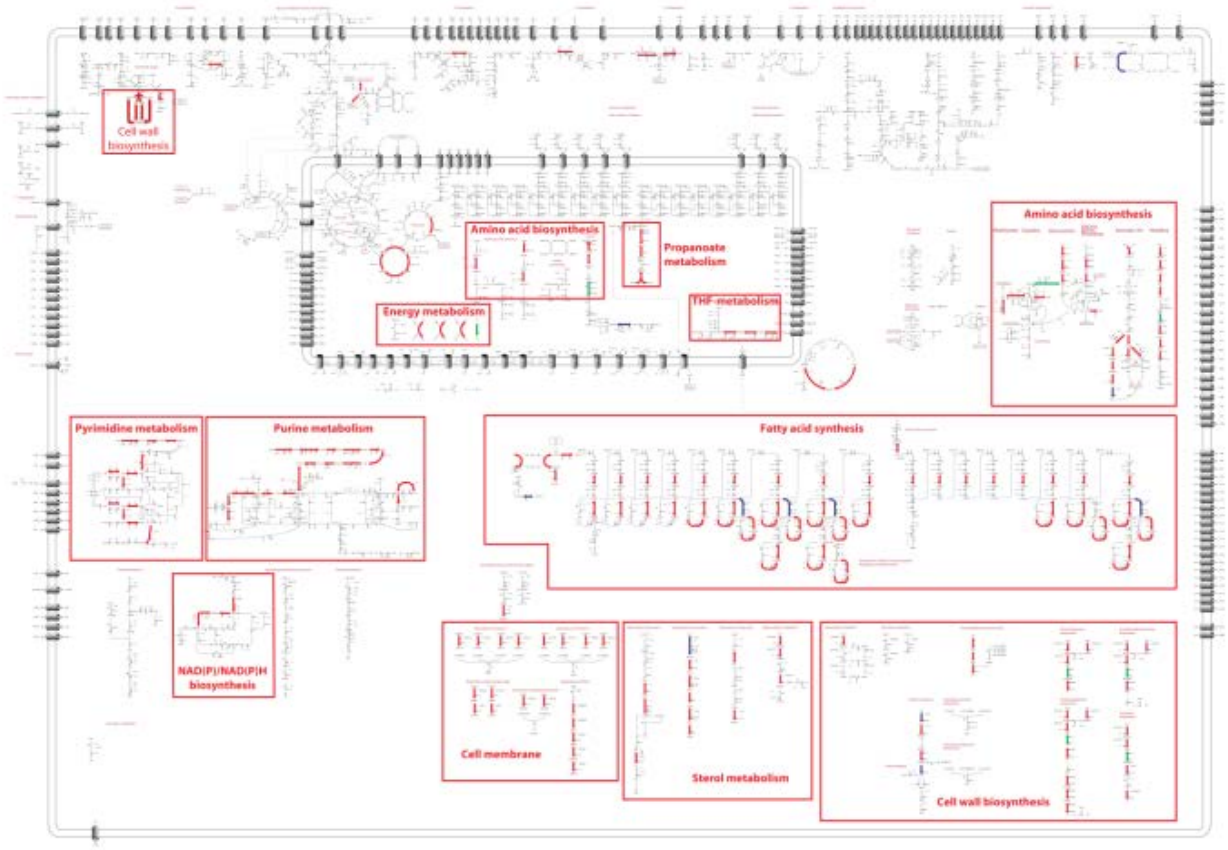

Based on the…
- Bibliome
- Genome
- Metabolome

Andersen, M. R.; Nielsen, M. L.; Nielsen, J. *Mol Syst Biol* **2008**, *4*, 178.

Metabolic model

- Computational modeling allows assessment of local and global metabolic pathways in identifying experimental targets to improve final compound formation
- Targets include both gene deletion and over-expression to improve metabolic flux to metabolites of interest
- Implementation of identified targets is possible through the experimental expertise of the remaining consortia team members
- Metabolic model generated and improved here will promote utilization of filamentous fungi in movement toward more advanced engineering and Synthetic biology

3 – Technical Accomplishments/ Progress/Results (cont'd)

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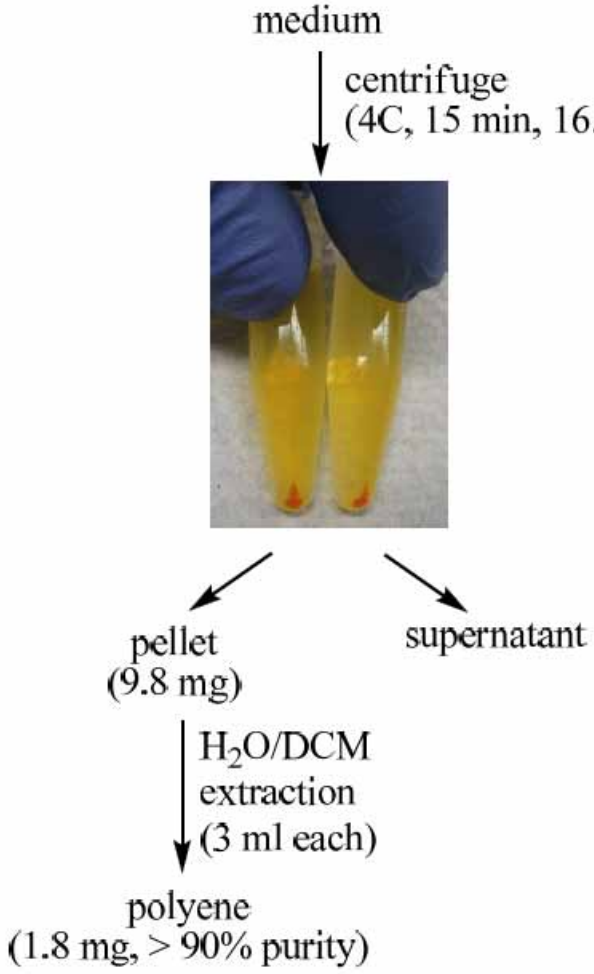
Production cost improvements as calculated On TechFin plan

- Start $27/dry lb
- Start Net diesel range fuel: $247/ gallon
- Current with production @ 400 mg/L on PCS
  - $9/dry lb
  - Net diesel range fuel $79/ gallon

-Pathways to conversion being explored in PNNL LDRD

-Newer compounds now available that will provide more conversion opportunities

-New compound that is not consumed by organism accumulates to point of precipitation



4 – Relevance

- *BETO Multi-Year Program Plan*
  - *This project will deliver novel pathways and organisms for the production of fuel and chemical precursors from biomass*
  - *This organism is proven to be useful at concentrations of pretreated corn stover that are inhibitory to other organisms*
- *Other applications*
  - *The product described is currently being studied at PNNL with internal funding for catalytic conversion to value added products*
  - *Novel compounds have been discovered that can be used to make other classes of compounds (longer carbon)*
- *Plans are underway to patent the process developed in order to be brought to a commercial partner*
  - *Key component will be increasing yield*

5 – Future Work

- *Thus project will end in FY15*
- *The metabolic model will be made available to other research groups*
- *We plan to continue to provide novel compounds to the catalytivc group at PNNL for experimentation*
- *Key Milestone will be demonstrating 500mg/L from pretreated corn stover in a 30 L Bioreactor*

(View the PowerPoint "Notes" page for additional information)

Summary

- **Key Points** :
  1. **Overview** –bringing Synthetic Biology approach to fungi with industry relevant objectives
  2. **Approach** – A team of capable researchers with expertise in fungi
  3. **Technical Accomplishments/Progress/Results** Increased titer AND on cheaper substrate
  4. **Relevance-**Novel pathways for lignocellulosic utilization
  5. **Future work** – Higher production, more compounds and new downstream conversion

Additional Slides

(Not a template slide – for information purposes only)

- *The following slides are to be included in your submission for Peer Evaluation purposes, but will **not** be part of your oral presentation –*
- *You may refer to them during the Q&A period if they are helpful to you in explaining certain points.*

Responses to Previous Reviewers' Comments

- If your project is an on-going project that was reviewed previously, address 1-3 significant questions/criticisms from the previous reviewers' comments (refer to the 2013 Peer Review Report, see notes section below)
- Also provide highlights from any Go/No-Go Reviews

Note: This slide is for the use of the Peer Reviewers only – it is not to be presented as part of your oral presentation. These Additional Slides will be included in the copy of your presentation that will be made available to the Reviewers.

Publications, Patents, Presentations, Awards, and Commercialization

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- List any publications, patents, awards, and presentations that have resulted from work on this project
- Use at least 12 point font
- Describe the status of any technology transfer or commercialization efforts

Note: This slide is for the use of the Peer Reviewers only – it is not to be presented as part of your oral presentation. These Additional Slides will be included in the copy of your presentation that will be made available to the Reviewers.

Cost Share

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	Total Costs FY 10 –FY 12	FY 13 Costs	FY 14 Costs	Total Planned Funding (FY 15-Project End Date
DOE Funded	0.00	360,366	1,452,661	589,584
Project Cost Share (Comp.)* KU	0.00	0.00	20,117	19,883
USC	0.00	49,567	0.00	50,433
SUNY	0.00	5,360	14,245	10,895
Novozymes	0	0	22,696	37,304
Bend	0	0	0	20,000
DTU	0	0	1,580	14,220

3. Gene overexpressions with MoMA

PF	Reaction ID	Reaction names	Reacrion formula
219.3166	r1694	Triosephosphate isomerase tpiA	T3P2[c] <=> T3P1[c]
104.8728	r901	Acetyl-CoA carboxylase ACC / biotin carboxylase	ACCOA[c] + CBCCP[c] <=> BCCP[c] + MALCOA[c]
104.8728	r899	Biotin carboxylase	H2O[c] + CO2[c] + ATP[c] + BCCP[c] <=> PI[c] + ADP[c] + CBCCP[c]
48.5131	r2004		MAL[c] + CITm[m] -> MALm[m] + CIT[c]
48.3564	r998	M ATP c:citrate oxaloacetate-lyase pro-3S-CH2COO- ->acetyl-CoA M ATP c-dephosphorylating	CIT[c] + COA[c] + ATP[c] -> OA[c] + PI[c] + ACCOA[c] + ADP[c]
32.1773	r1684	Glyceraldehyde 3-phosphate dehydrogenase gpdA	PI[c] + NAD[c] + T3P1[c] <=> NADH[c] + 13PDG[c]
32.1773	r1668	Phosphoglycerate kinase	ADP[c] + 13PDG[c] <=> ATP[c] + 3PG[c]
35.5032	r1653	Phosphoglycerate mutase	3PG[c] <=> 2PG[c]
35.5032	r1625	Phosphopyruvate hydratase enolase	2PG[c] <=> H2O[c] + PEP[c]
24.2127	r2036		PYR[c] -> PYRm[m]
23.6632	r1608	Pyruvate kinase pkiA	ADP[c] + PEP[c] -> PYR[c] + ATP[c]

4. Gene knockouts with FBA

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NO.	Gene	Enyme	Reaction	fPH	Note
1	'An08g02260'	Phosphoglycerate kinase	ADP[c] + 13PDG[c] <=> ATP[c] + 3PG[c]	9.8184	
2	'An02g05470'	M NADH c-ubiquinone oxidoreductase nad5, nuo51, nd4L /Proton pumping Mitochondrial M NADH c dehydrogenase that catalyzes the oxidation of cytosolic M NADH c /Respiratory-chain M NADH c dehydrogenase nad5, nuo51, nd4L	NADH[c] + Qm[m] -> NAD[c] + QH2m[m] /NADHm[m] + Qm[m] -> NADm[m] + QH2m[m] /NADH[c] + 4 HX_POm[m] + Qm[m] -> NAD[c] + 4 HX_PO[c] + QH2m[m] / NADHm[m] + 4 HX_POm[m] + Qm[m] -> NADm[m] + 4 HX_PO[c] + QH2m[m]	6.6	From gene 2 to 21, those genes are all involved in associated four reactions. And there relationships between each other is "and", means the reaction will not happen if one of there get deleted.
3	'An02g05880'				
4	'An02g09730'				
5-21 ◦ ◦ ◦	'An02g11200'				
22	'An02g01720'	Cytochrome c oxidase subunit I cox5	0.5 O2m[m] + 2 FEROm[m] + 4 HX_POm[m] -> H2Om[m] + 2 FERIm[m] + 4 HX_PO[c]	6.2554	Same as above, gene 22 to 29 work together to make this reaction feasible. And relationship also is "and"
23	'An02g09930'				
24	'An04g01560'				
25	'An07g07390'				
26	'An08g01550'				
27	'An09g03990'				
28	'An11g10200'				
29	'An14g04170'				
30	'An02g02930'	Ribose-5-phosphate isomerase	R5P[c] <=> RL5P[c]	6.0951	
31	'An16g09190'	Acetyl-CoA C-acetyltransferase, acetoacetyl-CoA thiolase	2 ACCOA[c] <=> COA[c] + AACCOA[c]	5.9645	
32	'An01g12210'	Ubiquinol-cytochrome c reductase	4 HX_POm[m] + 2 FERIm[m] + QH2m[m] -> 2 FEROm[m] + 4 HX_PO[c] + Qm[m]	5.5773	Work collectively
33	'An01g06180'				
34	'An04g05220'				
35	'An08g06550'				
36	'An14g04080'				

Gene knockouts with FBA

37	'An18g01670'	6-phosphofructokinase Phosphofructokinase I, pfkA	ATP[c] + F6P[c] -> ADP[c] + FDP[c]	2.6472	
38	'An16g05420'	Glucose-6-phosphate isomerase	G6P[c] <=> bDG6P[c] /bDG6P[c] <=> F6P[c] /G6P[c] <=> F6P[c]	2.2984	
39	'An04g04750'	alpha-ketoglutarate dehydrogenase	AKGm[m] + TDPE1m[m] -> CO2m[m] + AKGE1m[m]	2.0808	Work collectively
40	'An06g00390'				
41	'An11g11280'	Dihydrolipoamide M S c-succinyl transferase	AKGE1m[m] + LPSE2m[m] -> TDPE1m[m] + AKGE2m[m]	2.0808	
42	'An08g02970'	Succinate CoA ligase M GDP c forming	SUCCOAm[m] + GDPm[m] + PIm[m] <=> SUCCm[m] + COAm[m] + GTPm[m]	2.0808	Work collectively
43	'An08g02980'				
44	An01g11650'	5-formyltetrahydrofolate deformylase	H2O[c] + FTHF[c] -> FOR[c] + THF[c]	1.9786	
45	'An10g00230'	Formaldehyde dehydrogenase	H2O[c] + NAD[c] + FALD[c] <=> FOR[c] + NADH[c]	1.6118	
46	'An02g07650'	Phosphoglucomutase	G6P[c] <=> G1P[c]/ R5P[c] <=> R1P[c]	1.3485	
47	'An15g01860'	Malate synthase	H2Om[m] + GLXm[m] + ACCOAm[m] -> MALm[m] + COAm[m]	1.2995	
48	'An14g03500'	Dihydroxyacetone synthase	FALD[c] + XUL5P[c] <=> GLYN[c] + T3P1[c]	1.2557	
49	'An02g12430'	Isocitrate dehydrogenase icdA M NADP c+	ICITm[m] + NADPm[m] -> AKGm[m] + CO2m[m] + NADPHm[m]	1.2235	
50	'An15g07390'	Glycerol 3-phosphate dehydrogenase M NAD c+ dependent	NADH[c] + T3P2[c] -> NAD[c] + GL3P[c]	1.0449	
51	'An16g04160'	Galactokinase	GLAC[c] + ATP[c] -> ADP[c] + GAL1P[c]	1.0254	
52	'An02g03590'	M UDP c-glucose-hexose-1-phosphate uridylyltransferase M UTP c-hexose-1-phosphate uridylyltransferase M UDP c-galactose pyrophosphorylase	UDPG[c] + GAL1P[c] <=> UDPGAL[c] + G1P[c] UTP[c] + GAL1P[c] <=> UDPGAL[c] + PPI[c]	1.0254	These two reactions were processed by the same gene.
53	'An05g00160'	Cystathionine beta-synthase	SER[c] + HCYS[c] -> H2O[c] + LLCT[c]	1.0158	
54	'An12g01110'	Cystathionine gamma-synthase	CYS[c] + OAHSER[c] <=> AC[c] + LLCT[c]	1.0157	
55	'An04g05620'	Acetyl-CoA synthase acuA	AC[c] + COA[c] + ATP[c] -> ACCOA[c] + AMP[c] + PPI[c]	1.0024	
56	'An09g05840'	dTMP kinase/thymidylate kinase /Uridylate kinase	ATP[c] + DTMP[c] <=> ADP[c] + DTDP[c]	1.0013	

5. Gene deletions with EMA-GA

No.	Gene	Enyme	Reaction	Frequency
1	An01g13930	fumarate reductase	FUM + QH2 = SUCC + Q	0.991
2	An02g05410			
3	An01g09780	lactate dehydrogenase	PYR + NADH = LAC + NAD	0.835
4	An02g00080	formate dehydrogenase complex	FOR = CO2 + H2_ext	0.810
5	An04g03400	acetaldehyde dehydrogenase	ACoA + NADH = ACA + NAD + CoASH	0.802
6	An01g15170			
7	An08g07290			
8	An08g10820			
9	An10g00850			
10	An15g05890			
11	An18g04130			
12	An12g09810	alcohol dehydrogenase ; ethanol dehydrogenase	ACA + NADH = ETOH + NAD	0.798
13	An17g01530			
14	An01g12170			
15	An01g14590			
16	An02g02060			
17	An02g02870			
18	An03g01350			
19	An11g04290			
20	An12g09950			
21	An13g03330			
22	An14g02160			
23	An14g07180			
24	An16g00010			
25	An16g00400			
26	An16g06240			
27	An02g06420	acetate kinase	ACP + ADP = ACE + ATP	0.710

Tech Fin plan

Pacific Northwest NATIONAL LABORATORY
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	Description	Benchmark Process A. nidulans on Lactose	Intermediate Target (~6-8 months) A. nidulans	Near Final Actual (20 months) A. nidulans
Feed stock type		lactose	Corn stover	Corn stover
Feed Rate (dry US ton/day)	Dry rate of pure sugar or dry rate of untreated corn stover	1388	2205	2205
Line 1: Annual Aqueous Octatrienic Acid Production (MM lbs dry acid basis)		17	2.5	21
Line 1: Annual Diesel/Jet Production (MM gallons)		1.9	0.27	2.30
Equipment Costs (2007$) as applicable	**Description**	**Installed Capital Cost (MM$)**	**Installed Capital Cost (MM$)**	**Installed Capital Cost (MM$)**
Pure sugar plant battery limits	Per Bohlmann 2007 [2]	87	not applicable	not applicable
Feedstock Handling	Per Humbird et al, 2011 [1]	included in line 9	0.0	0
Pretreatment	Per Humbird et al, 2011 [1]	included in line 9	29.9	29.9
Neutralization/Conditioning	Per Humbird et al, 2011 [1]	not applicable	3.0	3.0
Enzyme Production (here or in operating costs)	Per Humbird et al, 2011 [1]	not applicable	18.7	18.7
Saccharification & Fermentation	Per Humbird et al, 2011 [1]	included in line 9	46.6	200.9
Product & Solids Recovery	Per Humbird et al, 2011 [1]	included in line 9	22.3	54.9
Ketonization and hydrotreating	Estimated: acid to a diesel range fuel [3]	7	3.9	8.3
Wastewater Treatment	Per Humbird et al, 2011 [1]	24	49.3	288.9
Storage	Per Humbird et al, 2011 [1]		5.0	5.0
Utilities (include steam/electricity here or in operating costs)	Per Humbird et al, 2011 [1]		72.7	72.7
Line 2: Total Installed Capital		**$117**	**$251**	**$682**
Total Installed Capital per Annual Gallon or lb (line 2 divided by line 1)		**$62**	**$932**	**$297**
Operating Costs (2007$) as applicable	**Description**	**MM$/yr**	**MM$/yr**	**MM$/yr**
Feedstock - Corn Stover ($/dry ton)	$58.50	not applicable	45	45
Feedstock - pure sugar	Lactose: $0.342/lb (PEP 2007 [4]); Glucose: $0.254/lb (Humbrid et al 2011 [1])	333	not applicable	not applicable
Organism Production Nutrients	Per Humbird et al, 2011 [1]		0	0
Fermentation Nutrients	Per Humbird et al, 2011 [1]	124	17.6	103.0
Enzymes (Cellulase)	Produced on-site	0.0	12.4	12.4
Fermentation Organism (include licensing fees)	*Engineered A. niger (or nidulans)*	0.0	0.0	0.0
Other Raw Materials	Per Humbird et al, 2011 [1]	0.4	9.8	9.8
Waste Disposal	Per Humbird et al, 2011 [1]	0.0	2.0	2.0
Hydrogen	calculated	1.0	0.1	1.2
Steam	Per Humbird et al, 2011 [1]			
Electricity	Per Humbird et al, 2011 [1]	1.0	-6.2	-6.2
Labor and Maintenance	Per Humbird et al, 2011 [1]	7.5	8.4	14.2
Line 3: Total Operating Costs		466	89.4	181.7
Line 4: Co-product Credits		0	0.0	0.0
Line 5: Net Operating Costs (line 3 minus line 4)		**466**	**89**	**182**
Net aqueous octatrienic acid ($/dry lb) (line 5 divided by line 1)		**$27**	**$36**	**$9**
Net diesel range fuel ($/gallon)		**$247**	**$331**	**$79**