Pathway Engineering via Synthetic Biology

Huimin Zhao
Energy Biosciences Institute

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Pathway Engineering via Synthetic Biology

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Department of Chemistry
Department of Biochemistry
Department of Bioengineering
Institute for Genomic Biology

ECI Metabolic Engineering Conference IX, Biarritz, France, June 6, 2012
Research Interests in Zhao Group

Pathways and Genomes
- Phloroglucinol biosynthesis
- FR900098 biosynthesis
- Xylitol biosynthesis
- Advanced biofuels
- Alkaloid biosynthesis

Tool Development
- Directed evolution
- Rational design
- High throughput methods
- Biosynthesis
- Genome engineering

Proteins
- Gene switches/circuits
- Artificial nucleases
- P450 enzymes
- Type III PKS
- N-Oxygenases

Synthesis
Analysis ↔ Modeling

Synthetic Biology

Grand Challenge #1 (Energy & Sustainability): Urgent need for oil replacement
→ Use renewable feedstocks to produce fuels, chemicals, and drugs

Grand Challenge #2 (Health): Need for new therapeutics
#1. Engineering Microbial Factories (Fuels and Natural Products @ UIUC)

Hemicellulose → L-Arabinose → L-xylitol (biorefinery building block) → PPP

Cellulose → D-Xylose → Glucose → PEP → Pyruvate → Acetyl-coA

Lignin → Cellulases → Cellulosomes → Heat/Electricity

PPP:
- Phloroglucinol (specialty chemical)
- Polyketides

Acetyl-coA:
- FR900098 (antimalarial drug)
- Butanol, Hydrocarbons (2nd generation biofuels)
- Ethanol (1st generation biofuel)
Overall Goal: Develop and apply systems and synthetic biology approaches to engineer microorganisms capable of cost-effectively producing industrial chemicals from renewable feedstocks.
First product: human insulin, produced in *E. coli* in 1978.
- Recombinant human growth hormone
- Recombinant blood clotting factor VIII
- ……

Global market size for recombinant proteins: ~$60B in 2009
Transformative Advances in DNA Sequencing and Synthesis

Carlson, Nat. Biotech. 27, 1091 (2009)

Carr and Church, Nat. Biotech. 27, 1151 (2009)

10-1000’s genes
complex chemicals and materials
organisms as products
...
Building Large DNA Molecules via One-step DNA Assembler

\[ \text{in vivo} \text{ assembly of DNA fragments in yeast} \]

Eight-gene Pathway: A Combined Xylose and Zeaxanthin Pathway
Broad Application of DNA Assembler

Pathway Engineering

Pathway Discovery

Synthetic Biology

Protein Engineering

Genome Assembly

Biofuels Production

Gibson et al. *PNAS*  
Online publication date: Dec. 10, 2008

Online publication date: Dec. 12, 2008

Discovering New Drugs
Activating Cryptic Pathways from Sequenced Genomes and Metagenomes

News & Views: Cobb and Zhao, *Nature Biotech* 2012
Engineering a Microbial Factory for Advanced Biofuels Production

Hemicellulose/Cellulose

Glucose, Xylose, Arabinose

PEP

Pyruvate

Acetyl-coA

yeast

Ethanol

Advanced biofuels (Butanol, Hydrocarbons)
Pentose Utilization in Yeast

Sugar Uptake

Heterologous Pathway

Redox Imbalance

Metabolic Flux
Glucose Repression in Mixed Sugar Fermentation

- Glucose repression occurs in *S. cerevisiae*
- Alternative carbon source fermentation is inhibited in the presence of glucose
- Lag time in xylose and arabinose consumption curve

Coexpression of Cellobiose Transporter and \( \beta \)-Glucosidase

- Cellobiose
- \( \beta \)-glucosidase
- Glucose
- Xylose
- Pentose
- Ethanol

Outside cell processes:
- Cellobiose transport
- \( \beta \)-glucosidase activity

Inside cell processes:
- Glucose metabolism
- Pentose repression

Repression mechanisms:
- Ethanol repression
- Pentose repression
Coexpression of Cellobiose Transporter and β-Glucosidase

- Cellohextrin transport system from *Neurospora crassa*
  - Cellohextrin transporters: NCU00801 (cdt1), NCU00809, NCU08114 (cdt2)
  - β-glucosidase: NCU00130 (gh1-1)

- *S. cerevisiae* with a heterologous cellohextrin transport system showed improved growth rate.

Coexpression of Cellobiose Transporter and β-Glucosidase

Genes
- 3 transporters: cdt-1, cdt-2, NCU00809
- 2 β-glucosidases: gh1-1 from N. crassa, bgl1 from A. aculeatus

Plasmids
- Use DNA assembler method to integrate genes into pRS425 plasmid

Strains
- 6 plasmids constructed were transformed into S. cerevisiae strain with an integrated xylose utilization pathway

<table>
<thead>
<tr>
<th>Strain</th>
<th>Transporter</th>
<th>β-glucosidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL01</td>
<td>cdt1</td>
<td>gt1-1</td>
</tr>
<tr>
<td>SL02</td>
<td>NCU00809</td>
<td>gt1-1</td>
</tr>
<tr>
<td>SL03</td>
<td>cdt2</td>
<td>gt1-1</td>
</tr>
<tr>
<td>SL04</td>
<td>cdt1</td>
<td>bgl1</td>
</tr>
<tr>
<td>SL05</td>
<td>NCU00809</td>
<td>bgl1</td>
</tr>
<tr>
<td>SL06</td>
<td>cdt2</td>
<td>bgl1</td>
</tr>
<tr>
<td>SL00</td>
<td>-</td>
<td>-</td>
</tr>
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</table>
Mixed Sugar Cultivation in Shake-flask: Cellobiose+Xylose

<table>
<thead>
<tr>
<th></th>
<th>SL01</th>
<th>SL00</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yield_{ethanol}</td>
<td>0.28</td>
<td>0.22</td>
</tr>
<tr>
<td>Productivity_{ethanol} (g/(L h))</td>
<td>0.23</td>
<td>0.07</td>
</tr>
</tbody>
</table>

cellobiose (■), xylose (▲), glucose(●), ethanol (▼), Dry cell weight (□)
Mixed Sugar Cultivation in Bioreactor: Cellobiose+Xylose

SL01

SL00

<table>
<thead>
<tr>
<th></th>
<th>SL01</th>
<th>SL00</th>
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</thead>
<tbody>
<tr>
<td>Yield_{ethanol}</td>
<td>0.39</td>
<td>0.24</td>
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<tr>
<td>Productivity_{ethanol (g/(L h))}</td>
<td>0.49</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Li et al. Mol Biosyst 2010
Balancing Metabolic Flux Remains a Big Challenge

- Production of value-added compounds usually requires introduction of multi-step metabolic pathways

- Metabolic flux in multistep metabolic pathways need to be optimized to avoid metabolic burden
  - Overexpression of certain genes,
  - Redox imbalance from unmatched cofactor specificity
  - Accumulation of unstable or toxic intermediates

- Traditional approaches
  - Overexpression and deletion of certain genes in metabolic pathways
  - Modulating the expression levels of individual enzymes
  - Protein engineering to improve performance of rate limiting enzymes
  - Targeting a specific enzyme instead of the overall pathway

- Simultaneous optimization of multiple metabolic genes remains a big challenge
Balancing Metabolic Flux Remains a Big Challenge

- Perturbation of global transcription machinery
- Genome-scale mapping of fitness altering genes
- Multiplex genome engineering
- Balance metabolic flux within the target pathway
  - Strengths of promoters
  - Ribosome binding sites
  - Intergenic regions
  - Synthetic scaffolds

Salis et al., Nat Biotechnol 27, 946-950 (2009)
Alper et al., Proc Natl Acad Sci U S A 102, 12678-12683 (2005)
Dueber et al., Nat Biotechnol 27, 753-759 (2009)
Alper et al., Metab Eng 9, 258-267 (2007)
Warnecke et al., Metab Eng 12, 241-250 (2010)
Pathway Optimization by COMPACTER

Customized Optimization of Metabolic Pathways by Combinatorial Transcriptional Engineering (COMPACTER)

Target pathway

Combinatorial assembly of a library of mutant pathways

Screening/selection of mutant pathways with desired phenotype
Promoter Mutants with Varying Strength

- Selected 6 yeast promoters
- Nucleotide analogue mutagenesis
- Isolating promoter mutants via FACS

Promoters used in cassette
- GPD
- GPM1
- TPI1
- TEF1

Mean Fluorescence of GFP

Alper et al. PNAS 102, 12678-12683 (2005)
Promoter Mutants with Varying Strength

**TEF1p Mutants**

**PDC1p Mutants**

**ENO2p mutants**

**FBA mutants**

**GPM mutants**
Pathway Optimization by COMPACTER

**Xylose Utilizing Pathway**

1. D-Xylose
2. XR → Xylose
3. XDH → D-Xylose
4. XKS → D-Xylose-5-phosphate
5. Non-oxidative PPP
6. Ethanol

**Cellodextrin Utilizing Pathway**

1. Extra-cellobiose
2. β-glucosidase → Intra-cellobiose
3. Celloextransin Transporter
4. Glycolysis
5. Ethanol
Optimization of the Xylose Utilizing Pathway in the INVSc1 Strain

- **Host strain**: INVSc1 (Invitrogen)
  - Diploid, auxotrophic mutation available
- **Control**
  - pRS416-PDC1p(WT)-csXR-TEF1p(WT)-ctXDH-ENO2p(WT)-ppXKS
- **Backbone**: pRS416
  - Single copy shuttle vector
- **Library size**: $10^4$~$10^5$
- **Fermentation**:
  - Initial OD~1
  - Oxygen limited condition
  - YP media

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>S3</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylose consumption rate</td>
<td>0.24</td>
<td>0.40</td>
<td>g/L/hr</td>
</tr>
<tr>
<td>Ethanol production rate</td>
<td>0.04</td>
<td>0.10</td>
<td>g/L/hr</td>
</tr>
<tr>
<td>Ethanol yield</td>
<td>0.16</td>
<td>0.25</td>
<td>g/g xylose</td>
</tr>
</tbody>
</table>

![Graph showing xylanase concentration and ethanol yield over time](image-url)
Optimization of the Xylose Utilizing Pathway in an Industrial Strain

- **Host Strain**
  - Still Spirits (Classic) Turbo Distiller’s Yeast

- **Control**
  - pRS-KanMX-PDC1p(WT)-csXR-TEF1p(WT)-ctXDH-ENO2p(WT)-ppXKS

- **Backbone**: pRS-KanMX
  - Single copy shuttle vector

- **Library size**: $10^3$~$10^4$

- **Fermentation**:
  - Initial OD~10
  - Oxygen limited condition
  - YP media

### YPD seed & YPX seed

<table>
<thead>
<tr>
<th></th>
<th>YPD seed</th>
<th>YPX seed</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Classic WT</td>
<td>Classic S7</td>
<td></td>
</tr>
<tr>
<td>Xylose consumption rate</td>
<td>0.06</td>
<td>0.74</td>
<td>0.92</td>
</tr>
<tr>
<td>Ethanol production rate</td>
<td>0</td>
<td>0.17</td>
<td>0.24</td>
</tr>
<tr>
<td>Ethanol yield</td>
<td>0</td>
<td>0.24</td>
<td>0.26</td>
</tr>
</tbody>
</table>

![Graph](image)
Host-specific Pathway Optimization

Switching optimized xylose utilizing pathways between laboratory and industrial strains

INVSc1 (switched)

Classic (switched)

xylose (■), ethanol (▼)
Host-specific Pathway Optimization

- qPCR analysis of the optimized xylose utilizing pathways between laboratory and industrial strains

![Graphs showing relative expression levels for different strains and pathways.](image)
Optimization of the Cellobiose Utilizing Pathway
Optimized Xylose Utilizing Pathways are Strain Specific

Open symbol: pathway optimized in INVSc1 strain, Solid symbol: pathway optimized in Classic strain, Red circle: cellobiose, Black square: OD (A_{600}), Blue down triangle: ethanol.
Optimized Cellobiose Utilizing Pathways are Strain Specific

- qPCR analysis of the optimized cellobiose utilizing pathways between laboratory and industrial strains
Directed Evolution for Strain Development

**Local Optimization**

Parent strain

promoter1 → Gene1 → promoter2 → Gene2

Error-prone PCR

Transformation - DNA Assembly

Screening

Global Optimization

Evolutionary cultivation

Single colony isolation/selection

Compare

If = control

If > control

Final optimized strain

Compare

If < control
Directed Evolution for Strain Development

- #9,#91 and #9118 have same final OD, ethanol concentration and glucose accumulation
- A#9118 has lower OD and higher ethanol
- A#9118 has much lower glucose accumulation
- No mutations were found in promoter regions in A#9118
Directed Evolution for Strain Development

Cellobiose fermentation performance of evolved yeast strains #9, #9-1, #9-1-18 and A#9-1-18

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>#9</th>
<th>#9-1</th>
<th>#9-1-18</th>
<th>A#9-1-18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose consumption</td>
<td>0.388</td>
<td>2.24</td>
<td>2.5</td>
<td>2.5</td>
<td>3.27</td>
</tr>
<tr>
<td>(g cellulose/L/h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethanol productivity</td>
<td>0.137</td>
<td>0.77</td>
<td>0.81</td>
<td>0.89</td>
<td>1.30</td>
</tr>
<tr>
<td>(g ethanol/L/h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yield</td>
<td>0.373</td>
<td>0.36</td>
<td>0.36</td>
<td>0.37</td>
<td>0.40</td>
</tr>
<tr>
<td>(g ethanol/g cellulose)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Consolidated Bioprocessing (CBP):

- Recombinant microbe:
  - Cellulolytic
  - Ethanologenic (yeast)

- Development towards more consolidated process

- Direct conversion of pretreated cellulosic biomass into ethanol

- Consolidated bioprocessing (CBP): save ~10-20 cents/gallon of ethanol

Direct Conversion of Cellulose to Ethanol by Engineered Mini-cellulosomes

- EG: Endoglucanase
- CBH: Exoglucanase
- BG: β-glucosidase

Yeast surface display of functional minicellulosomes
- Functional display of a mini-scaffoldin
- Successful assembly of minicellulosomes through cohesin-dockerin interaction
- Synergistic hydrolysis of cellulose
- Direct fermentation of hydrolysate (glucose) to ethanol
Direct Ethanol Production from PASC

Ethanol production

Residual PASC

Yield: 0.31 grams of ethanol per gram of PASC

62% of theoretical yield

Wen, F., Sun, J. and Zhao, H. *AEM* (2010)
Direct Conversion of Xylan to Ethanol by Engineered Hemicellulosomes

Direct Conversion of Xylan to Ethanol by Engineered Hemicellulosomes

Yield: 0.31 grams of ethanol per gram of birchwood xylan

Summary

- Developed a DNA assembler method for constructing large DNA molecules such as pathways, plasmids, and genomes.

- Developed a DNA assembler based synthetic biology method (COMPACTER) for optimizing the metabolic flux in a heterologous pathway.

- Engineered a yeast strain capable of simultaneously and efficiently utilizing C5/C6 sugars.

- Engineered yeast strains for consolidated bioprocessing of cellulose and xylan respectively.
The Zhao Group

Current Group Members

Postdocs
Zengyi Shao, Meng Wang, Xueyang Feng, Dan Coursolle

Graduate students
Carl Denard, Ning Sun
Jie Sun, Ryan Cobb, Dawn Eriksen, Jing Liang,
Yunzi Luo, Sijin Li, Luigi Chanco, Si Tong,
Todd Freestone, Guodong Rao
Zhanar Abigail, Jiazhang Lian
Jinglin Li, Sai Wen, Zehua Bao, Jonathan Ning,
Chao Ran, Sujit Jagtap

Alumni
Dr. Hua Zhao, Dr. Byoung-jin Kim, Dr. Jing Du,
Dr. Yongbo Yuan, Dr. Fei Wen

Collaborators
Wilfred van der Donk, Bill Metcalf, Satish Nair, Neil Kelleher, Nathan Price, Steve Long

Funding Support
NIH, Keck Foundation, BP EBI, ARPA-E

Undergraduate students
Lu Lu, Amy Oreskovic
Wei Yang, Patrick Lynn
Mark your calendars

For ECI’s Biochemical and Molecular Engineering Conference to be held in Beijing, China
June 16 to 20, 2013

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Frontiers in Biological Design, Synthetic Biology and Processing
East Meets West

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Co-chairs: Huimin Zhao (U. Illinois), David Robinson (Merck), and Tianwei Tan (Beijing)
Advisory Committee Co-Chairs: Weichang Zhou (USA), and Guoping Zhao (China)

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