Institute of Biological Engineering

2008 Annual Conference

A PLATFORM FOR PARTNERSHIPS AND PROGRESS
CIII Advances in Engineering
Metabolism & Microbial
Conversion

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Manipulation and Balance of Reducing Equivalents to Enhance Productivity of Chemicals in E. coli
Cofactor Engineering

- Coenzyme A and acetyl coenzyme-A (CoA and acetyl-CoA)
  - NAD(P)H/NAD(P)^+ Cofactor Pair
Recycle of cofactors necessary for cell growth

NAD(P)H/NADP⁺ Cofactor Pair

- Donor or acceptor of reducing equivalents
- Important in metabolism
  - Cofactor in >300 red-ox reactions
  - Regulates genes and enzymes
- Reversible transformation

Recycle of cofactors necessary for cell growth
NADH/NAD$^+$ cofactor pair

If product needs more reductant can use a NADH recycling system for increased availability
Simplified Fermentation Pathway of *E. coli*

- **Glucose**
- **Pyruvate**
- **Acetyl-CoA**
- **Succinate**
- **Formate**
- **Lactate**
- **Ethanol**
- **Acetate**

Some reducing equivalents are trapped in formate.
Methylotrophic yeasts grow on methanol and have an active NAD-Formate dehydrogenase in cytosol.

Candida boidinii

Diagram from Hartner & Glieder 2006
Strain study (Shake Tubes)

Control: GJT001 (pDHK29)
Mutant: BS1 (pSBF2)
Carbon source: glucose

pDHK29: cloning vector serve as control
pSBF2: pDHK29 carrying a NAD-dependent FDH
BS1: GJT001 lacking native FDH
% of Increase/Decrease for BS1 (pSBF2) relative to GJT001 (pDHK29)

- Glucose consumption: 3-fold increase
- Pyruvate: 2NAD+ 2NADH
- Acetyl-CoA: 8-fold increase, 15-fold increase in Ethanol
- Lactate: 91% decrease
- Succinate: 55% increase
- Formate: 8-fold increase in conversion
- O.D.600: 59%
- Et/Ac: 27-fold

Anaerobic Tube Experiment

New FDH competes effectively with native FDH for available formate (fdh- mutation not necessary)

- **pDHK29**: cloning vector serves as control
- **pSBF2**: pDHK29 carrying a NAD-dependent FDH
- **BS1**: GJT001 lacking native FDH

Effect of NADH regeneration (overexpressing NAD+-dependent FDH)

- Drastic increase in ethanol/acetate ratio
- The new FDH competes effectively with native FDH for available formate (*fdh*- mutation not necessary)
- Increase in intracellular NADH availability allows increase reduced product yields (such as ethanol)
NADPH/NADP+

Usually formed in quantity by pentose phosphate pathway or isocitrate conversion

\[
\beta-D\text{-glucose-6-phosphate} + \text{NADP}^+ = D\text{-glucono-\(\delta\text{-lactone-6-phosphate}\)} + \text{NADPH} + H^+ \\
D\text{-isocitrate} + \text{NADP}^+ = \text{NADPH} + 2\text{-ketoglutarate} + \text{CO}_2
\]

Exchange reactions in E coli

\[
\text{NAD}^+ + \text{NADPH} \leftrightarrow \text{NADH} + \text{NADP}^+
\]

pntAB system (membrane bound)
udh system sthA (soluble)
• Many reactions use this reductant
• Can engineer a specific protein that uses NADH instead of NADPH (sometimes modified protein works but may be less efficient)
• We are interested in overall cell network change and use in cell (more metabolic engineering than protein engineering)
Model Product Experiment

Poly(3-hydroxybutyrate) (PHB)
PHB Production (Shake Flasks)

Control: GJT001 (pDHK29, pAET29)
Mutant: GJT001 (pUDHAK, pAET29)

- **pDHK29**: cloning vector serve as control
- **pUDHAK**: pDHK29 carrying the soluble pyridine nucleotide transhydrogenase (udhA)
- **pAeT29**: plasmid carrying the PHB biosynthesis pathway
Production of PHB

- **pDHK29**: control plasmid
- **pUDHAK**: pDHK29 carrying the soluble pyridine nucleotide transhydrogenase (*udhA*)
- **pAeT29**: plasmid carrying the PHB genes

Strain carrying UdhA produces a significantly higher quantity of PHB – a product that requires NADPH for its biosynthesis.

This study suggested higher availability of NADPH could lead to observed change in metabolites

- The transhydrogenase offered a way to help convert part of the NADH pool to useful NADPH
- Optional to cell

- Would like to force cell to make more NADPH
- Connect to required carbon pathway
A Direct Approach

Metabolic engineer *E. coli* central metabolism to increase **NADPH** availability
**Pentose Phosphate Pathway**

1. Glucose → Glucose-6-P → F6P → G3P → 3PG
2. Ribulose-5-P → Ribose-5-P → Xylulose-5-P
3. S7P → G3P → F6P → E4P

**Glycolysis**

1. Glucose → Glucose-6-P → F6P → G3P → 3PG
2. Pyruvate → Lactate
3. Formate → H₂
4. Acetyl-CoA → CO₂
5. ATP → Acetate

**TCA cycle**

1. 2 CO₂ → 3 NADH → 1 FADH₂

**GapA**

Glyceraldehyde-3-phosphate dehydrogenase
Potential Sources of an NADPH dependent GAPDH

- Plants (small preference for NADPH, highly regulated) EC 1.2.1.13 and non-phosphorylating EC 1.2.1.9 types
- Methanothermus fervidus, Synechococcus PCC7942
- Streptococcus pyogenes
- Clostridium acetobutylicum
- Structure of NADH dependent GapN from Hyperthermophilic Archaeum *Thermoproteus tenax* (Pohl et al JBC 277, 19938-19945, 2002) NADH dependent
Strategy

- eliminate the native NAD\(^+\)-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from \textit{E. coli}

- replace it with an NADP\(^+\)-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from \textit{C. acetobutylicum}
**Strategy**

GapA: glyceraldehyde-3-phosphate dehydrogenase (E. coli)

GapN: glyceraldehyde-3-phosphate dehydrogenase (C. acetobutylicum)
Two moles of NADPH will be formed per mole of glucose passing through the glycolysis pathway.

GapA: glyceraldehyde-3-phosphate dehydrogenase (E. coli)

GapN: glyceraldehyde-3-phosphate dehydrogenase (C. acetobutylicum)
**Strains**

Control: MG1655 pDHC29

Mutant: MG1655 $\Delta$gapA pHL621

- pDHC29: cloning vector serve as control
- pHL621: pDHC29 carrying a NADP$^+$-dependent GAP
Metabolic Flux Analysis

using C-13 labeling
Control: MG1655 pDHC29
Mutant: MG1655 ΔgapA pHL621
So see quite a difference in partitioning through network

- See if this can be exploited with an appropriate sink for NADPH
Model Product Experiments

- Lycopene Production
- Poly(3-hydroxybutyrate) (PHB)
Model Product Experiments

Lycopene Production
Lycopene Synthesis

Non-mevalonate pathway

\[ 8 \text{ G3P} + 8 \text{ Pyr} + 16 \text{ NADPH} + 8 \text{ CTP} + 8 \text{ ATP} \]

\[ 1 \text{ Lycopene} + 8 \text{ CO}_2 + 16 \text{ NADP}^+ + 8 \text{ CMP} + 8 \text{ ADP} + 12 \text{ PPI} \]
Lycopene Production (Shake Flask)

Control: MG1655 (pDHC29, pK19-Lyco)

Mutant: MG1655 $\Delta$gapA (pHL621, pK19-lyco)

- pDHC29: cloning vector serve as control
- pHL621: pDHC29 carrying a NADPH-dependent GAP
- pK19-lyco: plasmid carrying the lycopene biosynthesis pathway
Lycopene Production (24 hr Shake Flask, OD about same)

Control: MG1655 (pDHC29 pK19-Lyco)
Mutant: MG1655 \( \Delta \)gapA (pHL621 pK19-lyco)

New strain reaches to a slightly higher final optical density in both LB and 2YT media.
Lycopene Production (Shake Flask)

Lycopene concentration

![Graph showing lycopene concentration in LB and 2YT media for Control and Mutant samples.]

- Final lycopene concentration increased by >250%
- Specific lycopene production increased by >200%
Model Product Experiments

Poly(3-hydroxybutyrate) (PHB)
Control: MG1655 (pDHC29, pAeT29)

Mutant: MG1655 \( \Delta \text{gapA} \) (pHL621, pAeT29)

- pDHC29: cloning vector serve as control
- pHIL621: pDHC29 carrying a NADPH-dependent GAP
- pAeT29: plasmid carrying the PHB biosynthesis pathway
• Higher final PHB production at the lower temperature
• Mutant strain yielded significantly higher PHB than the control strain
Model Product Experiments

Whole cell single step conversion involving a NADPH-dependent reaction
Whole Cell Single Step Conversion

\[
\text{cyclohexanone} + O_2 + \text{NADPH} + H^+ \xrightarrow{\text{CHMO}} \text{ε-caprolactone} + H_2O + \text{NADP}^+
\]

Whole Cell Single Step Conversion

Control: BL21(pDHC29, pMM4)

Mutant: BL21\Delta\text{gapA}(pHL621, pMM4)

- **pDHC29**: cloning vector serve as control
- **pHL621**: pDHC29 carrying a NADPH-dependent GAP
- **pMM4**: plasmid carrying the cyclohexanone monooxygenase from Stewart U Fla
Conclusions

- Various approaches to increase NAD(P)H availability
- Replacement of native GAPDH from E. coli with the NADP⁺-dependent GAPDH from C. acetobutylicum shows big changes
- We increased the synthesis of NADPH-dependent products PHB and lycopene.
- We have shown that the system is also applicable for single step conversion with improved rates and glucose yield
- This metabolic engineered strain will be useful for future applications where high levels of NADPH are required.
Acknowledgments

Susana Berrios-Rivera
Ailen Sanchez
Irene Martínez
Mary Harrison
Dr. Jiangfeng Zhu

Funding source:
The National Science Foundation