Metabolic Engineering

Eighth
Interagency Conference
on
Metabolic Engineering

Fred Heineken
Chair
Interagency Metabolic Engineering Working Group

February 11, 2008

Metabolic Engineering

Participating Agencies/Departments

- Department of Agriculture
- Department of Commerce
- Department of Defense
- Department of Energy
- Environmental Protection Agency
- National Aeronautics and Space Administration
- National Institutes of Health
- National Science Foundation

Metabolic Engineering

Metabolic Engineering Working Group

Goals

- Promote the Advancement of Metabolic Engineering
- Coordinate Federal Metabolic Engineering Research
- Identify and Address Gaps in Metabolic Engineering Research

Metabolic Engineering

Accomplishments (13 Years)

- Held Three Interagency Workshops on Metabolic Engineering
- Held Eight Interagency Conferences on Metabolic Engineering
- Issued Six Interagency Announcements of Opportunities in Metabolic Engineering
- Generated an Interagency Web Site (www.metabolicengineering.gov)
- Research Results have been Published in High Impact Journals

Metabolic Engineering

6 Interagency Announcements of Opportunities in Metabolic Engineering

Topic Areas of Interagency Interest

- Instrumentation, Tools, and Methods to facilitate the Study of Metabolic Pathways in Cells
- Quantitative and Conceptual Models
- Bioinformatics
- Engineering of Metabolic Pathways for a Desired Output

Metabolic Engineering

Responses to Announcements

Proposals	236
Awards	54
Award Total	$34M

Metabolic Engineering

Future Plans

- Issue New Interagency Announcements of Opportunities in Metabolic Engineering
- Sponsor Annual Interagency Conferences on Metabolic Engineering
- Maintain an Up-To-Date Interagency Web Site for Metabolic Engineering

Metabolic Engineering

Conference Theme

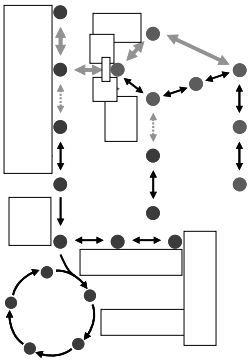
Metabolic Engineering for Biorefineries:
Alternative Fuels and Bioproducts

Foundational advances in RNA engineering applied to control of biosynthesis

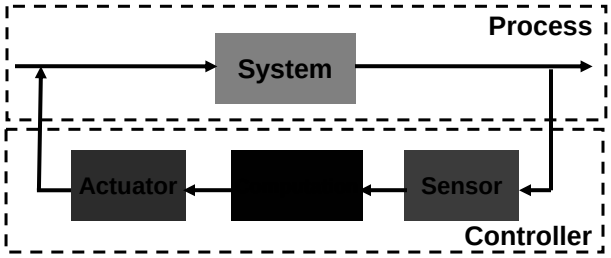
Christina D. Smolke
Division of Chemistry and Chemical Engineering
California Institute of Technology

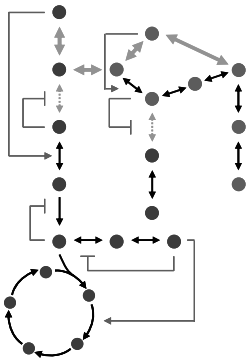
February 11, 2008
MEWG: Interagency Conference on Metabolic Engineering

The role of feedback control in biosynthesis



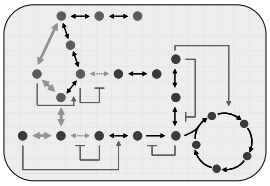
The role of feedback control in biosynthesis



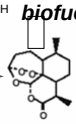


Applications of engineered biological systems

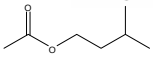
Metabolic engineering



Isobutanol
biofuels



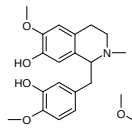
Artemisinin
drugs



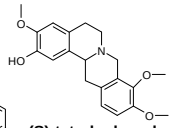
Isoamyl acetate
scents, flavors

Benzylisoquinoline alkaloid biosynthesis

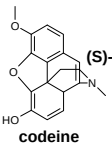
Papaver somniferum (opium poppy)



(S)-reticuline



(S)-tetrahydrocolumbamine

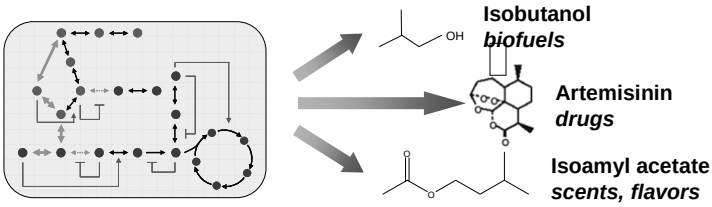


codeine

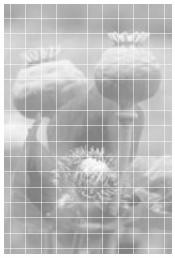
http://www.nature.com/news/2004/040816/images/yeast.jpg
http://www.westwindphotos.ca/images/101%20Opium%20Poppy.jpg

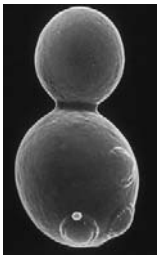
Applications of engineered biological systems

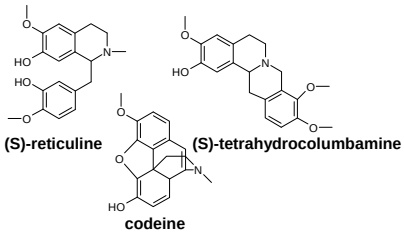
Metabolic engineering



Benzylisoquinoline alkaloid microbial synthesis

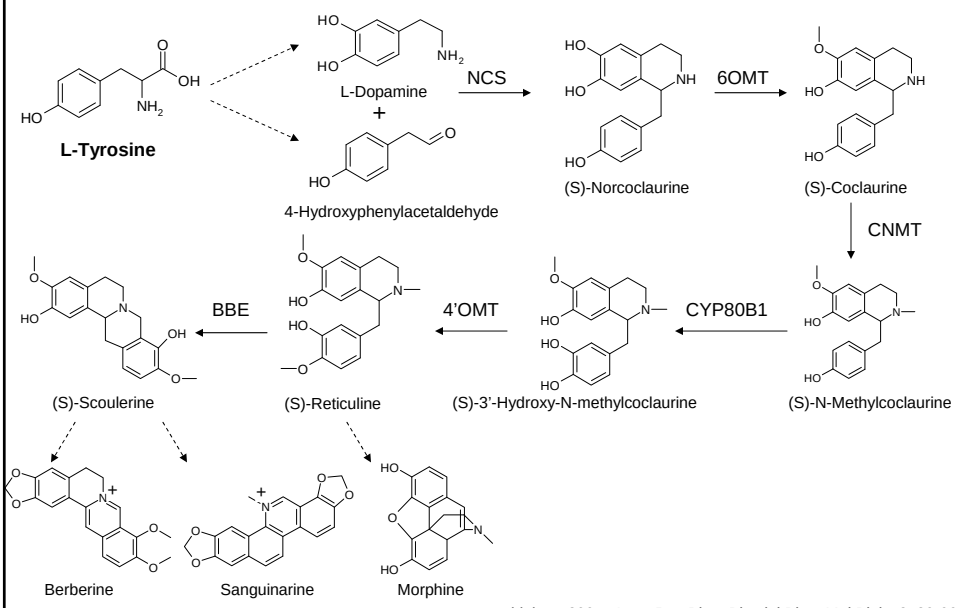






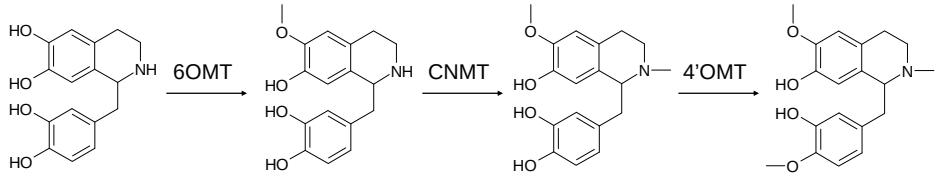
http://www.nature.com/news/2004/040816/images/yeast.jpg
http://www.westwindphotos.ca/images/101%20Opium%20Poppy.jpg

Native BIA pathway



Facchini PJ. 2001. *Annu Rev Plant Physiol Plant Mol Biol.* 52: 29-66

Late Synthetic BIA pathway

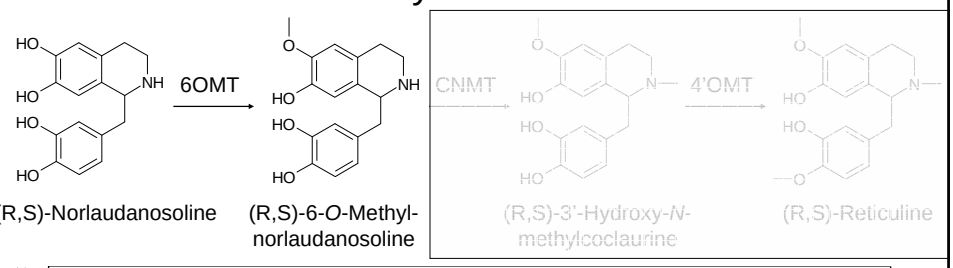


(R,S)-Norlaudanosoline (R,S)-6-*O*-Methyl-norlaudanosoline (R,S)-3'-Hydroxy-*N*-methylcoclaurine (R,S)-Reticuline

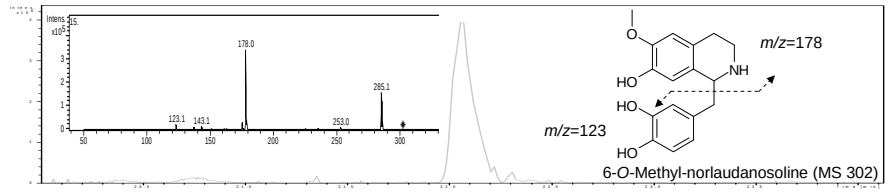
Hawkins KH, Smolke CD. 2007. *In preparation.*

Peter Facchini, U. Calgary
Ounaroon A, et al. 2003. *Plant J.* 36: 808-19

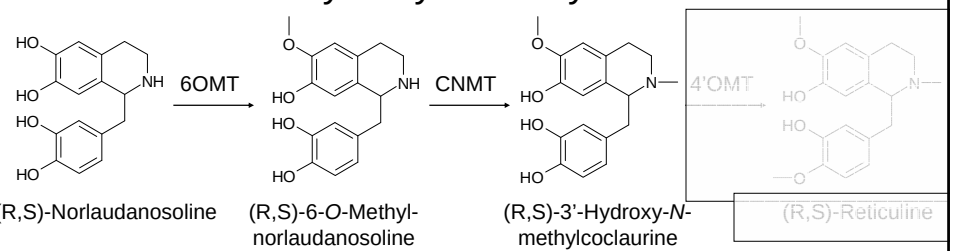
Production of 6-*O*-Methyl-norlaudanosoline

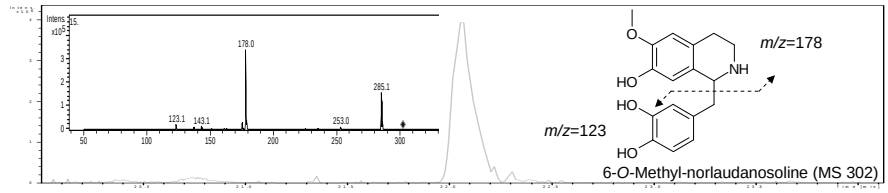


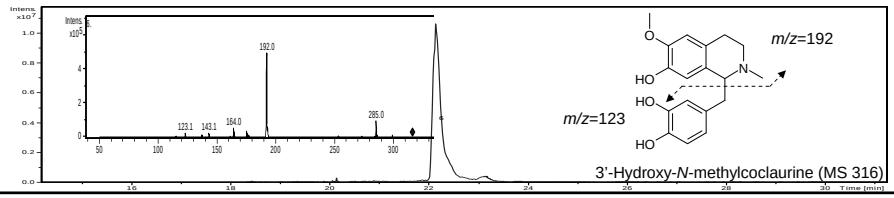
(R,S)-Norlaudanosoline (R,S)-6-*O*-Methyl-norlaudanosoline (R,S)-3'-Hydroxy-*N*-methylcoclaurine (R,S)-Reticuline



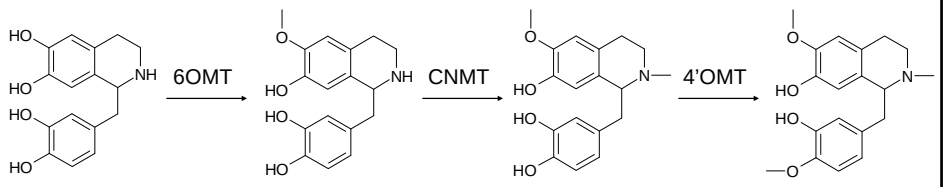
Production of 3'-Hydroxy-*N*-methylcoclaurine

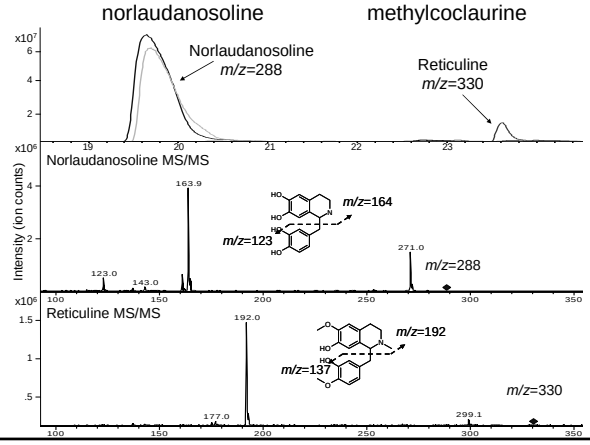




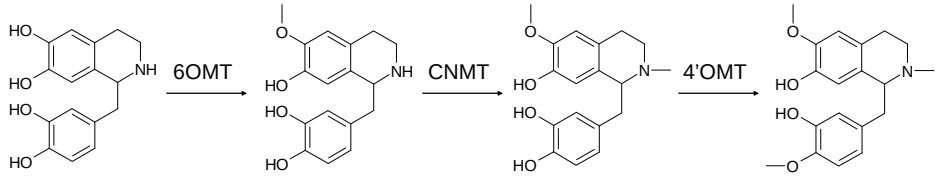


Production of (R,S)-Reticuline



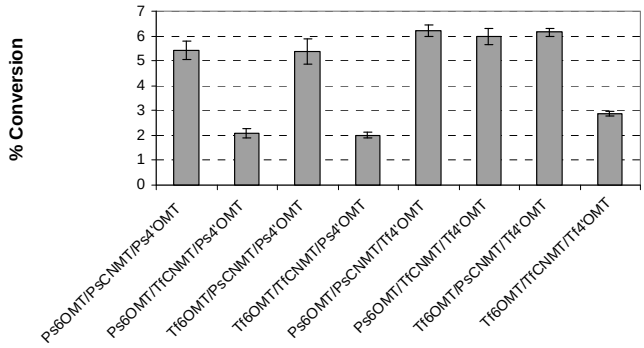


Optimization of (R,S)-Reticuline production

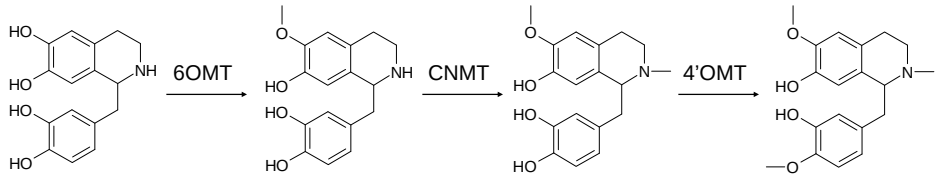


(R,S)-Norlaudanosoline (R,S)-6-*O*-Methyl-norlaudanosoline (R,S)-3'-Hydroxy-*N*-methylcoclaurine (R,S)-Reticuline

Enzyme variant dependence:

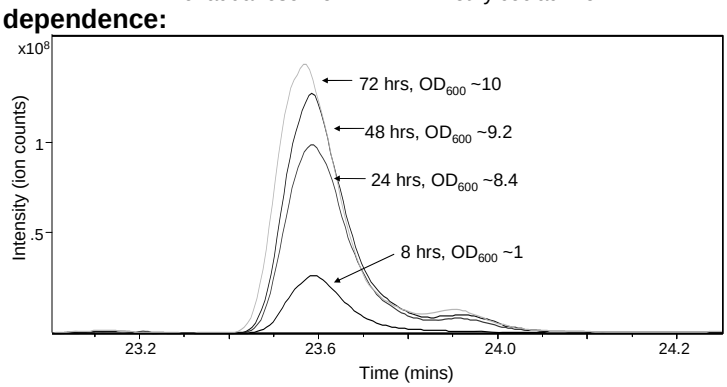


Optimization of (R,S)-Reticuline production



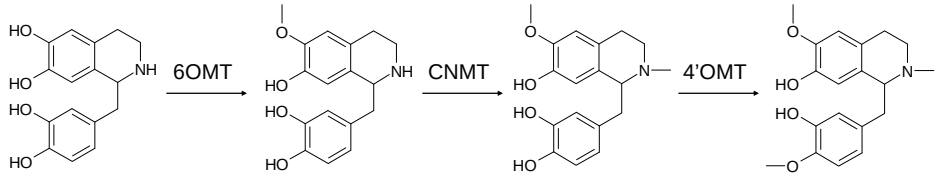
(R,S)-Norlaudanosoline (R,S)-6-*O*-Methyl-norlaudanosoline (R,S)-3'-Hydroxy-*N*-methylcoclaurine (R,S)-Reticuline

Time dependence:



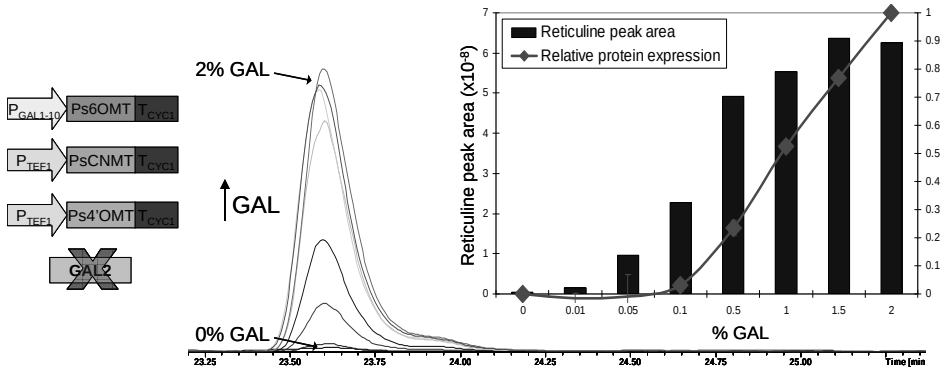
Strains continue to accumulate (R, S)-Reticuline during growth in stationary phase (OD_{600} ~ 10).

Optimization of (R,S)-Reticuline production

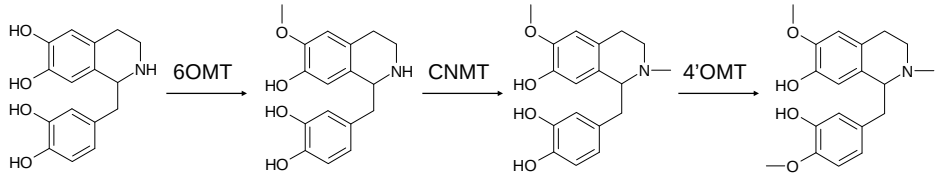


(R,S)-Norlaudanosoline (R,S)-6-*O*-Methyl-norlaudanosoline (R,S)-3'-Hydroxy-*N*-methylcoclaurine (R,S)-Reticuline

Enzyme level dependence:



Optimization of (R,S)-Reticuline production



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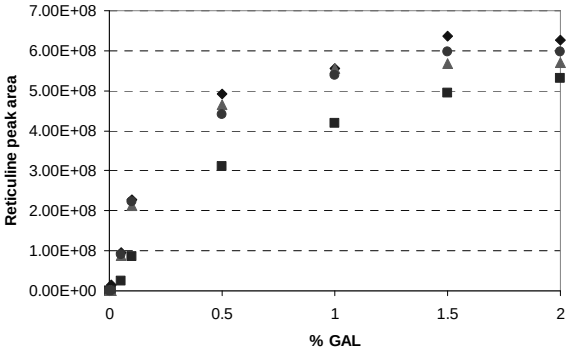
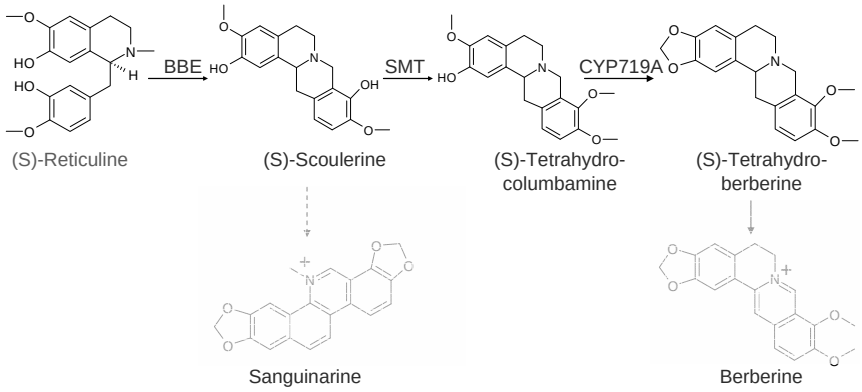


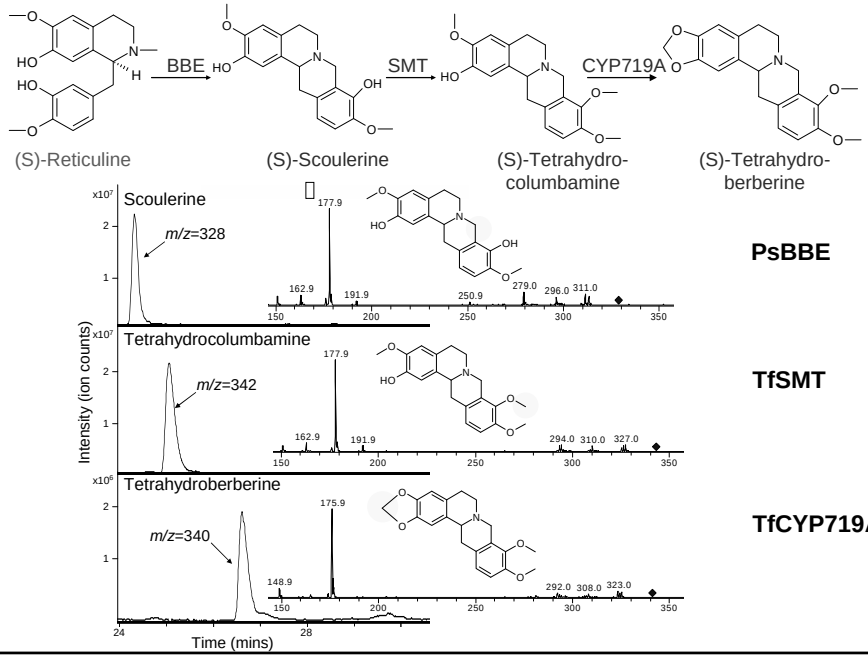
- P_{GAL}Ps6OMT
- P_{GAL}Ps4O'MT
- P_{GAL}PsCNMT
- P_{GAL}PsCNMT P_{TEF}Tf4'OMT

~15% full expression levels from P_{TET} sufficient for maximum conversion

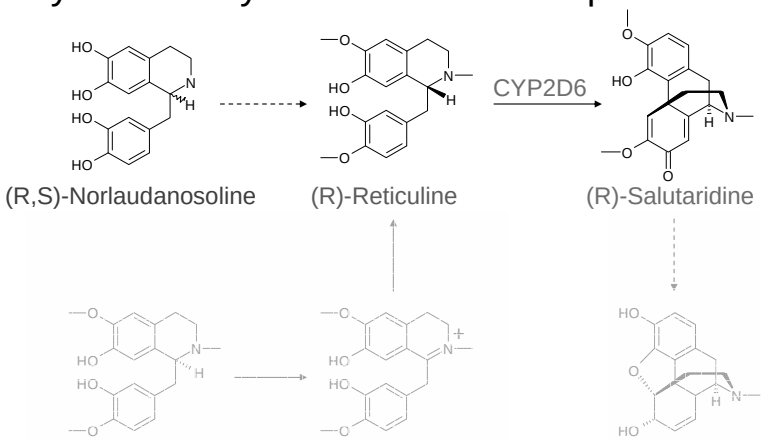
BIA synthesis beyond reticuline – berberine branch



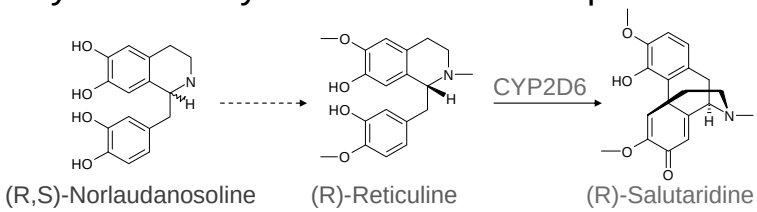
BIA synthesis beyond reticuline – berberine branch

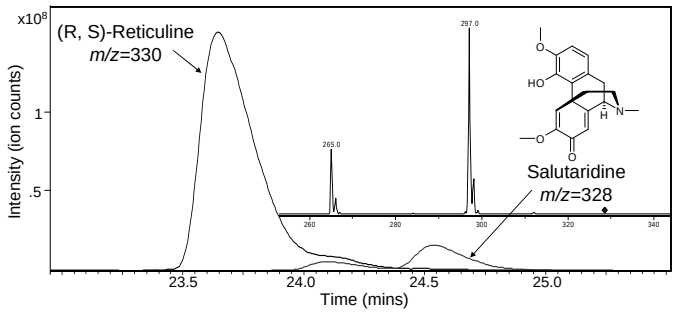


BIA synthesis beyond reticuline – morphine branch



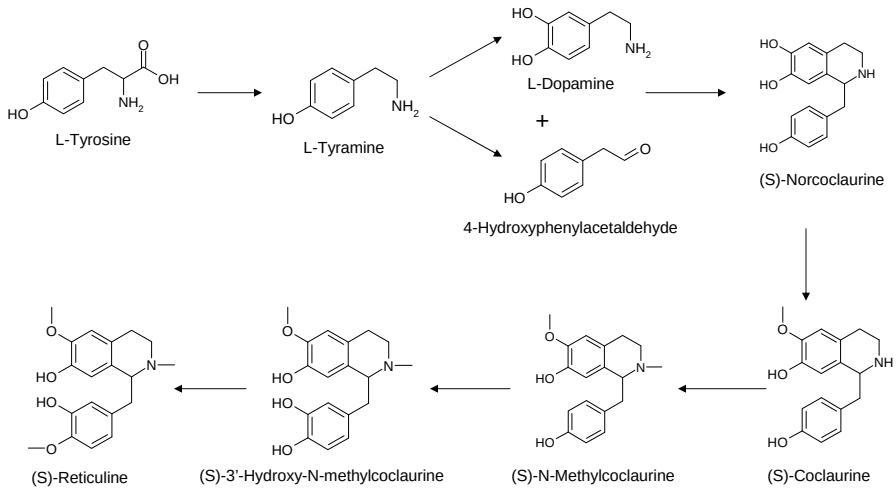
BIA synthesis beyond reticuline – morphine branch



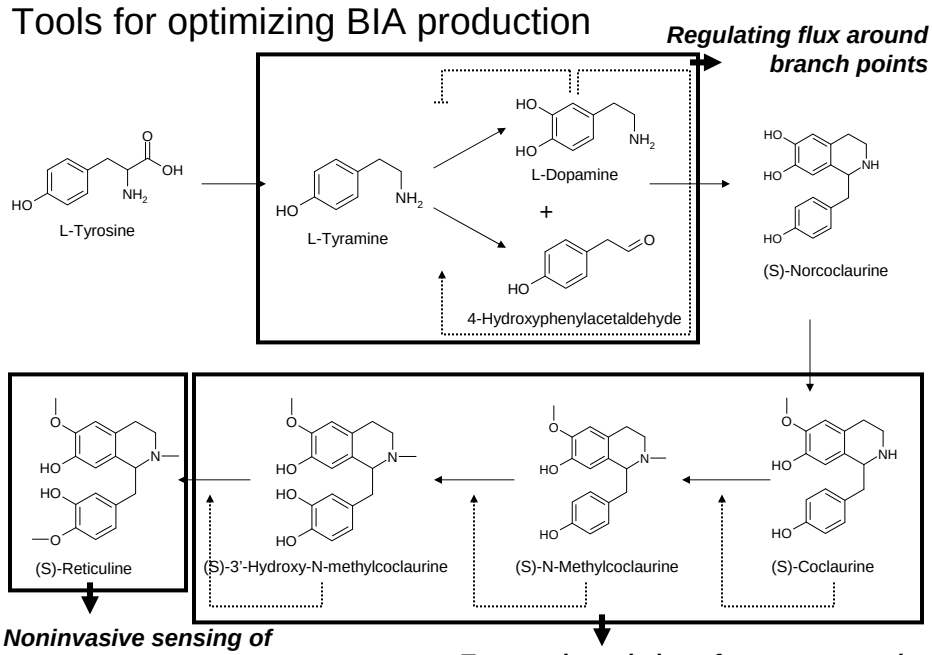


HsCYP2D6

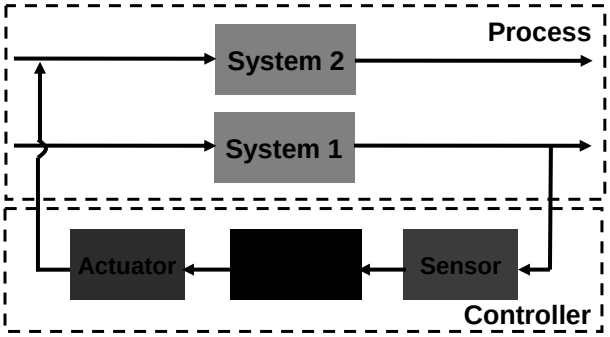
Tools for optimizing BIA production



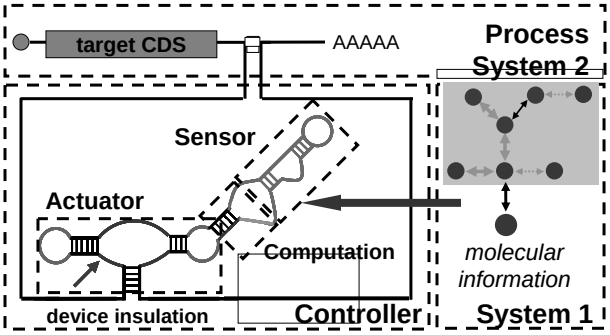
Tools for optimizing BIA production



General control system

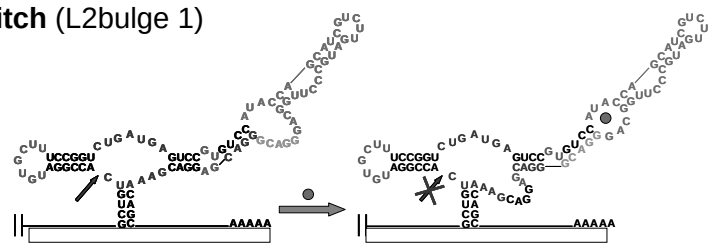


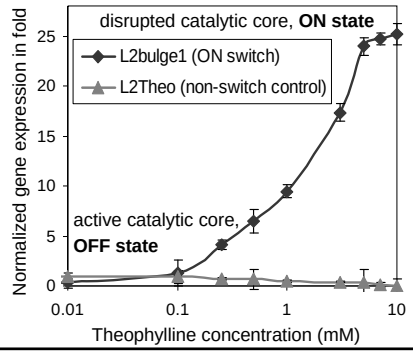
General biological control system



A ribozyme switch platform for up-regulating expression

ON switch (L2bulge 1)

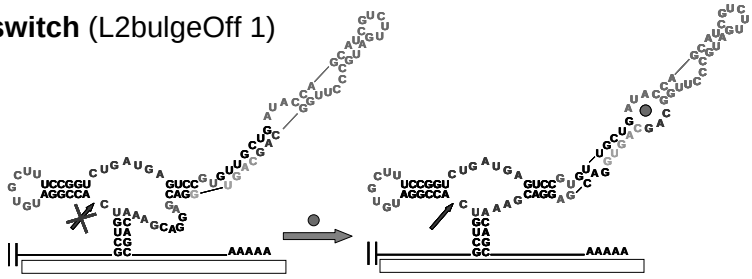


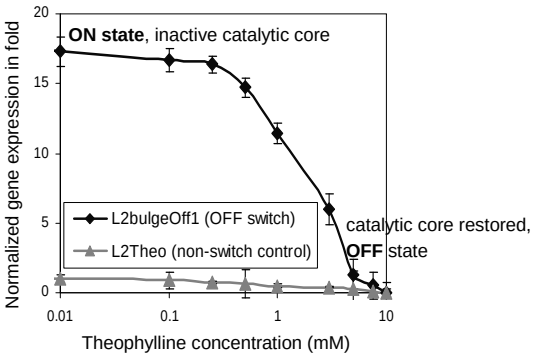


Win MN, Smolke CD. 2007. *PNAS*. 104: 14283-8

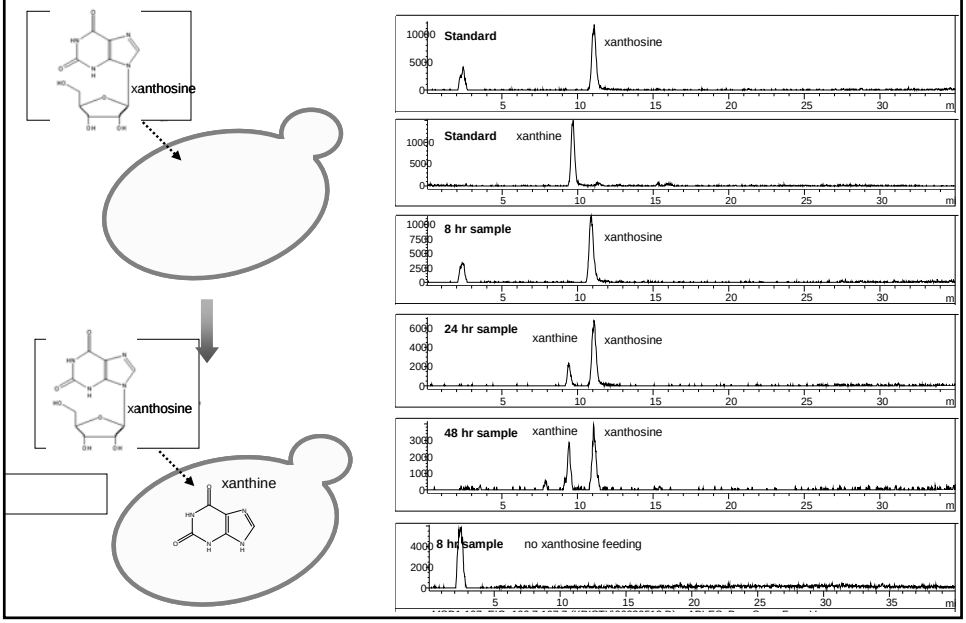
A ribozyme switch platform for down-regulating expression

OFF switch (L2bulgeOff 1)

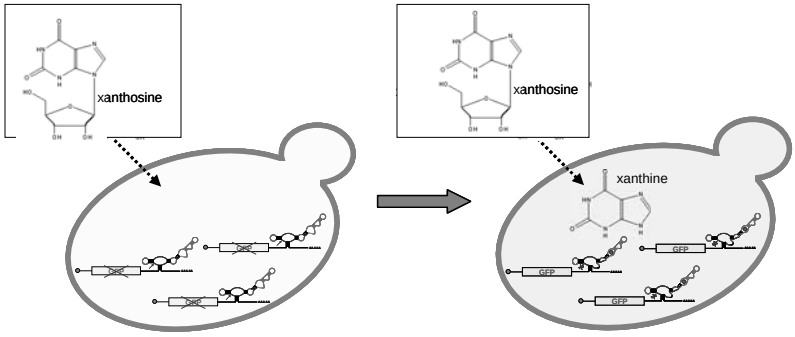




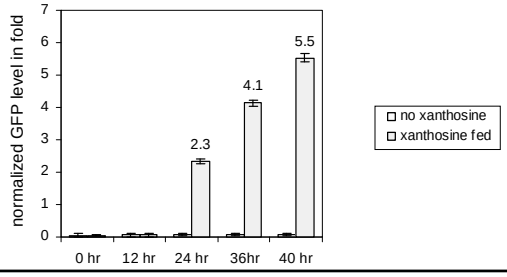
Integrating RNA devices as noninvasive sensors of metabolite concentration

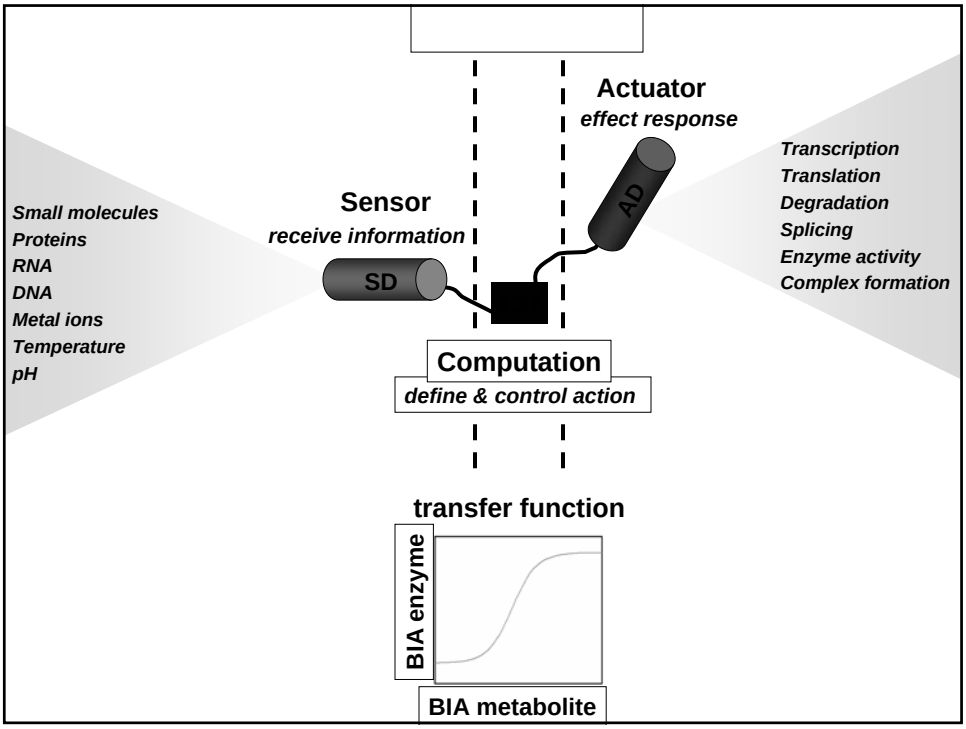


Integrating RNA devices as noninvasive sensors of metabolite concentration

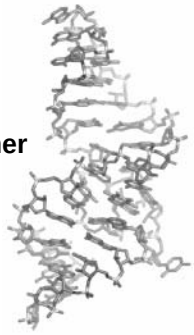


Implementation of ON ribozyme switch as an real-time noninvasive xanthine sensor



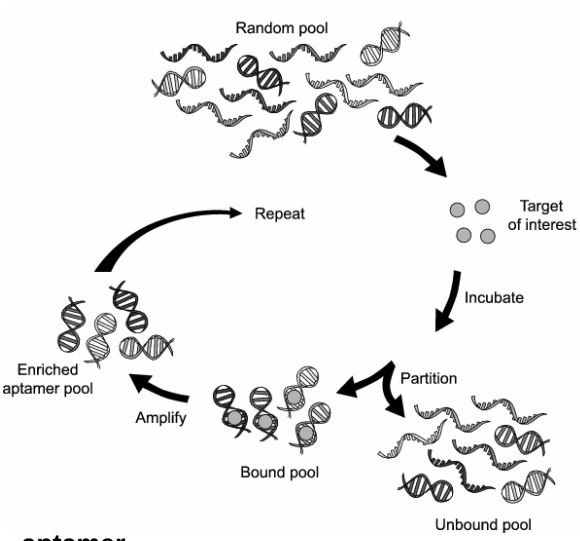


Scalability challenge: libraries of nucleic acid sensors



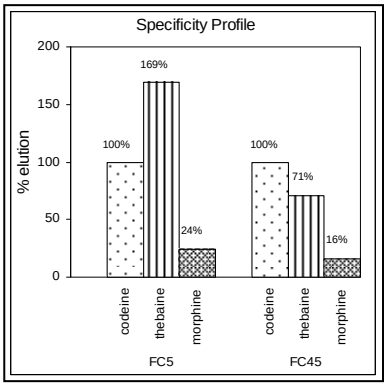
aptamer

- bind wide range of ligands
- high specificity and affinity
- generated through *in vitro* selection process

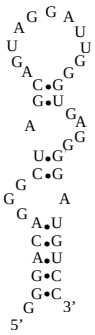


aptamer selection scheme

Specificities of BIA-binding aptamers

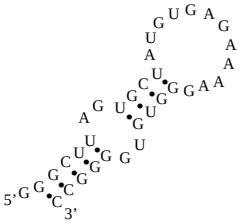


FC5

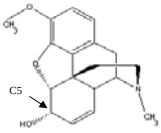


K_d (FC5L) = 4.55±0.14 μM

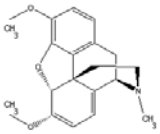
FC45



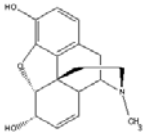
Kd (FC45L) = 2.59 ± 0.09 μM



codeine



thebaine



morphine

High-throughput SPR-based characterization strategy enables rapid screening for mini-aptamer sequences and structural analysis

Win MN, Klein JS, Smolke CD. 2006. *Nuc Acids Res.* 34: 5670-82.

Integrating synthetic metabolic networks and RNA-based control systems

- Metabolic pathway engineering requires a host of tools for optimizing flux and product accumulation
- User-programmed feedback control systems are useful for dynamically controlling flux through pathways
- Developing new genetically encoded tools for receiving, processing, and transmitting molecular information
- Response properties can be programmed to fit the performance specifications of a given application
- These technologies will advance the engineering of more robust cellular systems

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Large-scale Modeling of The Non-linear Enzymatic Reaction Kinetics to Optimize Engineered Pentose Fermentation in *Zymomonas mobilis*

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1. Chemical and Biological Engineering Dept, University of Colorado, Boulder
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Background

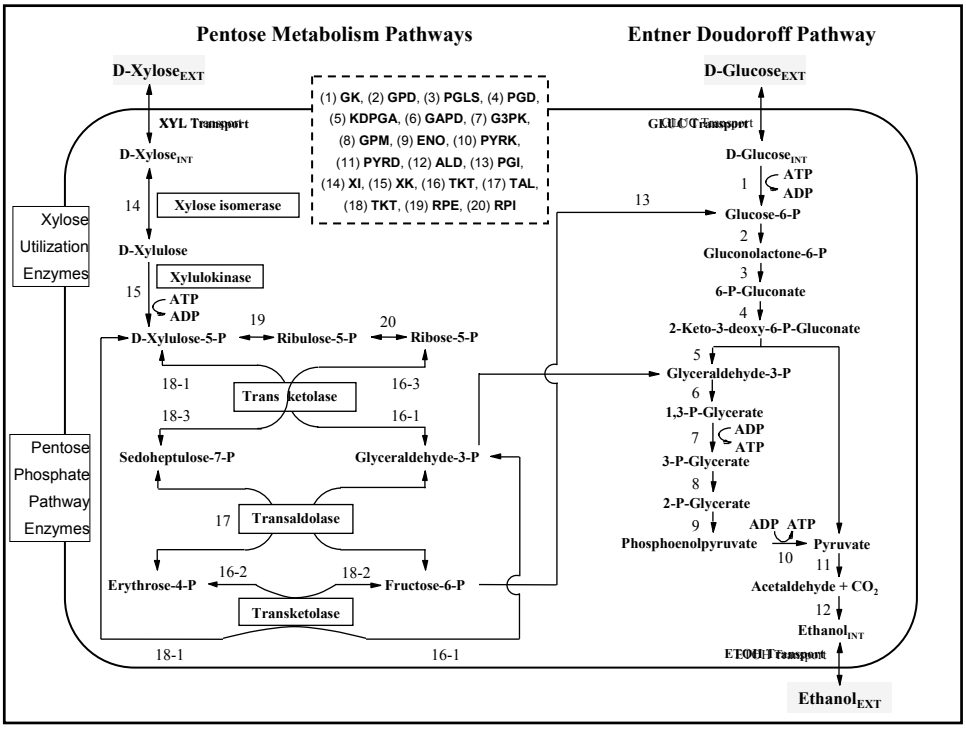
Zymomonas mobilis has been engineered with 4 new enzymes to ferment xylose along with glucose and a network of pentose pathway enzymatic reactions interacting with the native glycolytic Entner Doudoroff pathway has been hypothesized.

We investigated this proposed reaction network by developing a kinetic model for all the enzymatic reactions of the pentose phosphate and glycolytic pathways.

Kinetic data on different sugar metabolism rates and enzymatic activity data was used to refine the model parameters available in the literature and validate the proposed reaction network.

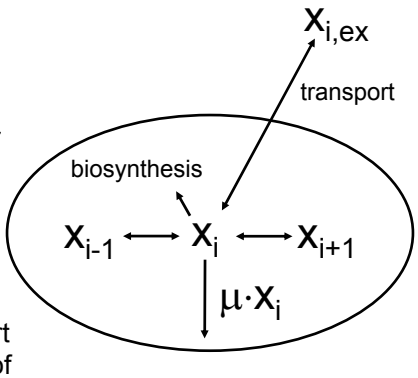
Objectives

- To investigate the assumed network of enzymatic reactions linking the pentose metabolism and glycolysis pathways.
- To incorporate the non-linear rate expressions for the feedback regulation of enzymatic reactions.
- To find an optimum combination of enzymes needed for maximizing ethanol concentration.



Features of Kinetic Modeling

- The mechanistic rate equations for each of the enzymatic reactions occurring inside the cell mass.
- The rate equations for the transport of major substrates into cells and of major products out of the cells.



The *Zymomonas* System

#	ENZYME	REACTANTS	PRODUCTS	INHIBITORS	MECHANISM
1 (→)	Glucokinase	GLUC + ATP	GLUC6P + ADP	GLUC6P	Michaelis-Menten
2 (→)	Glucose-6-P dehydrogenase	GLUC6P + NAD(P)	PGL + NAD(P)H	ATP	Michaelis-Menten
3 (→)	6-phosphogluconolactonase	PGL	PG	GLUC6P	Michaelis-Menten
4 (→)	6-phosphogluconate dehydratase	PG	KDPG	G3P	Michaelis-Menten
5 (→)	2-keto-3-deoxy-6-P-gluconate aldolase	KDPG	GAP + PYR		Michaelis-Menten
6 (↔)	Glyceraldehyde-3-P dehydrogenase	GAP + NAD	DPG + NADH		M.-Menten & Rev.
7 (↔)	3-phosphoglycerate kinase	DPG + ADP	G3P + ATP		M.-Menten & Rev.
8 (→)	Phosphoglycerate mutase	G3P	G2P		Michaelis-Menten
9 (→)	Enolase	G2P	PEP		Michaelis-Menten
10 (→)	Pyruvate kinase	PEP + ADP	PYR + ATP		Michaelis-Menten
11 (→)	Pyruvate decarboxylase	PYR	ACET		Michaelis-Menten
12 (↔)	Alcohol dehydrogenase	ACET + NADH	ETOH + NAD		M.-Menten & Rev.
13 (→)	Phosphoglucose isomerase	FRUC6P	GLUC6P	PG	Michaelis-Menten
14 (→)	Xylose isomerase	XYL	XYLU		Michaelis-Menten
15 (→)	Xylulokinase	XYLU + ATP	XYLU5P + ADP		Michaelis-Menten
16 (↔)	Transketolase	XYLU5P + RIB5P	SED7P + GAP		M.-Menten & Rev.
17 (↔)	Transaldolase	SED7P + GAP	FRUC6P + E4P		Ping-Pong Bi Bi
18 (↔)	Transketolase	XYLU5P + E4P	FRUC6P + GAP		M.-Menten & Rev.
19 (↔)	Ribulose 5-phosphate epimerase	XYLU5P	RIBU5P		M.-Menten & Rev.
20 (↔)	Ribose 5-phosphate isomerase	RIBU5P	RIB5P		M.-Menten & Rev.

Approach

- The kinetic model developed to describe the *Zymomonas* system is comprised of 24 rate expressions and 24 balance equations.
- All the derivatives of balance equations were set to zero to calculate a steady state. The resulting system of non-linear equations was solved numerically for the steady state metabolite concentrations.
- The amounts of 5 enzymes (PGI, XI, XK, TKT and TAL) were varied such that the total of ED and PP pathway enzymes ranges from 42% to 66% of the total cellular protein.
- The k_{cat} values reported at varying temperatures are normalized by using the 'glucose consumption vs. temperature' table presented by Scopes and Griffiths-Smith (1986) to estimate the k_{cat} values at 30°C.

Assumptions and Conditions

- The k_{cat} and K_m values used in the model were collected from the literature at varying pH's.
- The concentrations of ATP, ADP, NAD, NADH and NADP were assumed to be constant and equal to 2, 1, 1.5, 1 and 0.5 mM, respectively.
- A constant level of gene expression was assumed, i.e. the enzyme levels that were measured in the wild-type strain were used.
- For the heterologous enzymes, k_{cat} and K_m values from *E. coli* were used.

✯ ✯ ✯

- Xylose and glucose are constant at 5.0 and 0.875 g/L, respectively.
- The chemostat is operated at a constant dilution rate of 0.05 h^{-1}.
- The cell mass concentration in the bioreactor is constant at 1.0 g/L.
- The cytoplasmic volume of *Zymomonas* cells growing in the presence of glucose and xylose were taken to be 2.2 ml/g dcm.

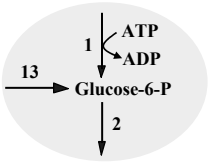
Sample Rate Expression

$$\upsilon_1 = \frac{k_{cat} \cdot [E]_T \times \left(\frac{GLUC}{K_{m,GLUC}}\right) \times \left[\frac{ATP}{K_{m,ATP} \times \left(1 + \frac{GLUC6P}{K_{i,GLUC6P}}\right)}\right]}{1 + \frac{GLUC}{K_{m,GLUC}} + \frac{ATP}{K_{m,ATP}} + \underbrace{\frac{GLUC \times ATP}{K_{m,GLUC} \times K_{m,ATP} \times \left(1 + \frac{GLUC6P}{K_{i,GLUC6P}}\right)}}_{\text{Competitive product inhibiton term}} + \frac{GLUC6P}{K_{i,GLUC6P}}}$$

2 Sub.s + 2 Prod.s
Michaelis-Menten Mech.
Irreversible Rxn.

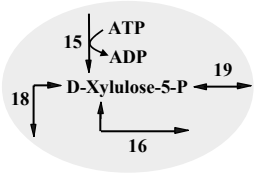
- K_{cat}, K_m and K_i values are reported in the literature
- $\upsilon_{max} = k_{cat} \cdot [E]_T$
- $[E_T]$ = (g enzyme/g total protein) x (g protein/g dry cell)

Representative Balance Equations



$$\frac{d[GLUC6P]}{dt} = (\upsilon_1 + \upsilon_{13} - \upsilon_2) - \mu \cdot [GLUC6P]$$

rate expressions — *dilution due to growth*



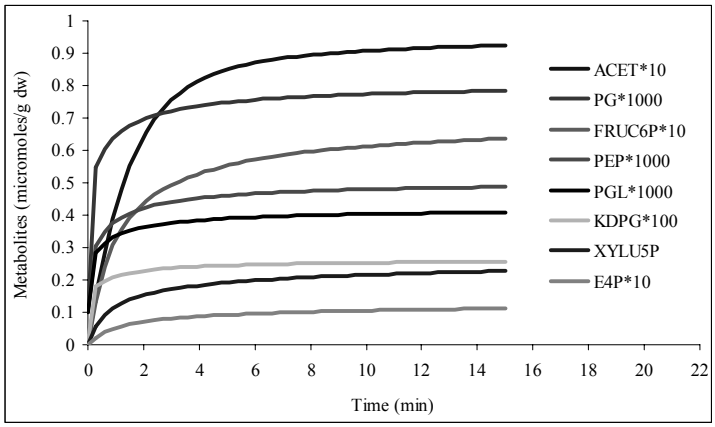
$$\frac{d[XYLU5P]}{dt} = (\upsilon_{15} - \upsilon_{16} - \upsilon_{18} - \upsilon_{19}) - \mu \cdot [XYLU5P]$$

METABOLITES

Metabolites	Dynamic Balance Equations (d[metabolite]/dt = …)	Steady State Intracellular Concentrations (mmol/g dry weight)
GLUC	$(\upsilon_{GLUC} - \upsilon_1) - (\mu \cdot GLUC)$	4.48×10^{-3}
GLUC6P	$(\upsilon_1 + \upsilon_{13} - \upsilon_2) - (\mu \cdot GLUC6P)$	4.70×10^{-3}
PGL	$(\upsilon_2 - \upsilon_3) - (\mu \cdot PGL)$	4.15×10^{-4}
PG	$(\upsilon_3 - \upsilon_4) - (\mu \cdot PG)$	7.97×10^{-4}
KDPG	$(\upsilon_4 - \upsilon_5) - (\mu \cdot KDPG)$	2.61×10^{-3}
GAP	$(\upsilon_5 + \upsilon_{16,1} - \upsilon_6 - \upsilon_{17} - \upsilon_{18,1}) - (\mu \cdot GAP)$	0.26
BPG	$(\upsilon_6 - \upsilon_7) - (\mu \cdot BPG)$	0.11
G3P	$(\upsilon_7 - \upsilon_8) - (\mu \cdot G3P)$	2.15×10^{-3}
G2P	$(\upsilon_8 - \upsilon_9) - (\mu \cdot G2P)$	4.68×10^{-3}
PEP	$(\upsilon_9 - \upsilon_{10}) - (\mu \cdot PEP)$	4.95×10^{-4}
PYR	$(\upsilon_5 + \upsilon_{10} - \upsilon_{11}) - (\mu \cdot PYR)$	1.28×10^{-2}
ACET	$(\upsilon_{11} - \upsilon_{12}) - (\mu \cdot ACET)$	0.14
ETOH	$(\upsilon_{12} - \upsilon_{ETOH}) - (\mu \cdot ETOH)$	192.10
XYL	$(\upsilon_{XYL} - v_{14}) - (\mu \cdot XYL)$	64.92
XYLU	$(\upsilon_{14} - \upsilon_{15}) - (\mu \cdot XYLU)$	7.92
XYLU5P	$(\upsilon_{15} + \upsilon_{18,1} - \upsilon_{16,1} - \upsilon_{19}) - (\mu \cdot XYLU5P)$	0.25
RIBU5P	$(\upsilon_{19} - \upsilon_{20}) - (\mu \cdot RIBU5P)$	0.27
RIB5P	$(\upsilon_{16,3} + \upsilon_{20} - \upsilon_{18,3}) - (\mu \cdot RIB5P)$	0.52
SED7P	$(\upsilon_{18,3} - \upsilon_{16,3} - \upsilon_{17}) - (\mu \cdot SED7P)$	7.94
E4P	$(\upsilon_{16,2} + \upsilon_{17} - \upsilon_{18,2}) - (\mu \cdot E4P)$	1.25×10^{-2}
FRUC6P	$(\upsilon_{17} + \upsilon_{18,2} - \upsilon_{13} - \upsilon_{16,2}) - (\mu \cdot FRUC6P)$	6.67×10^{-2}

Concentration Trajectories

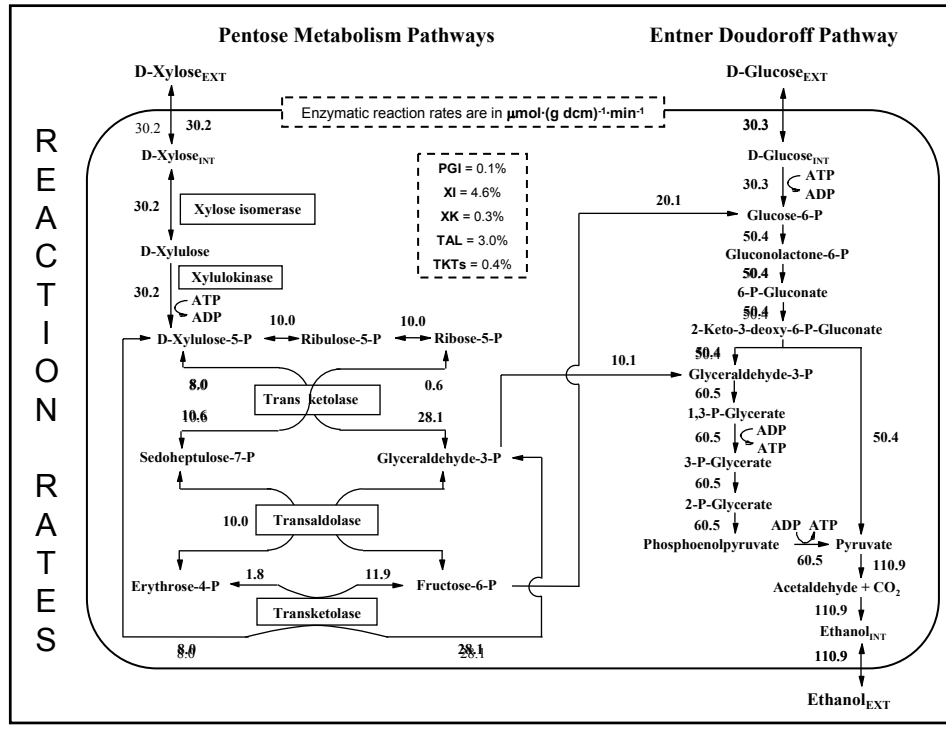
PGI = 0.1%
XI = 4.6%
XK = 0.3%
TAL = 3.0%
TKTs = 0.4%



ACET: acetaldehyde
PG: 6-phosphogluconate
FRUC6P: fructose-6-phosphate
PEP: phosphoenolpyruvate
PGL: 6-phosphogluconolactone
KDPG: 2-keto-3-deoxy-6-phosphogluconate
XYLU5P: xylulose-5-phosphate
E4P: erythrose-4-phosphate

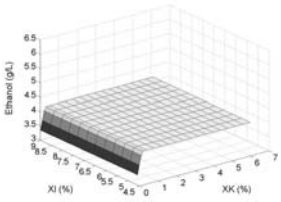
Maximum Ethanol Concentration & Optimal Ethanol Production Efficiency

PGI (%)	XI (%)	XK (%)	TAL (%)	TKTs (%)		Total Key Enzyme Expression Level (%)	ETOH (g/L)	ETOH / Key Enzyme Expression Level
				TKT	TKT-C_2			
Objective: Maximize ethanol concentration								
1.1	**9.0**	**3.7**	**6.1**	**0.8**	**4.1**	24.8	**6.1258**	0.2470
1.3	9.0	3.7	6.1	0.9	4.2	25.2	**6.1258**	0.2431
1.1	9.0	3.7	6.0	0.9	4.3	25.0	**6.1258**	0.2450
1.4	9.0	4.1	5.7	0.8	3.7	24.7	**6.1258**	0.2480
1.3	9.0	3.9	5.9	0.8	3.9	24.8	**6.1258**	0.2470
Objective: Optimize ethanol production efficiency								
0.1	**4.6**	**0.3**	**3.0**	**0.1**	**0.3**	8.4	6.1202	**0.7286**
0.1	4.6	0.3	3.1	0.1	0.2	8.4	6.1202	**0.7286**
0.1	4.6	0.3	2.9	0.2	0.3	8.4	6.1191	**0.7285**
0.1	4.6	0.3	3.1	0.1	0.3	8.5	6.1207	**0.7201**
0.1	4.6	0.3	3.2	0.1	0.2	8.5	6.1207	**0.7201**

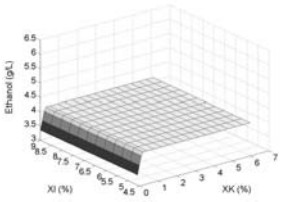


Impact of PGI, XI and XK Concentrations

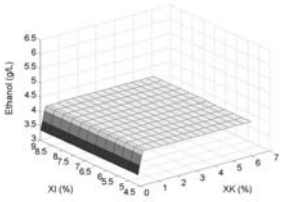
PGI=0%, TAL=0.1%, TKTs=0.1%



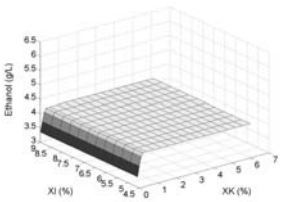
PGI=0%, TAL=0.1%, TKTs=0.1%



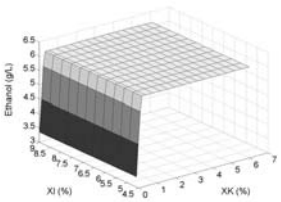
PGI=0%, TAL=0.1%, TKTs=0.1%



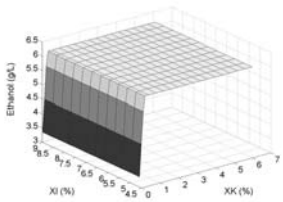
PGI=0.1-1.5%, TAL=0.1%, TKTs=0.1%



PGI=0.1-1.5%, TAL=0.1%, TKTs=0.1%



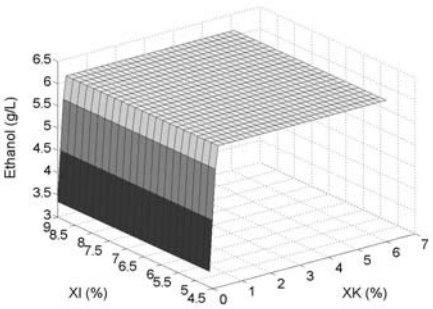
PGI=0.1-1.5%, TAL=0.1%, TKTs=0.1%

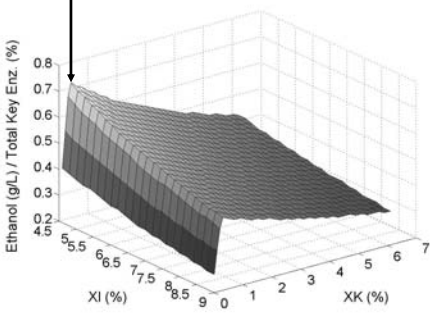


Impact of XI and XK Concentrations

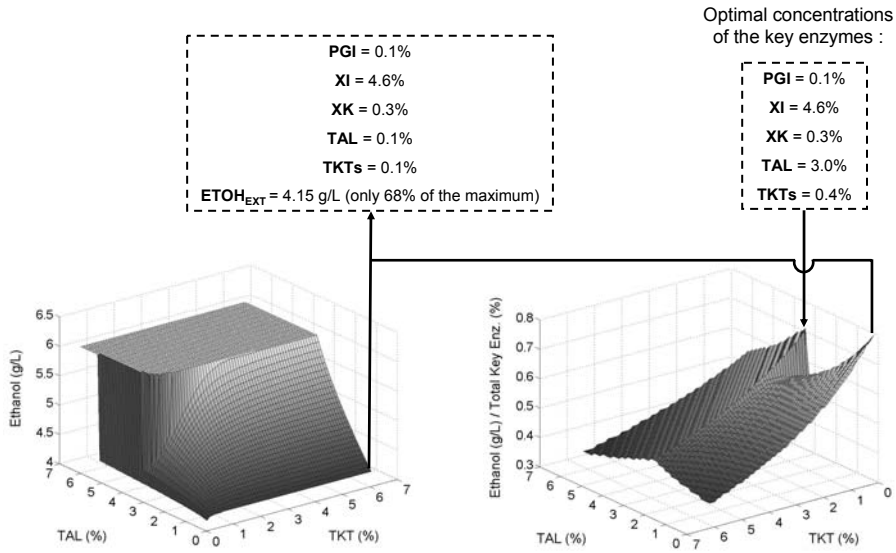
Optimal concentrations of the key enzymes :

PGI = 0.1%
XI = 4.6%
XK = 0.3%
TAL = 3.0%
TKTs = 0.4%





Impact of TAL and TKT Concentrations



Results

- The kinetic model simulations at each enzyme combination produce enormous amount of information, including the steady state metabolite concentrations as well as the dynamic variations in the individual reaction rates for each enzymatic reaction and metabolite.
- Amongst the 5 enzymes whose amounts were tested in terms of the ethanol production, it was found that TAL, XI and XK were the most important enzymes in terms of their effect on the extracellular ethanol concentration. Their presence at sufficient levels eliminates pathway bottlenecks and significant accumulation of intermediate metabolites.
- The relatively low amounts of native PGI and heterologous TKT are sufficient to enable maximal ethanol production.

Results

- Since the reaction rates in the glycolysis pathway are higher than the rates in PP pathway, ‘glyceraldehyde 3-P’ is channeled quickly into the glycolysis pathway.
- Following the flow of ‘glyceraldehyde 3-P’ into the glycolysis pathway, the amounts of ‘erythrose 4-P’ and ‘fructose 6-P’ drops dramatically when TAL and TKT enzymes are available.
- The relatively slow rate of reactions in the PP pathway makes XI and XK enzymes important in terms of their ability to control the reactions immediately following xylose transport into the cell.

Conclusion

- The kinetic modeling strategy allows us to incorporate known information about non-linear rate equations and regulation by other metabolites in determining the optimum combination of heterologous enzymes to maximize pathway efficiency.
- The model enables us to compare the enzymatic reaction rates between the pentose utilization (PP) and glycolytic (ED) pathways and calculate the metabolic flux for each enzymatic reaction at all the assumed levels of heterologous enzymes.

Acknowledgements

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USDA UNITED STATES DEPARTMENT OF AGRICULTURE

Development of tolerant and other complex phenotypes for biofuel production

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Complex phenotypes

- **Involve many genes, which are frequently or mostly not known in their entirety (class I)**
- **Even if we know the genes involved, we do not understand their dynamic behavior to allow us to alter or develop the phenotype (Class II)**

Examples?

- Cells that can tolerate harsh bio-processing conditions beyond what may currently exist in nature? (Class I)
- Like ethanol toxicity: Can we create cells that can tolerate 30% ethanol? Why not? What would it take? What do we know? What is the problem? Biophysical?

More examples…

- Plants with dramatically improved photosynthetic machinery that produce cellulosic biomass twice or thrice as fast as what is currently possible? (Class II)
- Cells that utilize quickly and effectively *(5-10x better than currently known)* cellulose or xylans to produce one major product (such as ethanol or butanol or other biofuels or commodity chemicals) (Class II or I?)

Which way to solve such problems?

- An *ab initio* cellular design?
  - How far can we go based on what we know?
  - What is holding us back? Synthetic biochemistry/biology or knowledge?
- A hybrid first approach: some knowledge based, some empirical or semi- empirical?
  - What is the evidence that it will work? What hypotheses must be tested to that effect?
- Luck, and trial & error empiricism like mostly currently practiced?

Our Research Goals

- Identify genes and cellular programs in clostridia affected by solvent (e.g., butanol) and carboxylic acid (butyrate & acetate) stress in order to identify:
  - Specific and general stress regulons
  - Genes which may impart solvent/acid tolerance

Applications: Bioprocessing for:

- Solvent-production: fermentations
- Biocatalysis
- Bioremediation

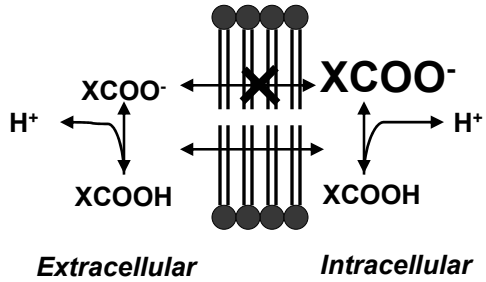
Production of solvents via fermentation provides a green alternative to petrochemicals

- 1910s-1950s: butanol produced by anaerobic fermentation: *C. acetobutylicum* (acetone, butanol, ethanol)
  - Fermentation yields 1.5% butanol (very low)
  - A limiting step in final titers: product inhibition (toxicity)
- Two phase fermentations
  - Exponential growth: production of butyrate, acetate
  - Stationary phase: uptake of butyrate and acetate, production of acetone, butanol, and ethanol
- TOXICITY: butanol (but also butyrate & acetate & interactions)

THEORY: Mechanisms of Solvent Toxicity

- Inhibition of growth and glucose uptake
- Disruption of membrane integrity
  - Loss of membrane ΔpH and $\Delta\Psi$
  - Loss of ATP production efficiency
  - Cells adapt slowly by altering membrane fluidity
    - Adaptation may inhibit membrane function

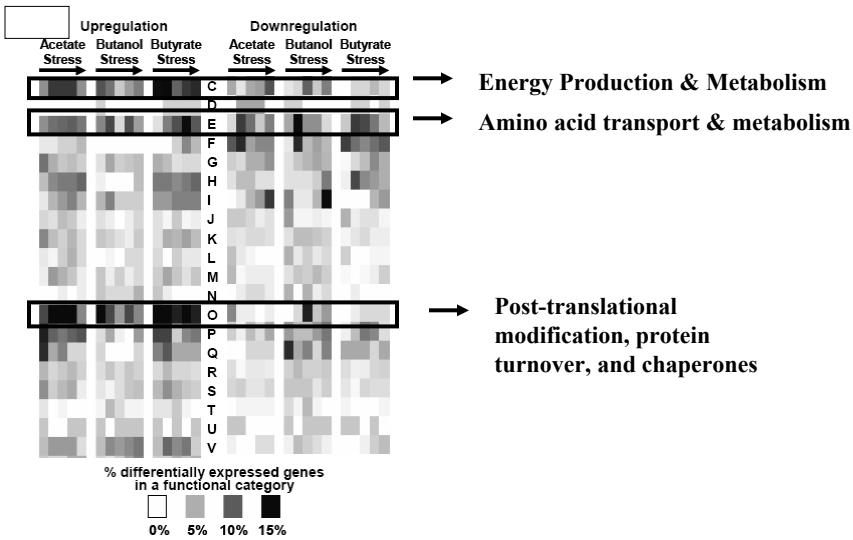
Uncoupling THEORY of Carboxylic Acid Toxicity: Undissociated acids cross cellular membrane and acidify the cytoplasm



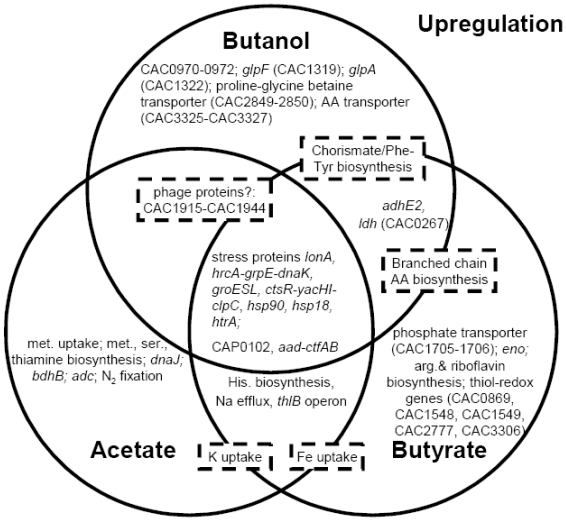
Russell and Diez-Gonzalez, *Adv. Microb. Phys.*, 1998, 208

- **Is it that simple?**

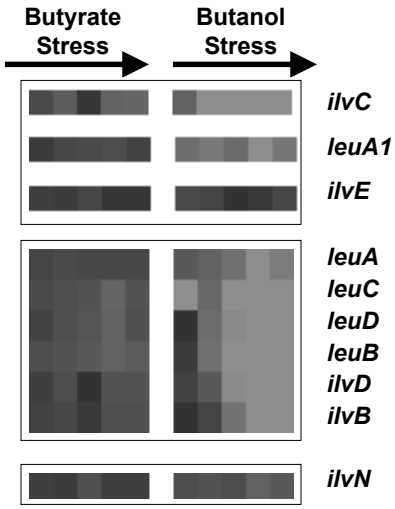
Ontological Analysis of multi-stress responses (based on Genome-scale μ-array analysis)



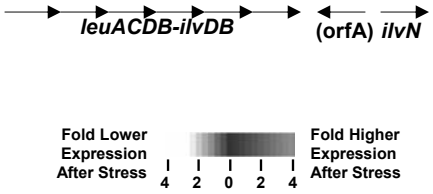
Common & Specific Stress Responses
(based on Genome-scale μ-array analysis)



Differential Expression of Branched Chain Amino Acid Synthesis Genes

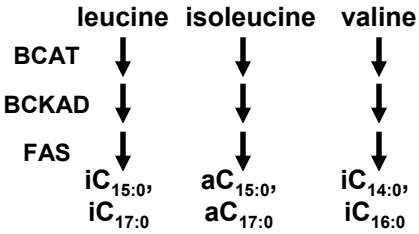


- Has this specific gene induction been seen in other microarray stress studies in other organisms?



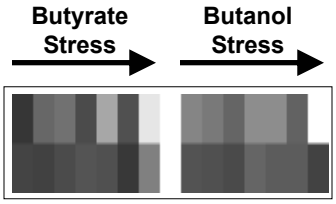
- Adaptation due to cold-shock of *B. subtilis* includes:
  - decreased membrane fluidity
  - downregulation of branched-chain amino acids (Kaan et al. *Microbiol.*, 2002)

- *Bacillus subtilis* can convert branched-chain amino acids into branched-chain fatty acids



- *C. acetobutylicum* increase membrane fluidity in response to butanol (Vollherst-Schneck et al. *J. Bacteriol.*, 1984)
- Synthesis of branched-chain fatty acids in *C. acetobutylicum* may help cells adapt to metabolite stress

Genes Specifically Induced by Butanol Stress



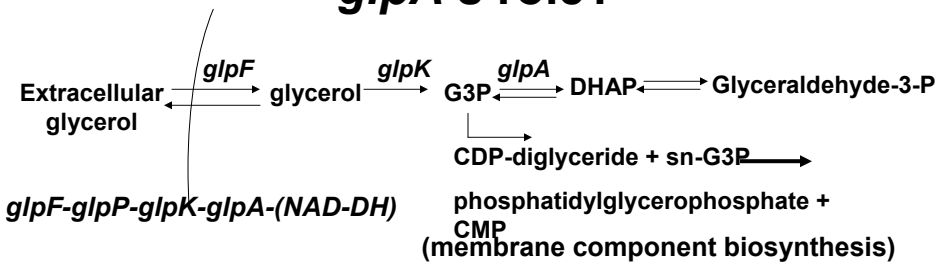
glpF-glpP-glpK-glpA-(NAD-DH)

Glycerol-3-phosphate dehydrogenase, *glpA*

NAD-dependent dehydrogenase

- In *E. coli*, homolog *glpC* is associated with solvent tolerance (Shimizu, *AEM*, 2005), although cannot act alone…

glpA's role?



- **What is the common role of glycerol-3-phosphate dehydrogenase in solvent tolerance?**
  - **First step in P-lipid biosynthesis as the need for an altered membrane composition develops (H. Goldfine et al.)**
  - **Yeast: *glp* genes play role in osmotolerance (Brisson, *BioEssays*, 2001)**

Universally Upregulated Genes by Acetate, Butyrate, and Butanol

- **Stress proteins**
  - **Chaperones: *dnaKJ*, *groESL*, *clpC, hsp90, hsp18***
  - **Protease: *lonA***
  - **Benefits of *groESL* overexpression in enhancing solvent tolerance has been established by our group[1,2]**
- ***Establishing protein stability and functionality seems to be key in responding to toxic stress***

- ***THUS, stress response can (sometimes) predict stress tolerant phenotypes***

1. Tomas, CT, NE Welker, ET Papoutsakis. 2003. *Appl. Environ. Microbiol.* 69: 4951-4965.
2. Tomas, CT, J Beamish, ET Papoutsakis. 2004. *J. Bacteriol.* 186: 2006-2018.

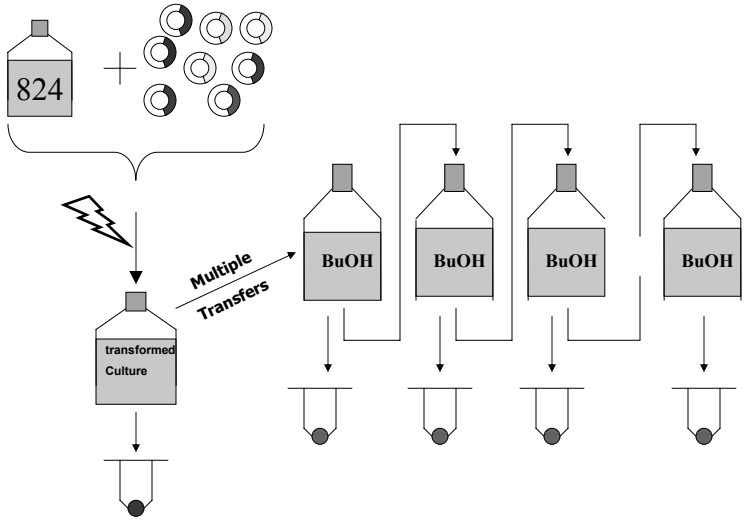
A lot of new information…

- **But no obvious high-throughput way to test and use such information for achieving strong tolerant phenotypes…**
- **Need then for such (empirical for now) tools, that can be more broadly used for developing other complex phenotypes**
  - **Genomic or expression libraries have been used extensively to identify genes or loci (or their variants) imparting a selectable phenotype, incl. tolerant ones**
  - **What is NOT known is how well and completely that works for either SINGLE loci/genes (let alone for INTERACTING MULTIPLE LOCI) and if these genes/loci are related to stress regulons**

Library approach

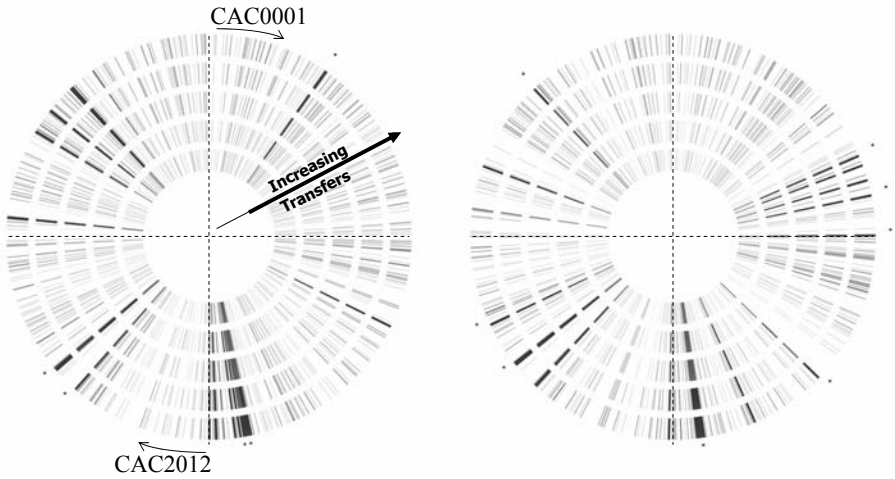
- Several types of libraries
- Have applied them for identifying tolerance to butanol and butyrate and have tested some of the identified genes

Library Selection



Assessment by high resolution HT (μ-array or deep sequencing analysis): stochasticity vs determinism

Biological Replicate 1 **Biological Replicate 2**



Genes color coded according to percentile rank in a given transfer: the top 5% are red, 5-33% are green, and 33-100% are gray

Several of the identified genes

- Impart solvent tolerance alone…
- But better as a group in a mixed population rather than in pure recombinant strains (extracellular molecules? Cell-to-cell communication?)
- Novel transcriptional regulators…

Other interesting findings

- **Butyrate-tolerance studies lead consistently to enrichment of IR (non-coding) DNA of the rRNA locus. Regulon has been determined by μ-array analysis but mechanism not known yet: a srRNA or what?**
- **Based on these identified DNAs, tolerance to butyrate imparts also tolerance to all tested carboxylic acids and leads to higher butanol formation (and tolerance?)**

To summarize, Library approach

- **Useful for identifying many loci, but not all**
  - **Little overlap with BuOH stress regulon**
  - **Significantly, it missed chaperons (why?) and likely more tolerance genes…**
  - **Appears to identify more transcriptional regulators (solo players)**
- **Misses large, multigenic programs…**
- **It is not strictly deterministic, and depends on**
  - **Selection assay (select for growth only?)**
  - **Application mode of selection assay**
  - **Insert size and regulation of gene expression**

Stress-regulon approach works best…

- When there is a dormant adaptive response such as in, e.g., clostridial adaptation to butanol and butyrate accumulation
- For identifying potential target programs and pathways

Current & future work in my lab: methods

- **HT methods to capture and combine distant multi-locus effects and allogeneic traits (for tolerant and other phenotypes, …**
- **Coupled with "enhanced" , "temporarily-induced" recombination, …**
- **to develop strains that quickly and simultaneously directly and efficiently ferment cellulose and xylans without enzymatic pre-treatment**

…and exploration of the relationship between differentiation (sporulation) and tolerance?

- Mature or even immature spores are extremely resistance to chemicals, radiation, everything,…
- Can we use "Differentiation Engineering" to freeze "differentiation" stage at cellular type which is tolerant and a good producer?

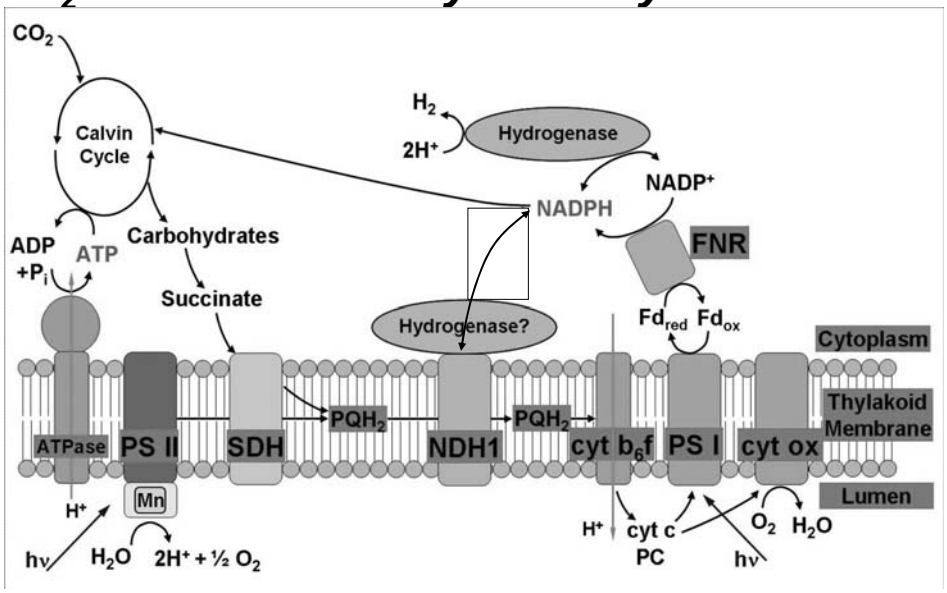
Metabolic Engineering of Light and Dark Biochemical Pathways in Wild-Type and Mutant *Synechocystis* PCC 6803 Strains for Maximal, 24-Hour Production of Hydrogen Gas

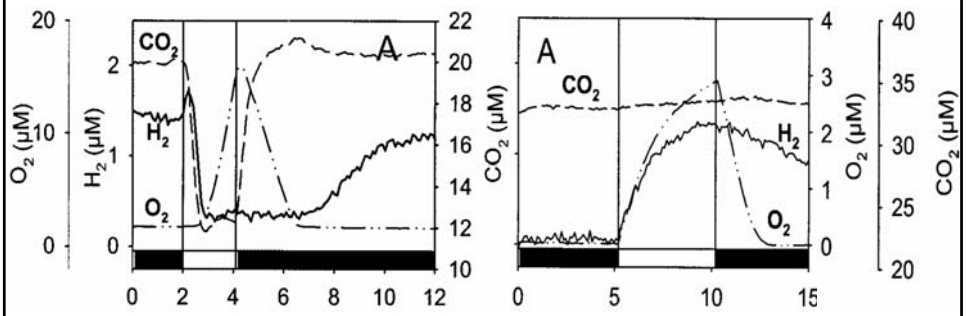
Roger L. Ely
Frank W.R. Chaplen

Oregon State University

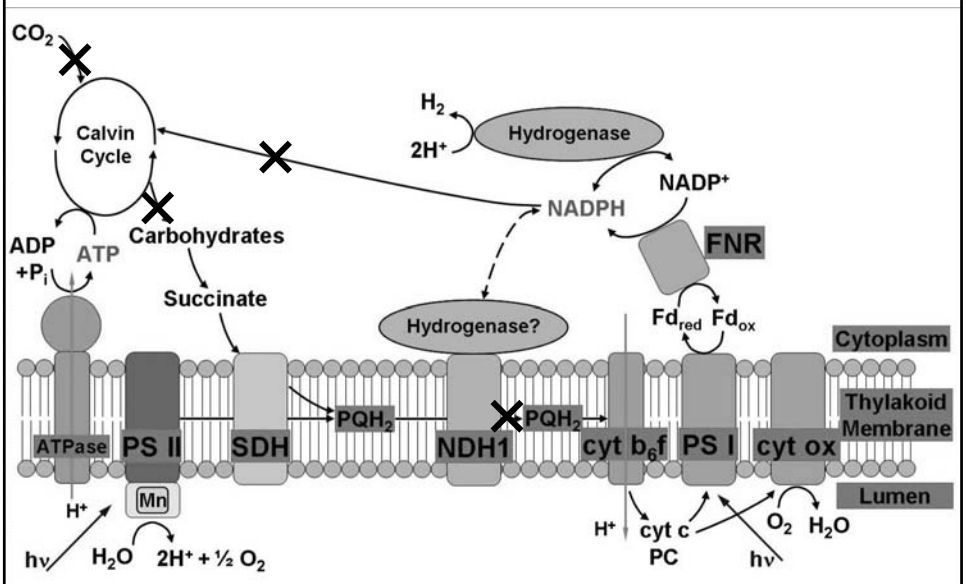
DOE-BER GTL-MEWG Annual Awardee Workshop
Feb 11, 2008

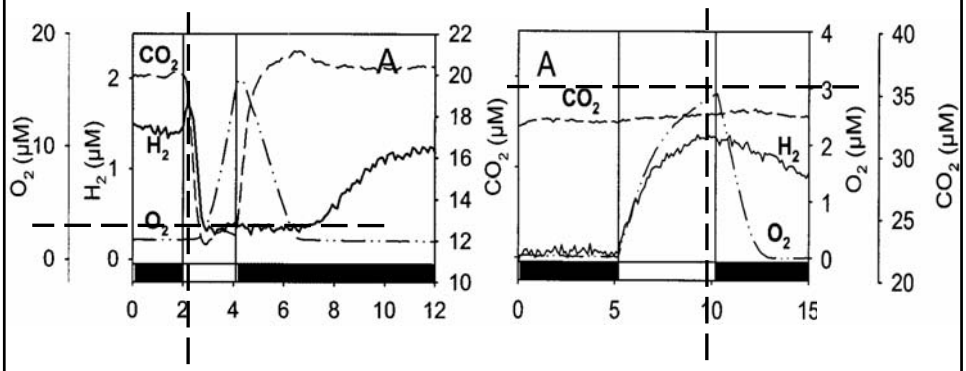
H_2 Production in *Synechocystis* PCC





Source: Cournac, L., G. Guedeney, G. Peltier, and P. M. Vignais. 2004. J. Bacteriol. 186(6):1737–1746

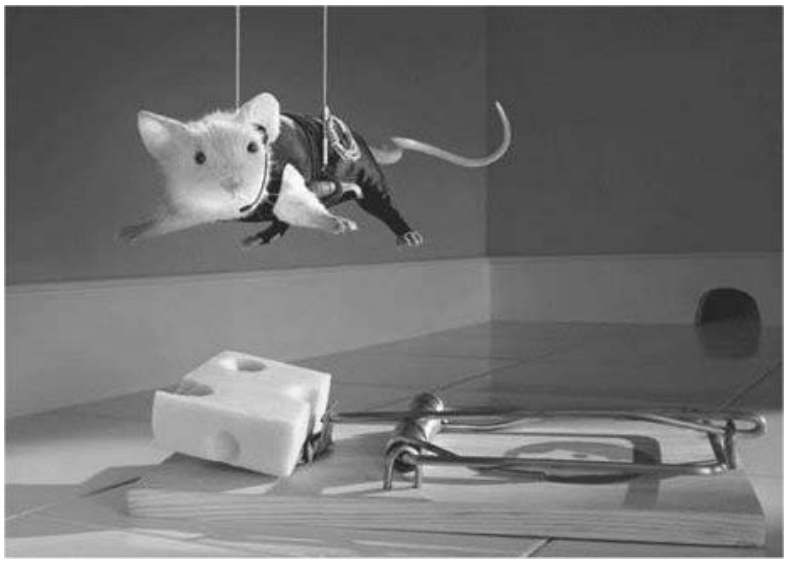


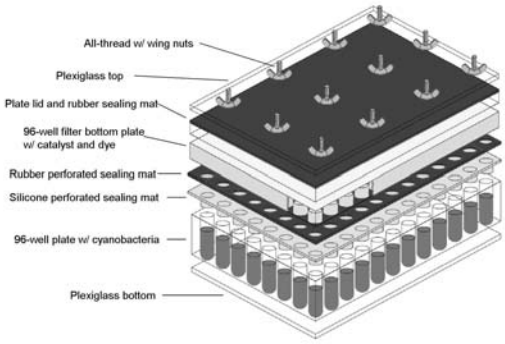


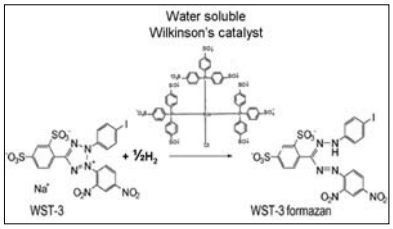
Source: Cournac, L., G. Guedeney, G. Peltier, and P. M. Vignais. 2004. J. Bacteriol. 186(6):1737–1746

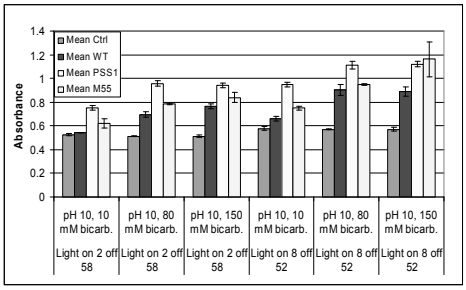
Approach

- **Reductant Supply**
  - **Initial Screening**
  - **H_2 Production Profiling**
  - **Metabolic Flux Analyses**
  - **Specific Inhibitors**
- **Oxygen Sensitivity**
  - **Rational Enzyme Modifications**
  - **Directed Evolution**

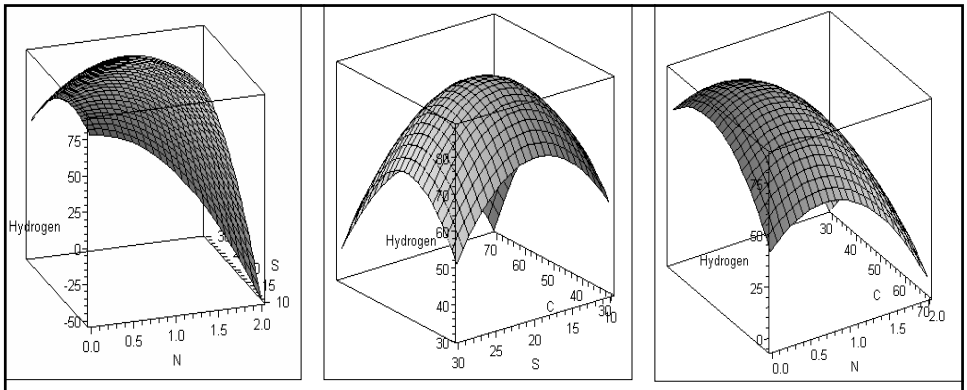



All-thread w/ wing nuts
Plexiglass top
Plate lid and rubber sealing mat
96-well filter bottom plate w/ catalyst and dye
Rubber perforated sealing mat
Silicone perforated sealing mat
96-well plate w/ cyanobacteria
Plexiglass bottom


Water soluble
Wilkinson's catalyst
+ ½H2
WST-3
WST-3 formazan

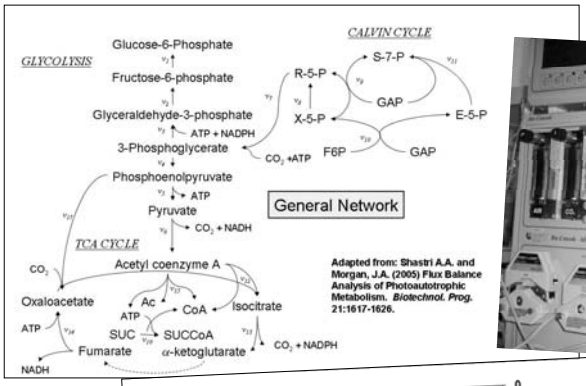

Mean Ctrl
Mean WT
Mean PSS1
Mean M55
Absorbance
1.4
1.2
1
0.8
0.6
0.4
0.2
0
pH 10, 10 mM bicarb.
pH 10, 80 mM bicarb.
pH 10, 150 mM bicarb.
pH 10, 10 mM bicarb.
pH 10, 80 mM bicarb.
pH 10, 150 mM bicarb.
Light on 2 off
Light on 2 off
Light on 2 off
Light on 8 off
Light on 8 off
Light on 8 off
58
58
58
52
52
52

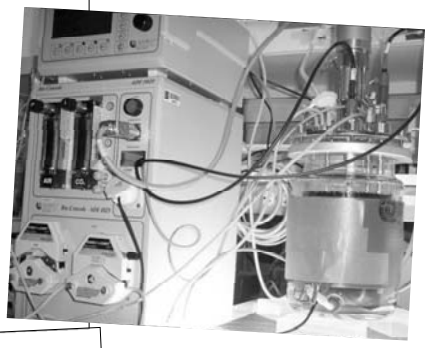
Poster # 23

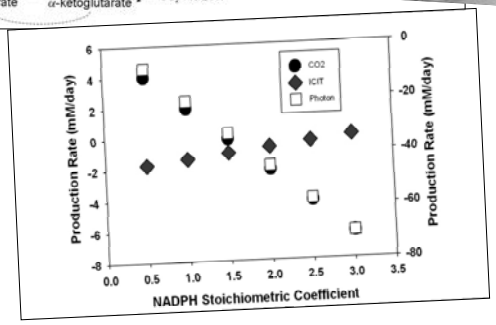


	H_2 accumulation in headspace (μM)	
	14 hours	40 hours
Predicted maximum	85 (23)	210 (54)
S-deprived cultures	0 (0)	0.16 (0.3)

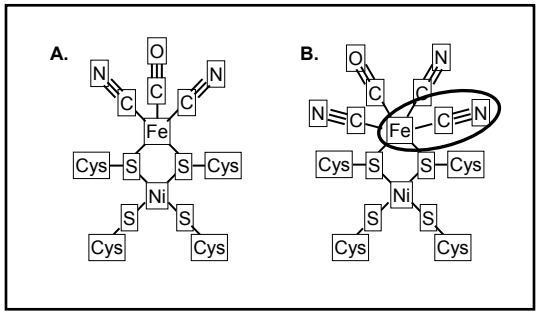
Poster # 26

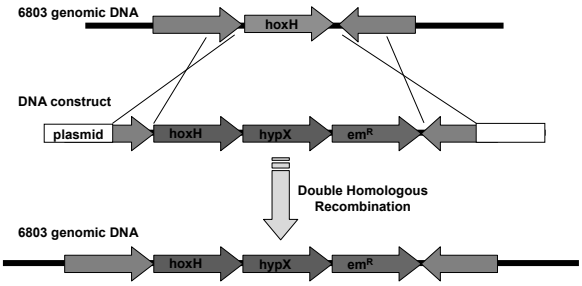


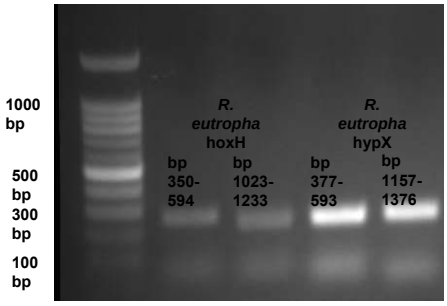


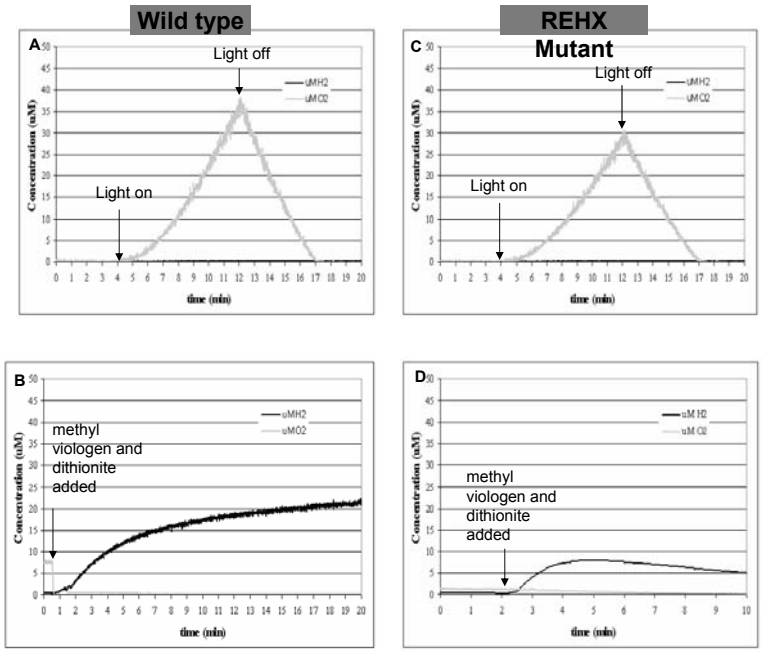


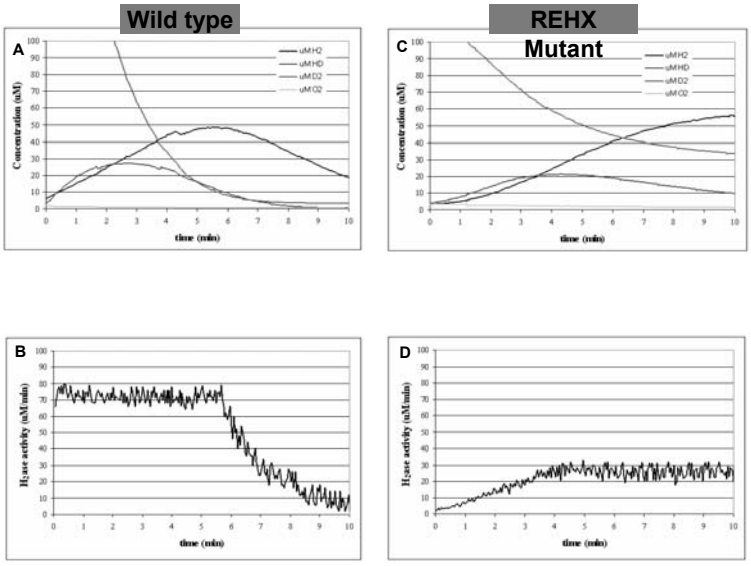
Poster # 25


A.
B.
O
N
C
Fe
Cys
S
Ni

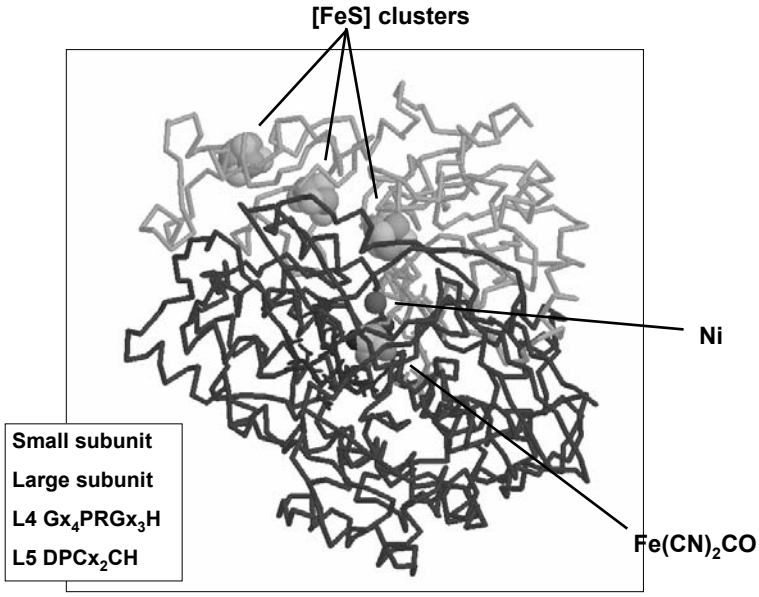

6803 genomic DNA
hoxH
DNA construct
plasmid
hypX
emR
Double Homologous
Recombination
6803 genomic DNA

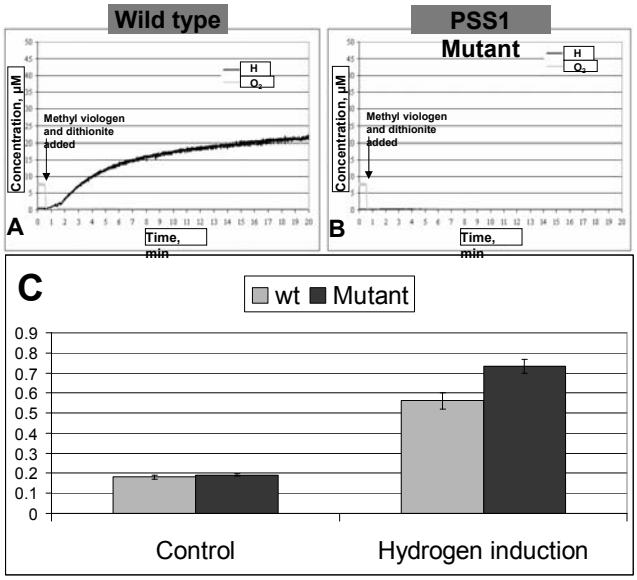

1000 bp
500 bp
300 bp
100 bp
R. eutropha hoxH
bp 350-594
bp 1023-1233
R. eutropha hypX
bp 377-593
bp 1157-1376

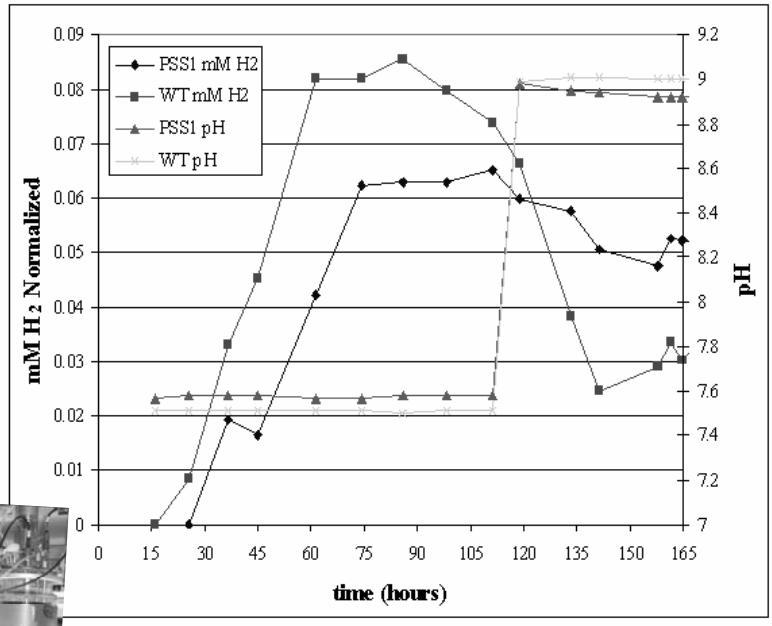

Wild type
REHX Mutant
A
B
C
D
Light off
Light on
methyl viologen and dithionite added
uMH2
uMO2
Concentration (uM)
time (min)

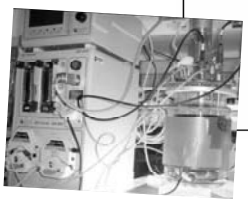

Wild type
REHX Mutant
A
B
C
D
uMH2
uMHD
uMD2
uMO2
Concentration (uM)
H2ase activity (uM/min)
time (min)

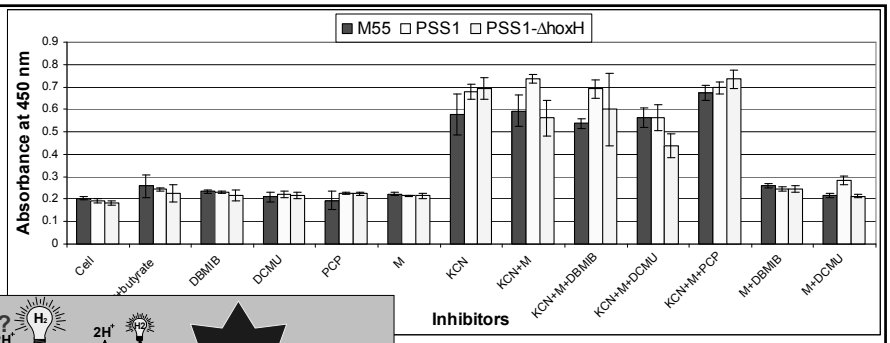
Poster # 27

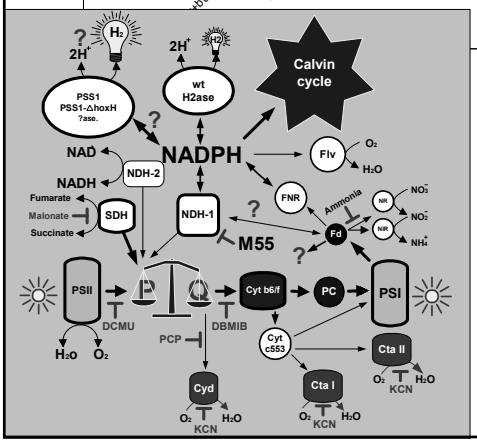

[FeS] clusters
Ni
Fe(CN)$_2$CO
Small subunit
Large subunit
L4 Gx$_4$PRGx$_3$H
L5 DPCx$_2$CH


Wild type
PSS1 Mutant
Concentration, µM
Methyl viologen and dithionite added
H
O$_2$
Time, min
A
B
C
wt
Mutant
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
Hydrogen induction


PSS1 mM H2
WT mM H2
PSS1 pH
WT pH
mM H2 Normalized
pH
time (hours)
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
9.2
9
8.8
8.6
8.4
8.2
8
7.8
7.6
7.4
7.2
7
0 15 30 45 60 75 90 105 120 135 150 165




M55
PSS1
PSS1-ΔhoxH
Absorbance at 450 nm
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Cell
DBMIB
DCMU
PCP
M
KCN
KCN+M
KCN+M+DBMIB
KCN+M+DCMU
KCN+M+PCP
M+DBMIB
M+DCMU
Inhibitors


H2
2H+
PSS1
PSS1-ΔhoxH
?ase.
wt
H2ase
Calvin
cycle
NADPH
NAD
NADH
NDH-2
Fumarate
Malonate
Succinate
SDH
NDH-1
M55
FNR
Ammonia
Fd
Flv
O2
H2O
NO3-
NO2-
NH4+
PSII
DCMU
H2O
O2
PCP
DBMIB
Cyt b6/f
PC
PSI
Cyt
c553
Cta II
Cta I
Cyd
KCN

Poster # 24

Recap

- High-Throughput Screening Assay
- Optimization
- Metabolic Flux Analysis
- REHX Mutant
- MV-DT Assay and Performance of Mutants

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Hatem Mohamed

PhD Students:
Paul Schrader
Liz Burrows

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THANK YOU!

Creating a Pathway for the Biosynthesis of 1,2,4-Butanetriol

John Frost
Michigan State University

Collaborators:

Wei Niu
Man Kit Lau
Mapitso Molefe

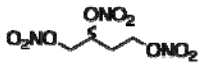
February 11, 2008 MEWG: Interagency Conference on Metabolic Engineering

Draths

Okemos, Michigan
Minneapolis, Minnesota

Research Areas:

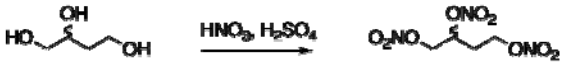
- synthetic chemistry
- catalysis
- materials science
- biocatalysis
- metabolic engineering



1,2,4-butanetriol trinitrate

BTTN

- less volatile than nitroglycerine (NG)
- more thermally stable than NG
- lower shock sensitivity relative to NG



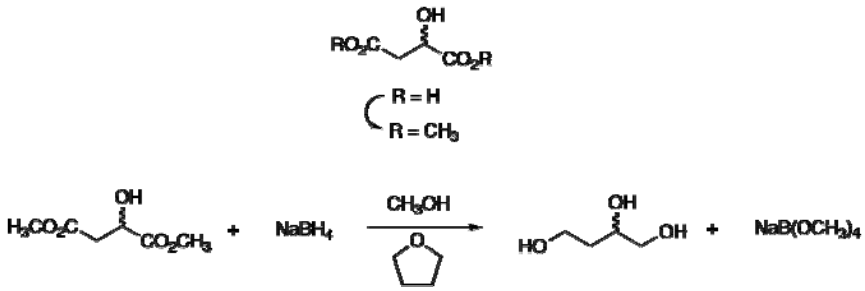
1,2,4-butanetriol

BT

1,2,4-butanetriol trinitrate

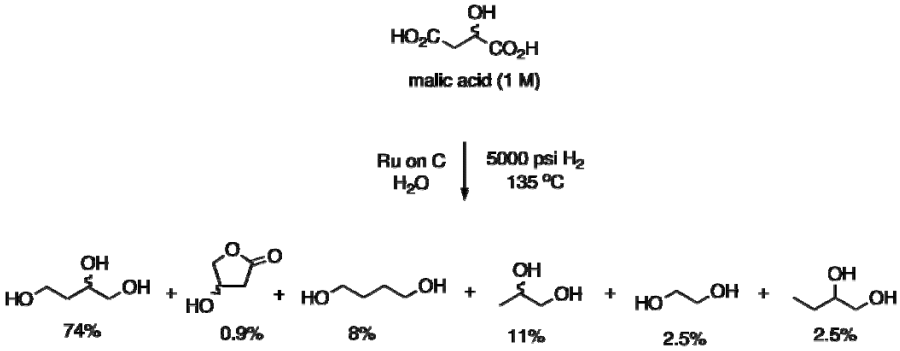
BTTN

BT Manufacture:Stoichiometric Reduction

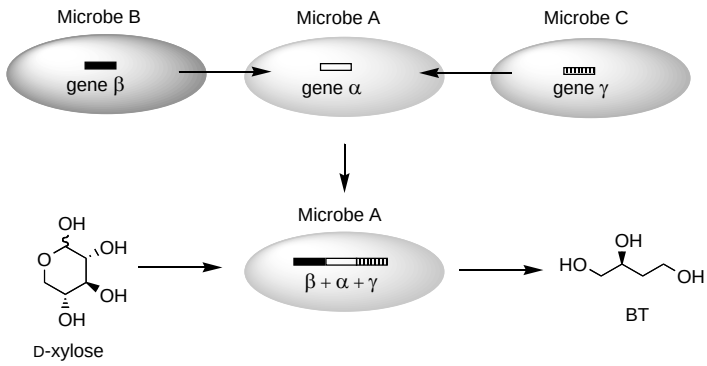


For every ton of BT produced, 2-6 tons of byproduct borate salts are generated. (WO 98/08793).

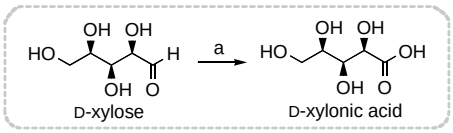
BT Synthesis: Catalytic Reduction



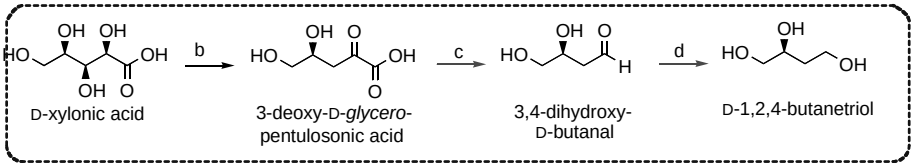
Creation of a Microbial Catalyst



Microbial Synthesis of D-BT



Pseudomonas fragi ATCC 4973 70% Yield



E. coli DH5α/pWN6.186A 25% Yield

a. D-xylonate dehydrogenase (*P. fragi*); b. D-xylonate dehydratase (*E. coli*);
c. benzoylformate decarboxylase (*P. putida*); d. alcohol dehydrogenase (*E. coli*)

→ enzymes catalyzing reactions on native substrates
→ enzymes catalyzing reactions on non-native substrates

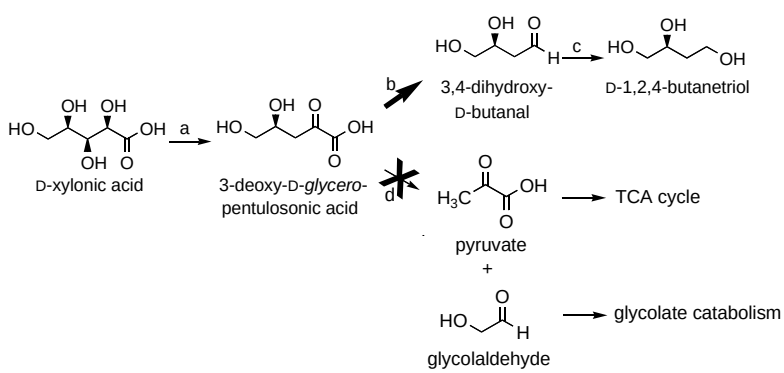
Escherichia coli Synthesis of D-1,2,4-Butanetriol from D-Xylonic Acid in Rich Medium and Minimal Salts Medium

host strain	plasmid and encoding genes	1,2,4-BT (g/L)	yield (mol%)	culture medium and carbon source
DH5α	pWN6.186A P_{tac}-*mdlC*, Kan^R, *lacI*^{Q}	1.6	25	rich medium[a] and glucose[c]
W3110 serA	pWN7.126B P_{tac}-*mdlC*, *serA*, *lacI*^{Q}	0.020	0.3	minimal medium[b] and glucose[c]

[a] tryptone (20 g/L), yeast extract (10 g/L), NaCl (5 g/L), K_2HPO_4 (3.75 g/L); [b] K_2HPO_4 (7.5 g/L), ammonium iron citrate (III) (0.3 g/L), citric acid monohydrate (2.1 g/L); [c] when cell growth entered mid-log phase, IPTG and D-xylonic acid were added into the culture medium.

Niu, W.; Molefe, M. N.; Frost, J. W. *J. Am. Chem. Soc.* **2003**, *125*, 12998.

Escherichia coli Biosynthesis of D-1,2,4-Butanetriol from D-Xylonic Acid



a. D-xylonate dehydratase (*E. coli*); b. benzoylformate decarboxylase (*P. putida*); c. alcohol dehydrogenase (*E. coli*); d. aldolase (*E. coli*).

Purification of *Pseudomonas fragi* D-Xylonate Dehydratase

205
116
97
66
45
29

← ~ 60 kDa

QuickTime™ and a TIFF (Uncompressed) decompressor are needed to see this picture.

	total protein (mg)	total activity (units)	specific activity (unit/mg)	yield (%)	purification fold
cell lysate	7274	215	0.03	100	1.0
DE-52	527	76	0.14	35	4.8
hydroxyapatite	219	66	0.30	31	10
phenylsepharose	24	50	2.10	23	70
resource Q	11	30	2.91	14	97

N-Terminal Sequences of Trypsin-Digested *Pseudomonas fragi* D-Xylonate Dehydratase

estimated position [a]	amino acid sequence	degenerate primers
1	TDSTPKRGRAQL[b]	JWF1006-JWF1009
70	DAGGIPMEFPVHPIAEQSR	N/A
150	IGSGTVLWHAR	N/A
300	HSGVELSLEDWQRVG[b]	JWF1057-JWF1059
400	MSVVGEAFR	N/A
430	AXVFEGPEDYTAR[b]	JWF1004-JWF1005

[a.] Positions of peptide fragments were assigned using the positions of the highest scored homologs derived from blasting the NCBI database;

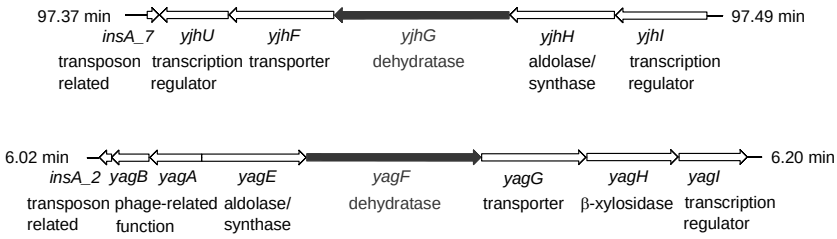
[b.] Degenerate primers were designed based on the underlined sequences.

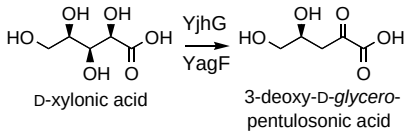
Partial DNA Sequence of *P. fragi* (ATCC 4973) D-Xylonate Dehydratase

```
  1  CTC GAG GAT TGG CAG CGC GTG GGT GAA GAC GTG CCC TTG CTG GTC AAC TGC ATG CCT GCC
  1▶  L   E   D   W   Q   R   V   G   E   D   V   P   L   L   V   N   C   M   P   A

 61  GGC GAG TAC CTG GGC GAA AGC TTC CAC CGC GCC GGT GGC GTA CCG GCG GTG ATG CAT GAG
 21▶  G   E   Y   L   G   E   S   F   H   R   A   G   G   V   P   A   V   M   H   E

121  CTG GAC AAA GTG GGC CGC CTG CAC CGC GAT TGC CTC ACG GTC AGT GGC CGC AAC ATG GGT
 41▶  L   D   K   V   G   R   L   H   R   D   C   L   T   V   S   G   R   N   M   G

181  GAA GTG GTC GCC GAC TGC GTC ACC GGC GAC CGC GAC GTG ATC CGC TCC TAC GAA GAC CCG
 61▶  E   V   V   A   D   C   V   T   G   D   R   D   V   I   R   S   Y   E   D   P

241  CTG ATG CAC CGC GCT GGT TTT ATT GTG CTC AGC GGC AAC TTC TTC GAC AGC GCG ATC ATG
 81▶  L   M   H   R   A   G   F   I   V   L   S   G   N   F   F   D   S   A   I   M

301  AAA ATG TCG GTG GTG GGC GAA GCC TTC CGC AAG ACC TAC CTC AGC GAC CCG CTG CAA CCC
101▶  K   M   S   V   V   G   E   A   F   R   K   T   Y   L   S   D   P   L   Q   P

361  AAC AGC TTC GAG GCG CGG GCC ATT GTG TTC GAA GGC CCC GAA GAC TAC AC
121▶  N   S   F   E   A   R   A   I   V   F   E   G   P   E   D   Y
```

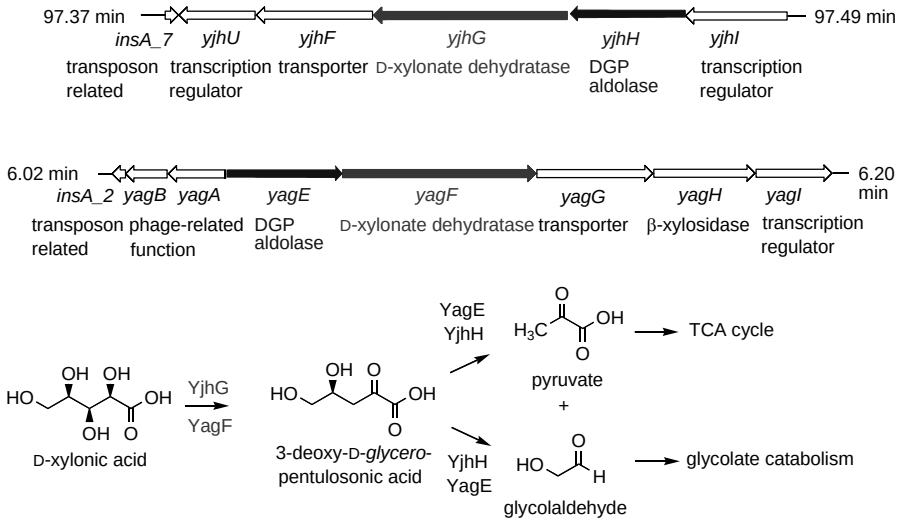
- The DNA sequence was isolated using PCR primer JWF1058 and JWF1004.
- DNA encoding the internal N-terminal sequence at position 400 is colored in red.
- The peptide has homology to hypothetical proteins from *Bradyrhizobium japonicum* USDA 110 and *Burkholderia fungorum* LB400.

Elucidation of the *Escherichia coli* D-Xylonic Acid Catabolic Pathway





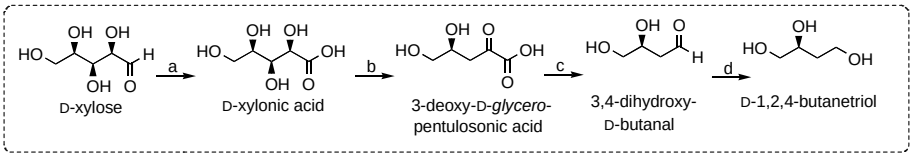
Elucidation of the *Escherichia coli* D-Xylonic Acid Catabolic Pathway



Microbial Synthesis of D-1,2,4-Butanetriol from D-Xylonic Acid Under Fermentor-Controlled Conditions

entry	*E. coli* construct	plasmid inserts	xylonic acid	BT titer	BT yield
1	W3110 *serA* pWN7.126	P_{tac}*mdlC* *serA* *lacI*^{Q}	18 g	0.08 g/L	1 %
2	W3110 *serA* Δ*yjhH* Δ*yagE* pWN7.126	P_{tac}*mdlC* *serA* *lacI*^{Q}	28 g	8 g/L	45 %

Synthesis of D-BT from D-Xylose



a. D-xylonate dehydrogenase

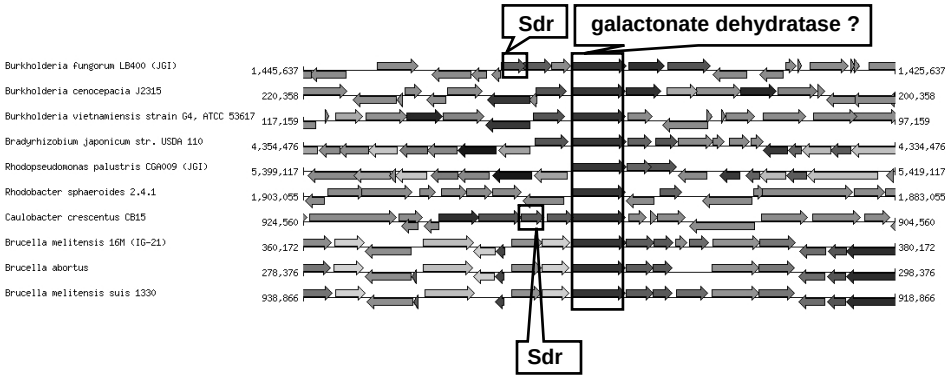
b. D-xylonate dehydratase (*E. coli*)

c. benzoylformate decarboxylase (*P. putida*)

d. alcohol dehydrogenase (*E. coli)*

Identification of a D-Xylose Dehydrogenase

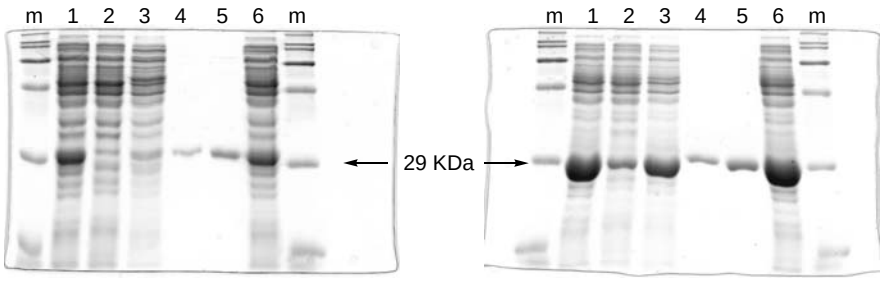
- BLAST search the ERGO database using the partial amino acid sequence of the previously isolated *P. fragi* D-xylonate dehydratase revealed Orfs with 50-70% of sequence identity.



Characterization of Putative D-Xylose Dehydrogenases

- The *xdh* gene from *Burkhoderia fungorum* LB400 and the *xdh* gene from *Caulobacter crescentus* CB15 were respectively cloned into vector pQE-30. The two D-xylose dehydrogenases were expressed in *E. coli* and purified as 6×His-tagged fusion proteins.

- SDS-PAGE for 6xHis-Xdh-LB400
- SDS-PAGE for 6xHis-Xdh-CB15



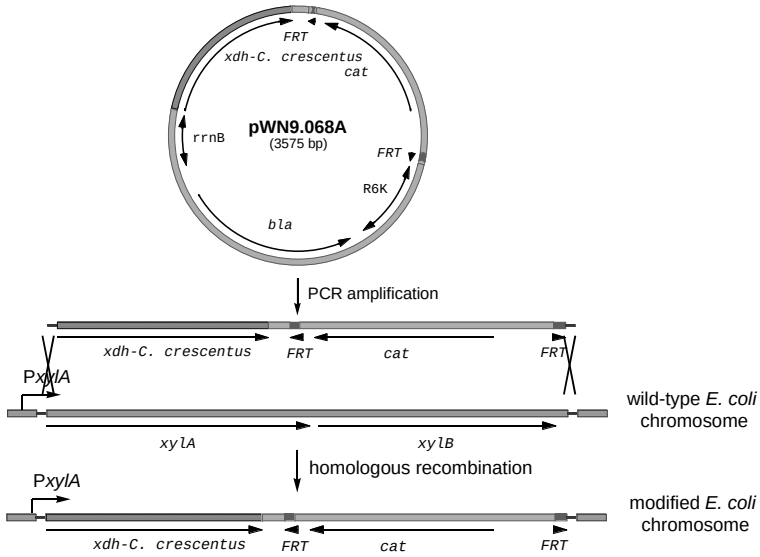
m, molecular weight markers; 1, cell lysate; 2, flow-through; 3, wash; 4, elution 1; 5, elution 2; 6, cell lysate.

Characterization of D-Xylose Dehydrogenases

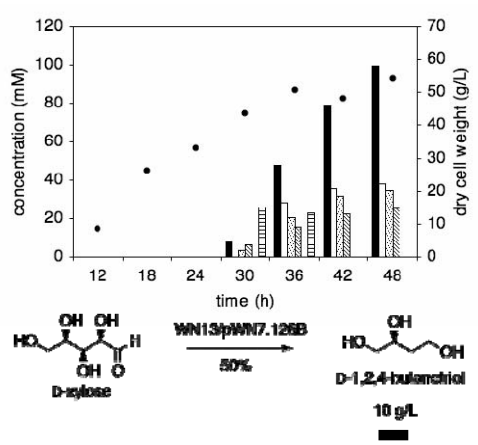
	NAD	D-xylose		D-glucose		L-arabinose	
	K_m (mM)	K_m (mM)	k_{cat}[c] (s^{-1})	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})
Xdh-*B. fungorum*	0.26[a]	0.97	29	176	12	43	13
Xdh-*C. crescentus*	0.13[a]	0.099	41	538	24	34	40

D-fructose, D-galactose, D-mannose and D-ribose were not substrates for either *B. fungorum* or *C. crescentus* xylose dehydrogenase

Insertion of *C. crescentus xdh* into the *E. coli* Chromosome

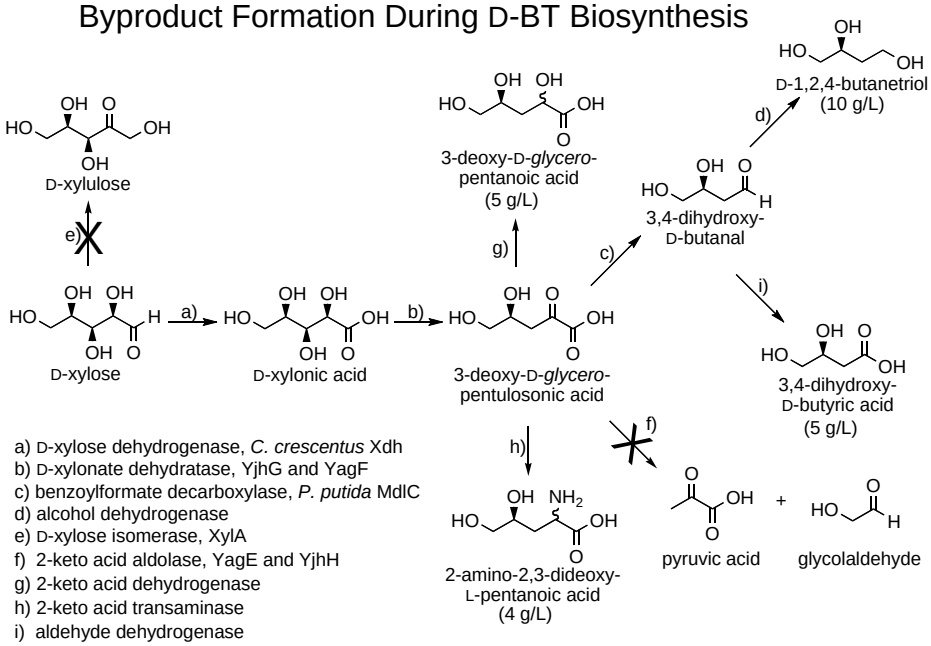


Fermentor-Controlled Synthesis of BT from Xylose



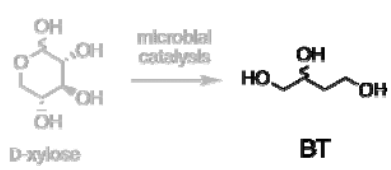
WN13: E. coli W3110*serA*Δ*yjhH*Δ*yagExylAB*::*xdh*-Cm^R pWN7.126B: *serA*, *lacI*^{Q}, P_{tac} *mdlC*

Byproduct Formation During D-BT Biosynthesis



a) D-xylose dehydrogenase, *C. crescentus* Xdh
b) D-xylonate dehydratase, YjhG and YagF
c) benzoylformate decarboxylase, *P. putida* MdlC
d) alcohol dehydrogenase
e) D-xylose isomerase, XylA
f) 2-keto acid aldolase, YagE and YjhH
g) 2-keto acid dehydrogenase
h) 2-keto acid transaminase
i) aldehyde dehydrogenase

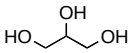
Green Synthesis of BT



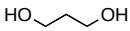
- nontoxic, renewable xylose
- reduction of salt waste streams
- avoidance of elevated temperatures and pressures
- single step
- domestic manufacture

Dual-Use Markets

C-3 Biobased



glycerol

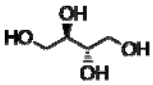


1,3-propanediol

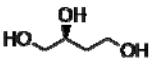
1,2-propanediol

C-4 Biobased

1,4-butanediol



erythritol



1,2,4-butanediol