
Institute of Biological Engineering
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AND PROGRESS
08

CIII Advances in Engineering Metabolism & Microbial Conversion

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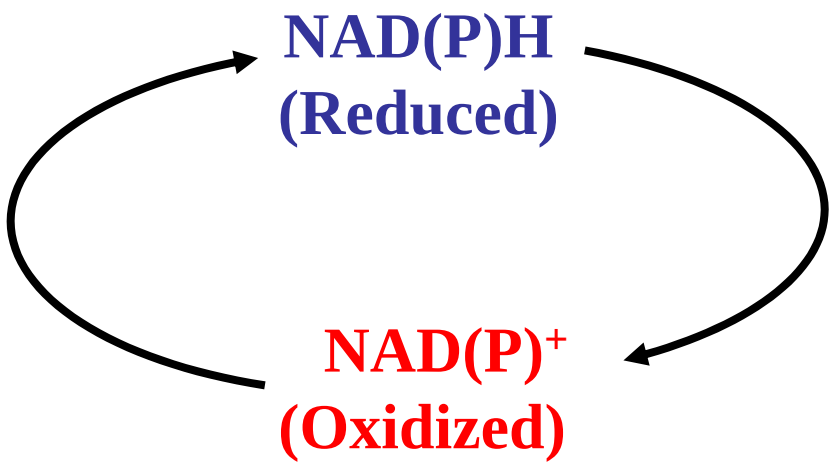
Manipulation and Balance of Reducing Equivalents to Enhance Productivity of Chemicals in E. coli

Cofactor Engineering

- Coenzyme A and acetyl coenzyme-A (CoA and acetyl-CoA)
- NAD(P)H/NAD(P)$^+$ Cofactor Pair 

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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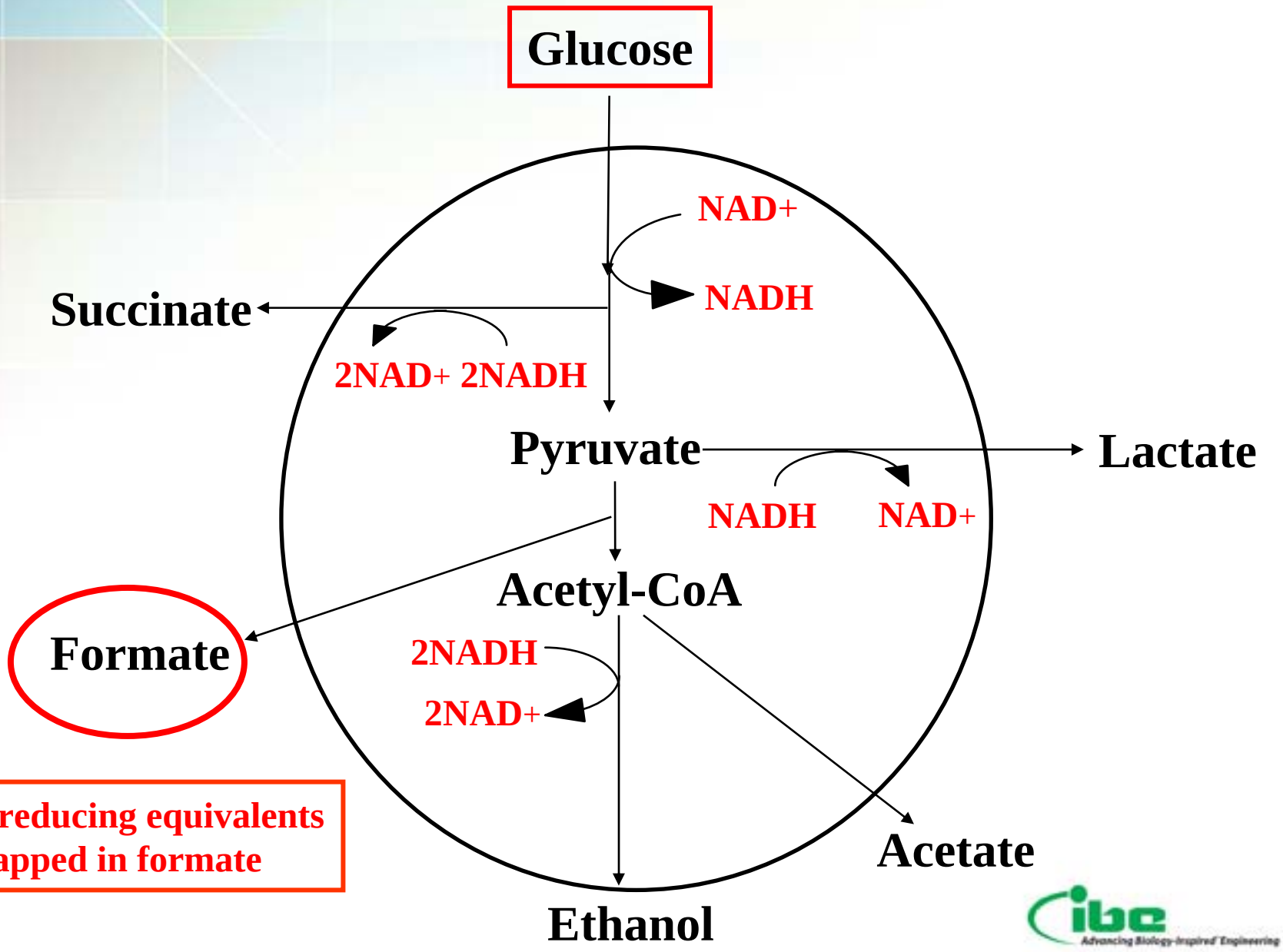
NADH/NAD$^+$ cofactor pair

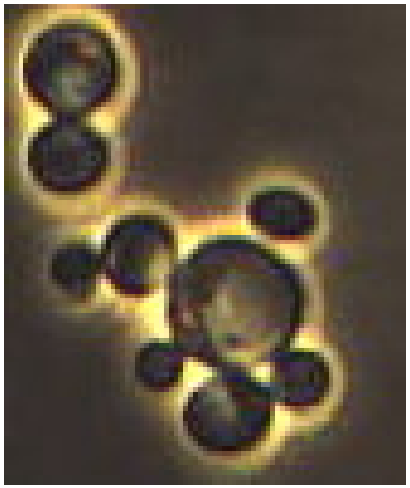
If product needs more reductant can use a NADH recycling system for increased availability

Simplified Fermentation Pathway of *E. coli*



Some reducing equivalents are trapped in formate

Methylotrophic yeasts grow on methanol and have an active NAD-Formate dehydrogenase in cytosol



Candida boidinii

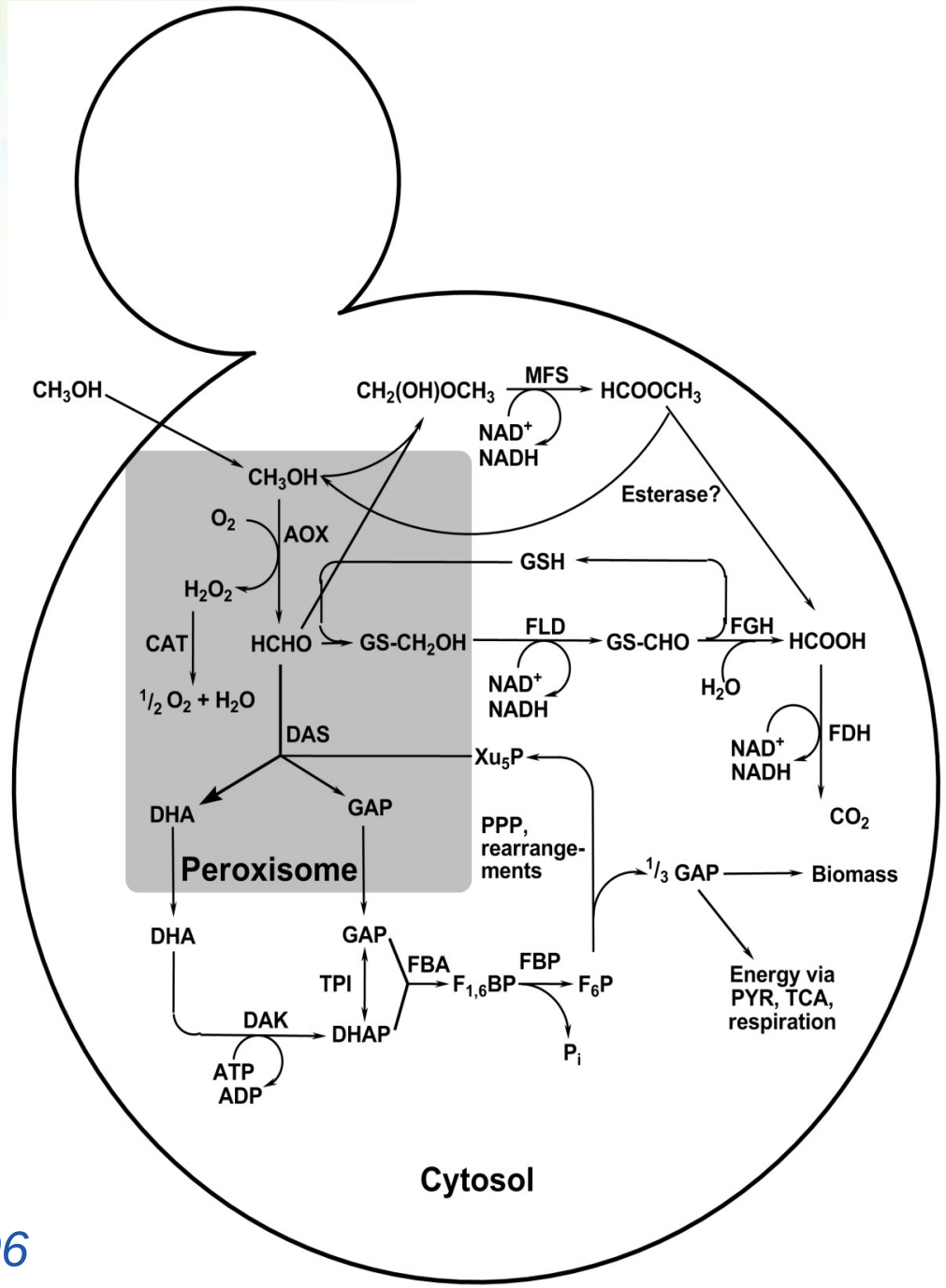


Diagram from Hartner & Glieder 2006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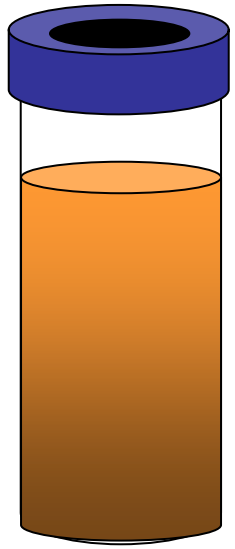
Strain study (Shake Tubes)

Control: GJT001 (pDHK29)

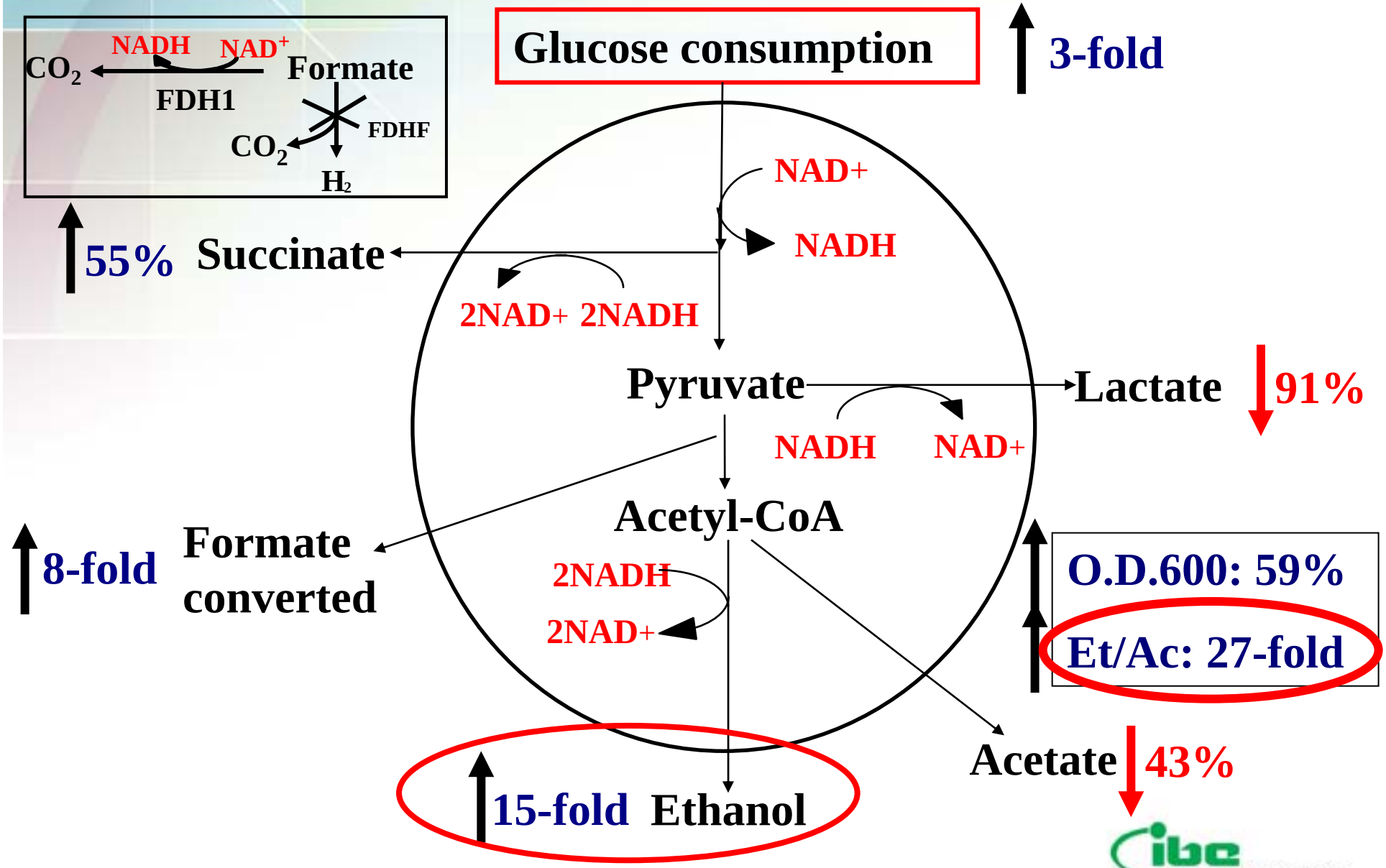
Mutant: BS1 (pSBF2)

Carbon source: glucose



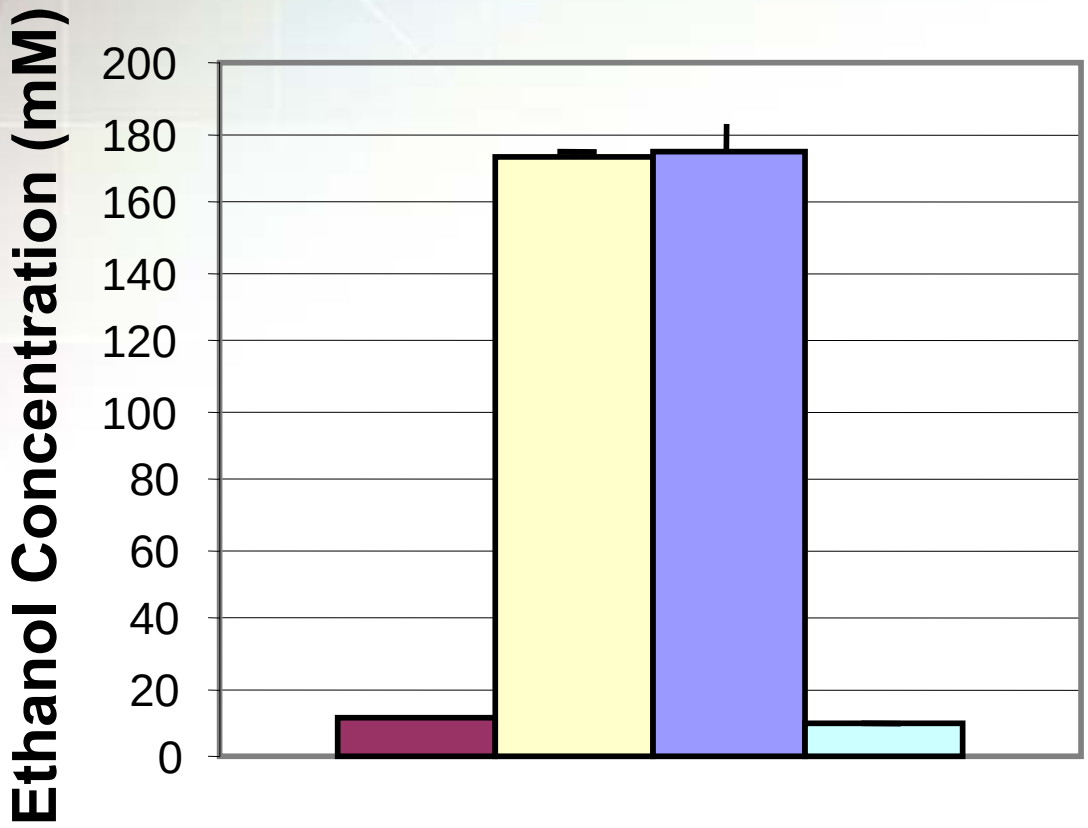
pDHK29: cloning vector serve as control
pSBF2: pDHK29 carrying a NAD-dependent FDH
BS1: GJT001 lacking native FDH

% of Increase/Decrease for BS1 (pSBF2) relative to GJT001 (pDHK29)



Berríos-Rivera et al., Metabolic Engineering, 4: 217-229 (2002)

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

Berríos-Rivera et al., Metabolic Engineering, 4: 217-229 (2002)

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)
- Other applications using in vivo FDH have included fructose to mannitol conversion (Kaup B, Bringer-Meyer S, Sahm H. Metabolic engineering of Escherichia coli: construction of an efficient biocatalyst for D-mannitol formation in a whole-cell biotransformation. Appl Microbiol Biotechnol. 2004 Apr;64(3):333-9).

NADPH/NADP+

Usually formed in quantity by pentose phosphate pathway or isocitrate conversion

β-D-glucose-6-phosphate + NADP+ = D-glucono-δ-lactone-6-phosphate + NADPH + H+

D-isocitrate + NADP+ = NADPH + 2-ketoglutarate + CO2

Exchange reactions in E coli

NAD+ + NADPH <=> NADH + NADP+

pntAB system (membrane bound)

udh system sthA (soluble)

NADPH

- 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)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Model Product Experiment

Poly(3-hydroxybutyrate) (PHB)


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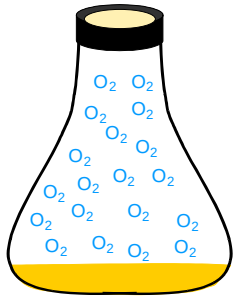
PHB Production (Shake Flasks)

Control: GJT001 (pDHK29, pAET29)

Mutant: GJT001 (pUDHAK, pAET29)

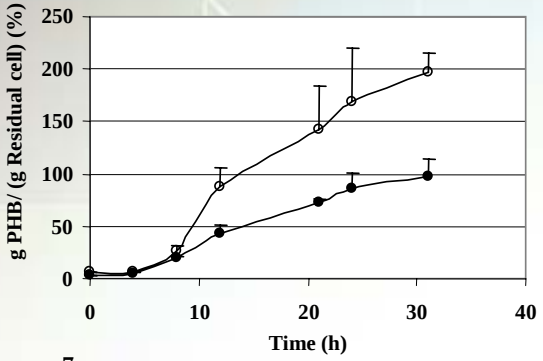


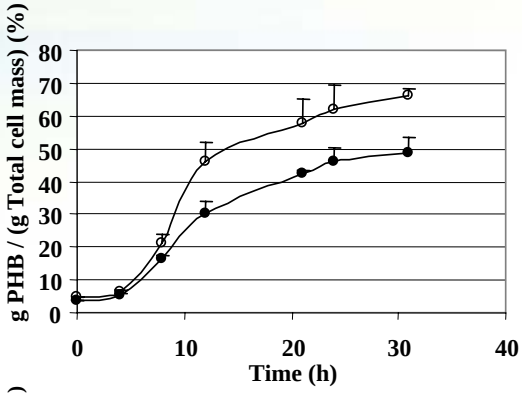
pDHK29: cloning vector serve as control

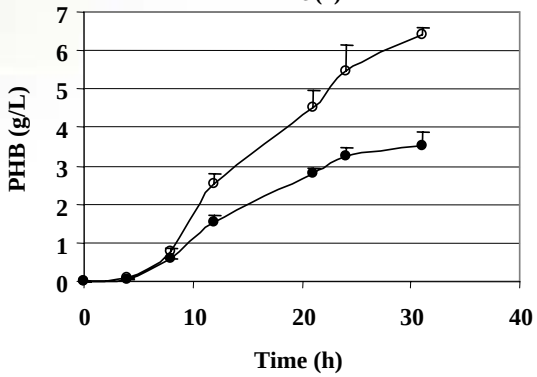
pUDHAK: pDHK29 carrying the soluble pyridine nucleotide transhydrogenase (udhA)

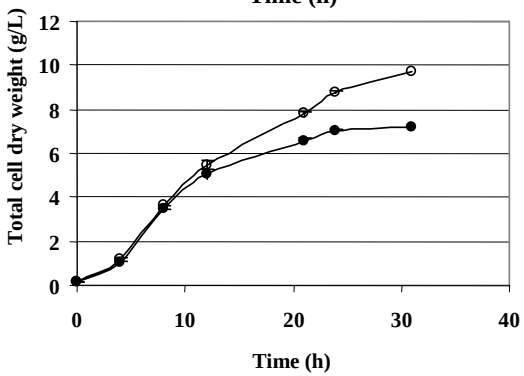
pAeT29 : plasmid carrying the PHB biosynthesis pathway

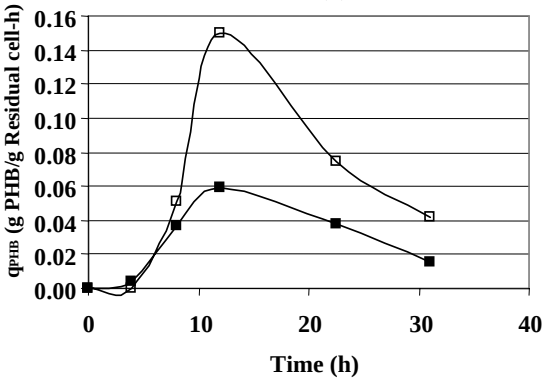
Production of PHB

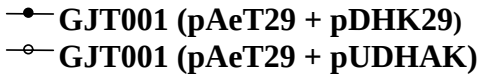












Strain carrying UdhA produces a significantly higher quantity of PHB – a product that requires NADPH for its biosynthesis

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

Sánchez et al., Biotechnology Progress, 22(2):420-5 (2006)

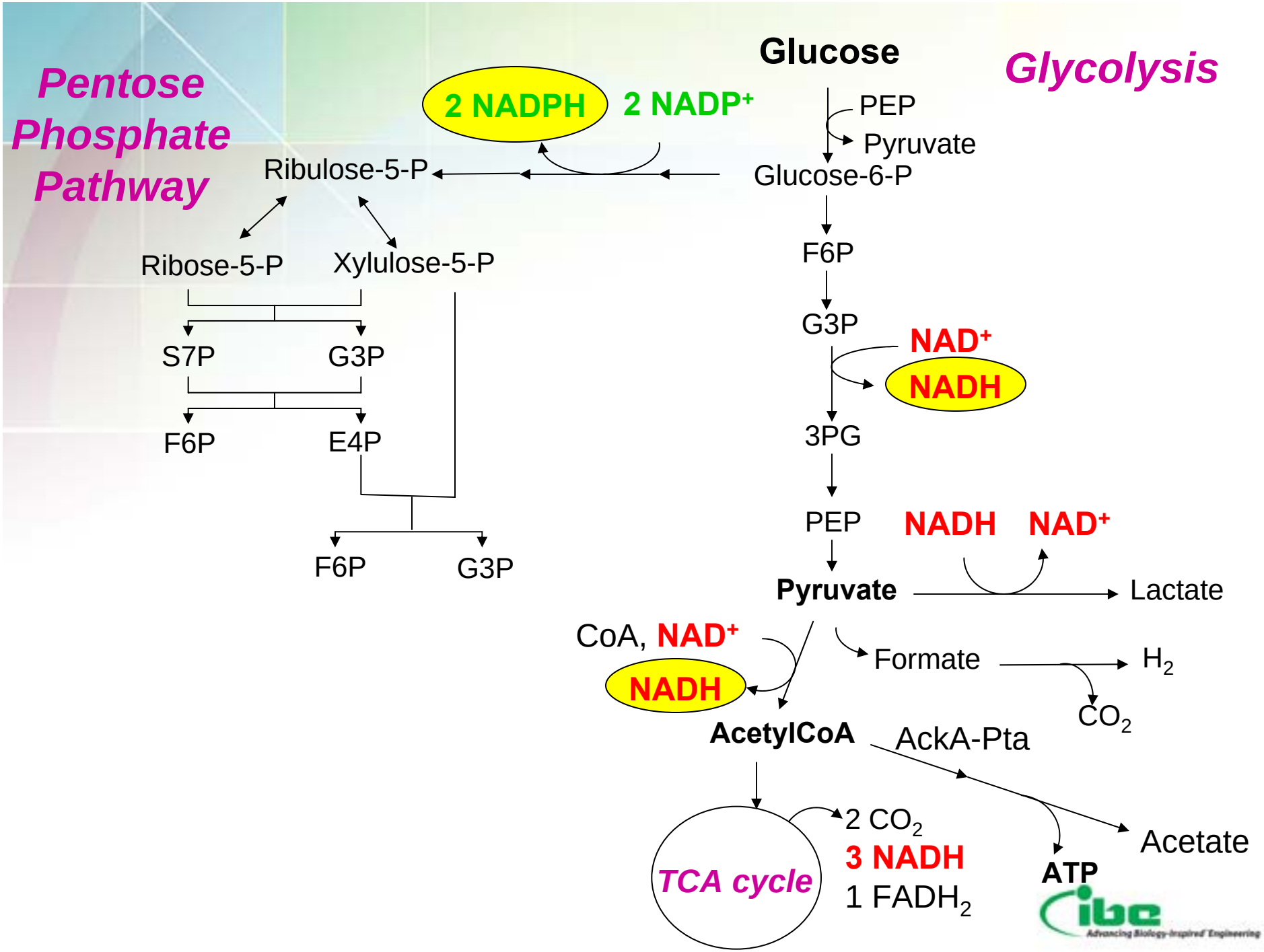
This study suggested higher availability of NADPH could lead to observed change in metabolites

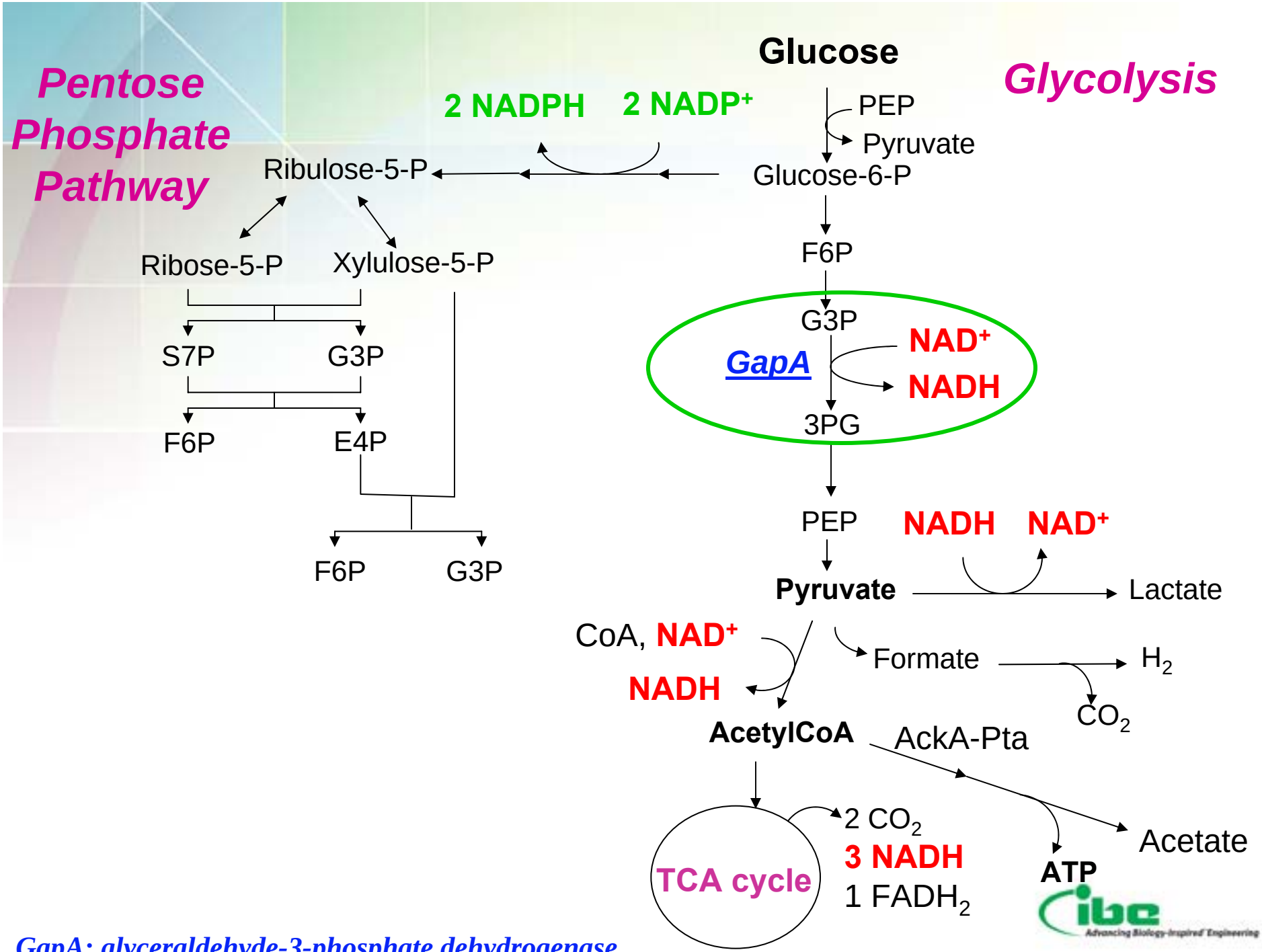
- The transhydrogenase offered a way to help convert part of the NADH pool to useful NADPH
- Optional to cell

- 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

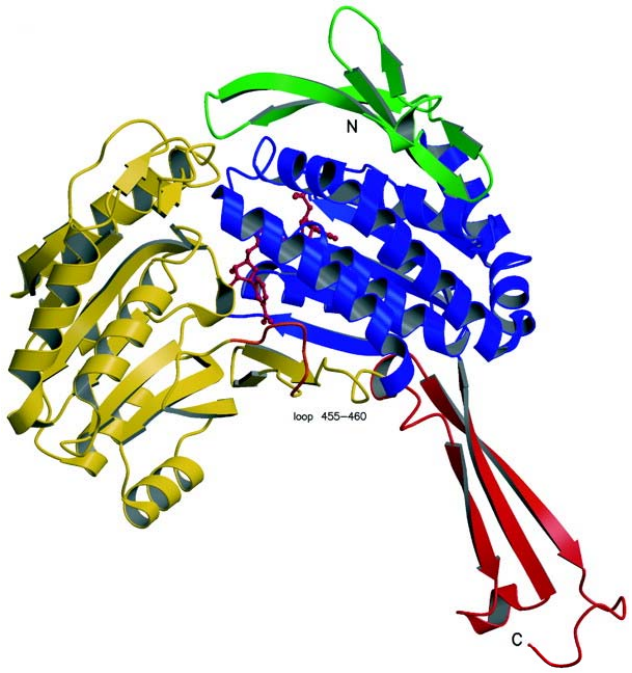

Pentose Phosphate Pathway
Glycolysis
Glucose
2 NADPH
2 NADP+
PEP
Pyruvate
Ribulose-5-P
Glucose-6-P
Ribose-5-P
Xylulose-5-P
F6P
G3P
NAD+
NADH
S7P
G3P
3PG
F6P
E4P
PEP
NADH
NAD+
Pyruvate
Lactate
F6P
G3P
CoA, NAD+
NADH
Formate
H2
CO2
AcetylCoA
AckA-Pta
2 CO2
3 NADH
1 FADH2
TCA cycle
Acetate
ATP



GapA: glyceraldehyde-3-phosphate dehydrogenase

Potential Sources of an NADPH dependent GAPDH

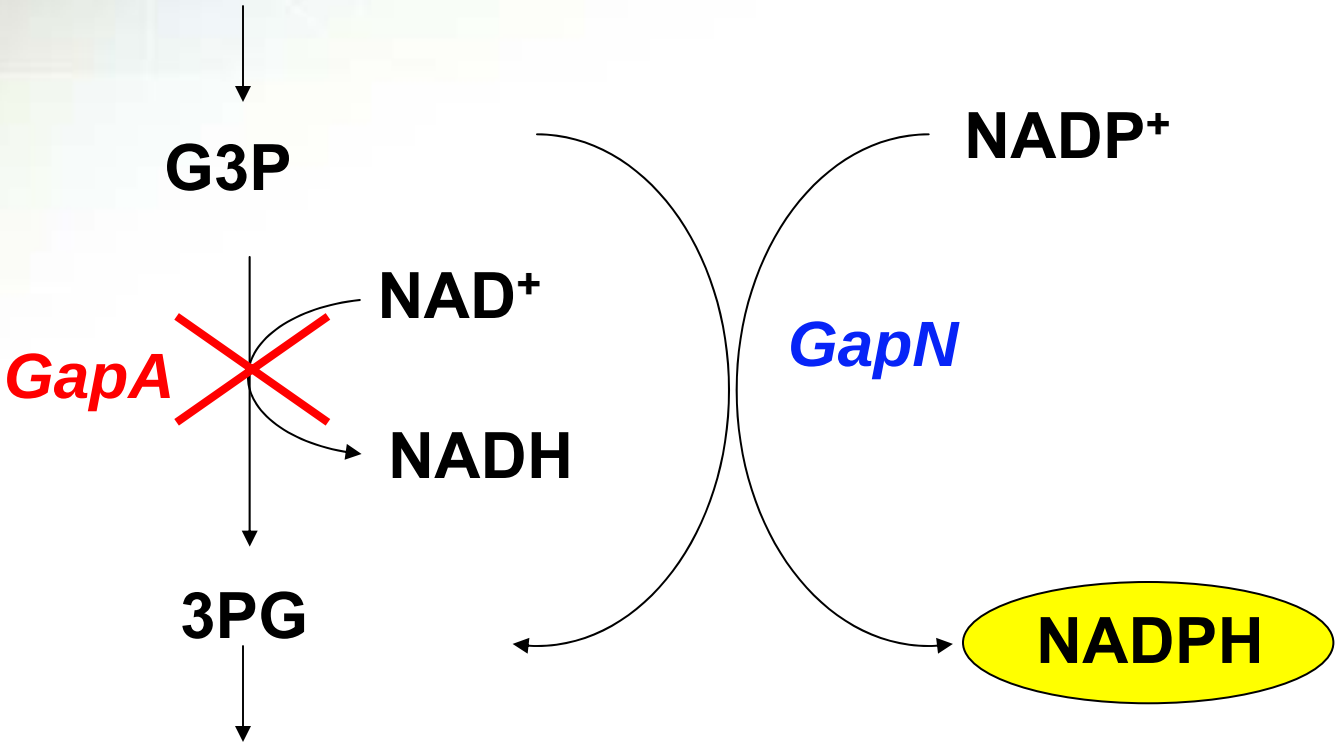
- **Plants (small preference for NADPH, highly regulated) EC 1.2.1.13 and non-phosphorylating EC 1.2.1.9 types**
- **Methanothermus fervidus, Synechococcus PCC7942**
- **Streptococcus pyrogenes**
- **Clostridium acetobutylicum**
- **Structure of NADH dependent GapN from Hyperthermophilic Archaeum *Thermoproteus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



Strategy

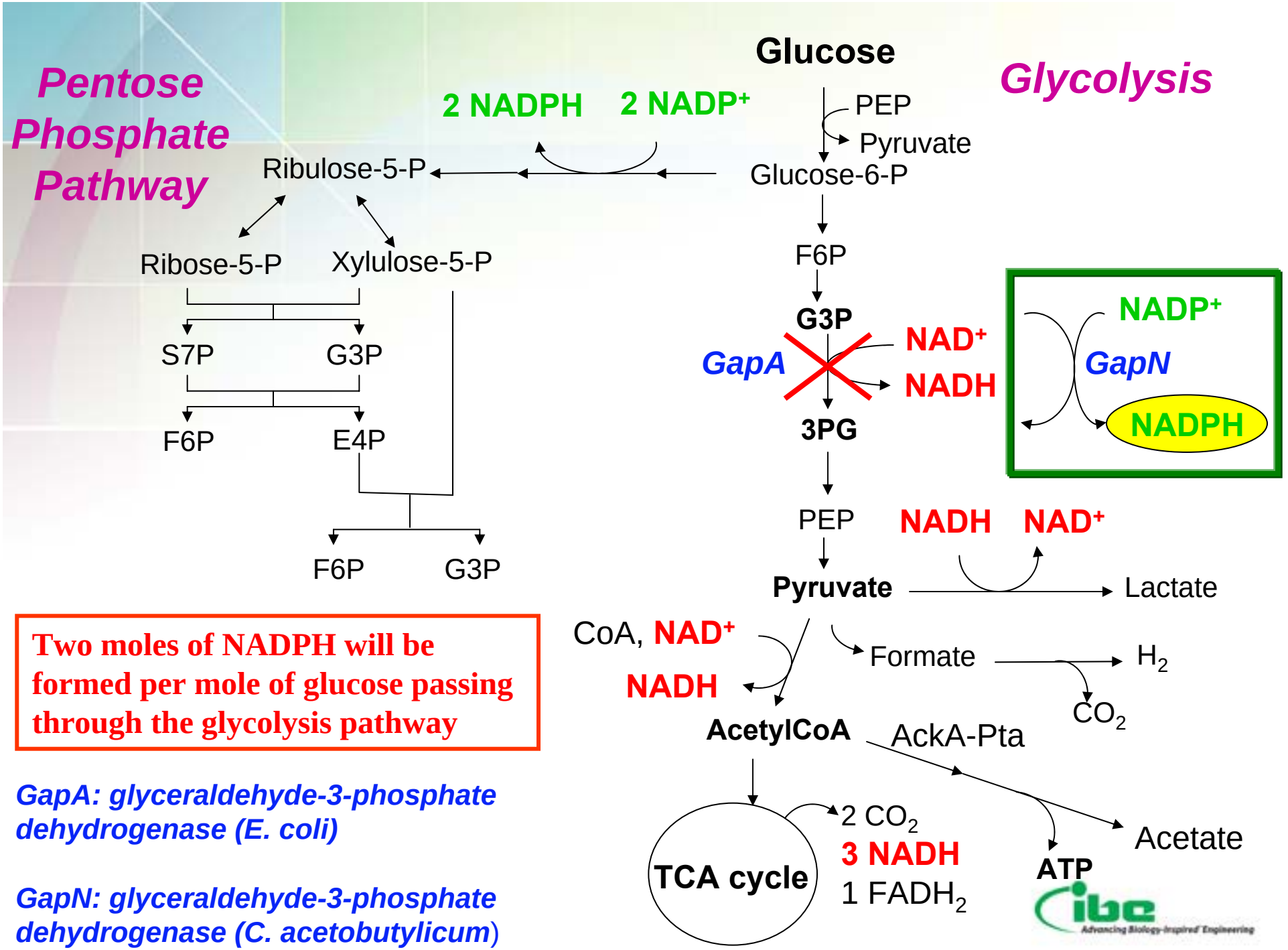
- eliminate the native NAD^+-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *E. coli*
- replace it with an $NADP^+$-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *C. acetobutylicum*

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 *ΔgapA* pHL621

pDHC29: cloning vector serve as control

pHL621: pDHC29 carrying a $NADP^+$-dependent GAP

Metabolic Flux Analysis

using C-13 labeling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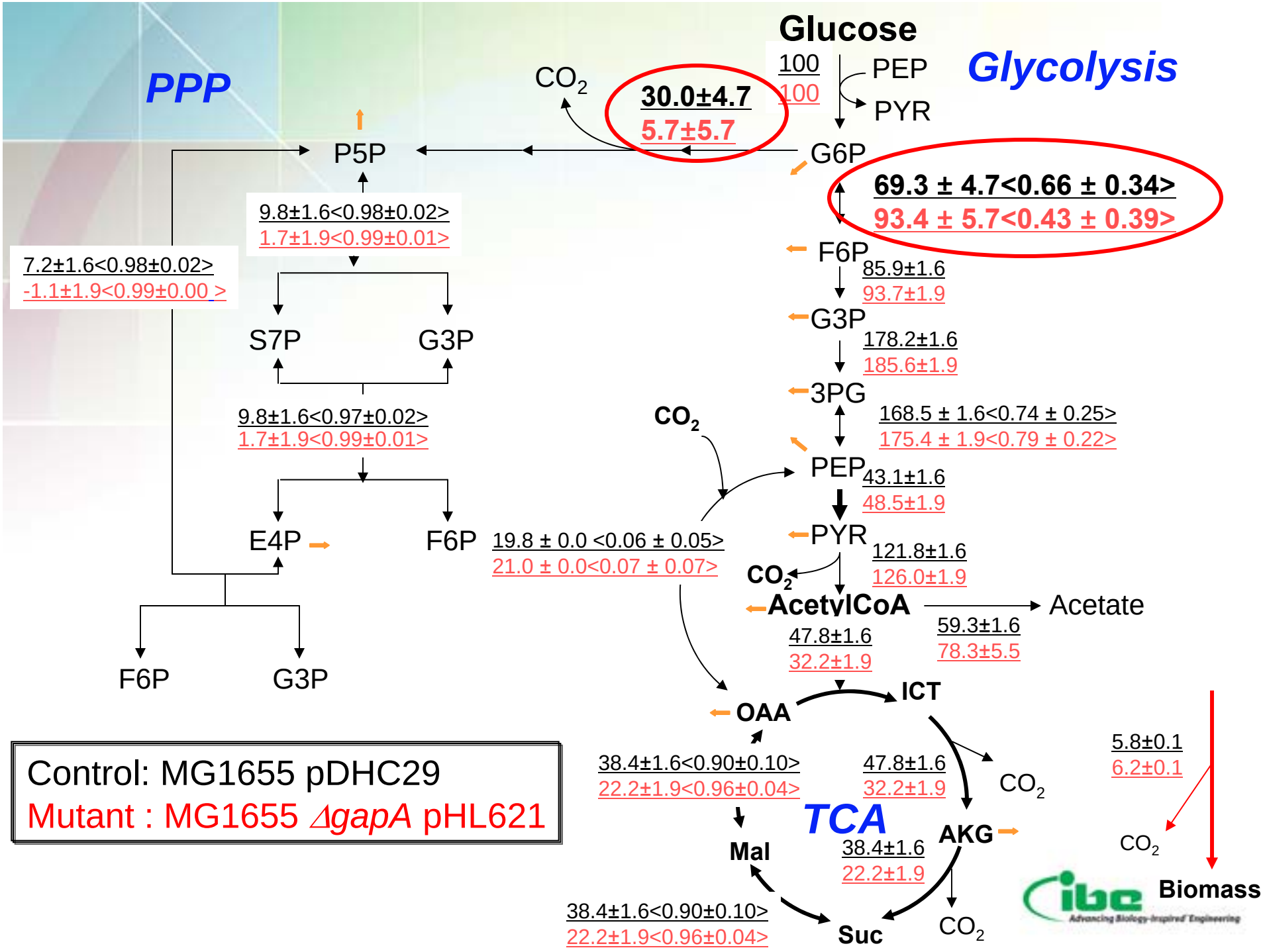

PPP
Glycolysis
Glucose
100
100
PEP
PYR
CO2
30.0±4.7
5.7±5.7
P5P
G6P
69.3 ± 4.7<0.66 ± 0.34>
93.4 ± 5.7<0.43 ± 0.39>
9.8±1.6<0.98±0.02>
1.7±1.9<0.99±0.01>
7.2±1.6<0.98±0.02>
-1.1±1.9<0.99±0.00 >
F6P
85.9±1.6
93.7±1.9
S7P
G3P
G3P
178.2±1.6
185.6±1.9
3PG
9.8±1.6<0.97±0.02>
1.7±1.9<0.99±0.01>
168.5 ± 1.6<0.74 ± 0.25>
175.4 ± 1.9<0.79 ± 0.22>
CO2
PEP
43.1±1.6
48.5±1.9
E4P
F6P
19.8 ± 0.0 <0.06 ± 0.05>
21.0 ± 0.0<0.07 ± 0.07>
PYR
121.8±1.6
126.0±1.9
CO2
AcetylCoA
Acetate
59.3±1.6
78.3±5.5
47.8±1.6
32.2±1.9
F6P
G3P
OAA
ICT
5.8±0.1
6.2±0.1
38.4±1.6<0.90±0.10>
22.2±1.9<0.96±0.04>
47.8±1.6
32.2±1.9
CO2
TCA
Mal
AKG
38.4±1.6
22.2±1.9
CO2
Biomass
38.4±1.6<0.90±0.10>
22.2±1.9<0.96±0.04>
Suc
CO2
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)*

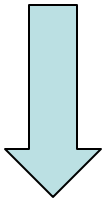
Model Product Experiments

Lycopene Production

Lycopene Synthesis

Non-mevalonate pathway

8 G3P + 8 Pyr + 16 *NADPH* + 8 *CTP* + 8 *ATP*

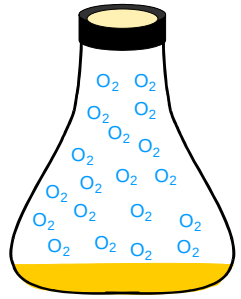


1 Lycopene + 8 CO_2 + 16 *$NADP^+$* + 8 *CMP* + 8 *ADP* + 12 *PPi*

Lycopene Production (Shake Flask)

Control: MG1655 (pDHC29, pK19-Lyco)

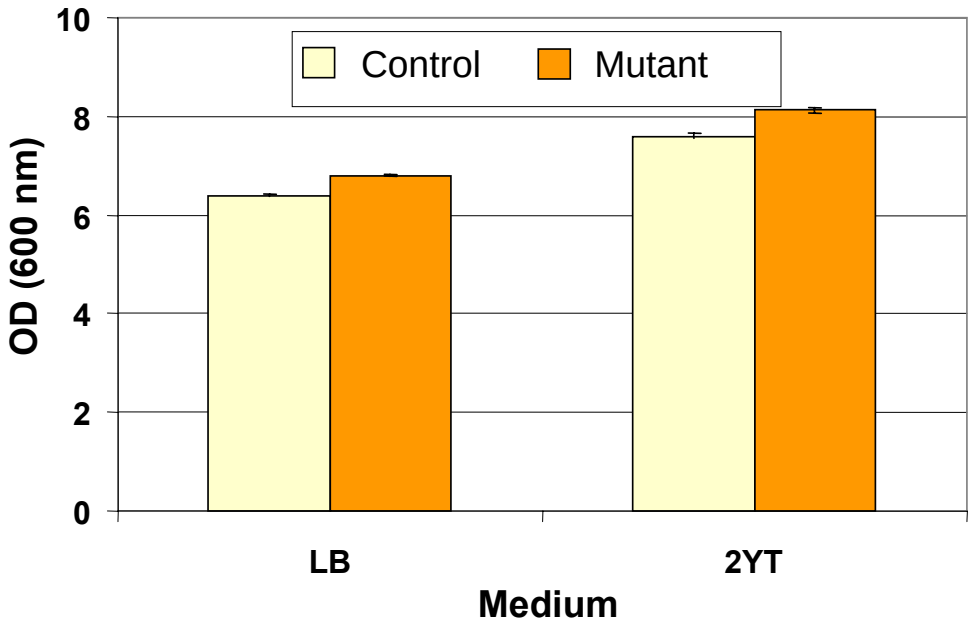
Mutant: MG1655 *ΔgapA* (pHL621, pK19-lyco)



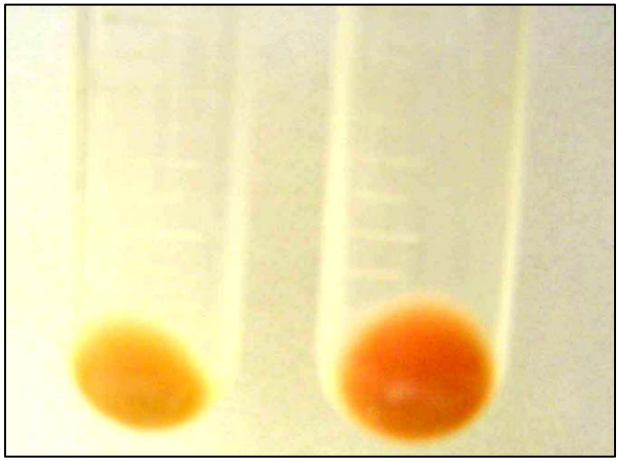
pDHC29: cloning vector serve as control
pHL621: pDHC29 carrying a NADPH-dependent GAP
pK19-lyco: plasmid carrying the lycopene biosynthesis pathway

Lycopene Production (24 hr Shake Flask, OD about same)

OD (24h, 30°C, 250 rpm)



Control Mutant

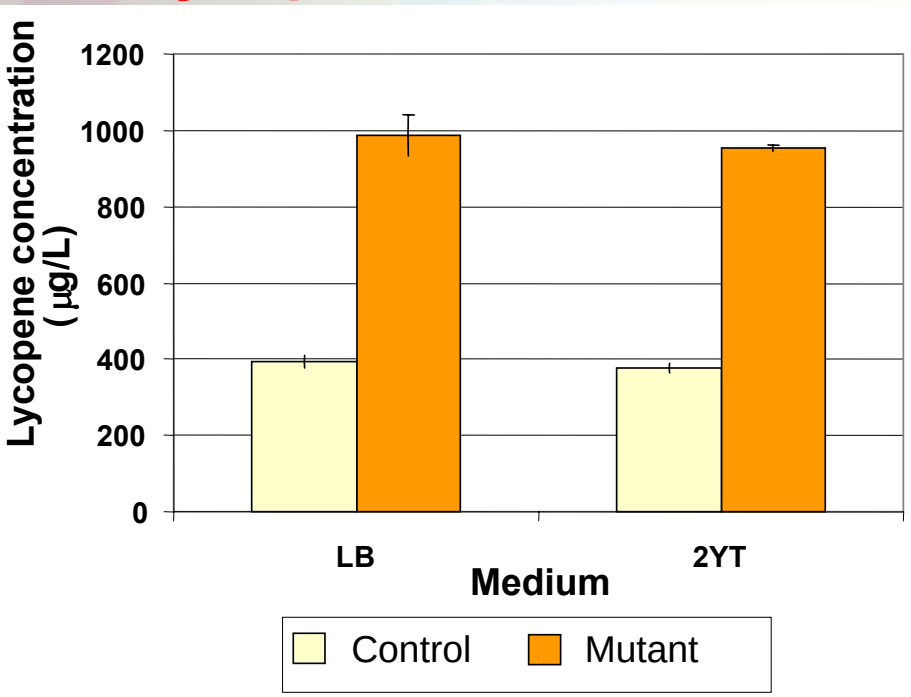


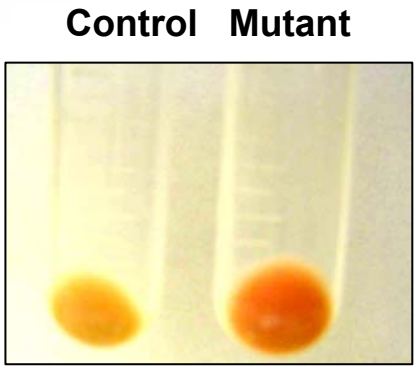
New strain reaches to a slightly higher final optical density in both LB and 2YT media

Control: MG1655 (pDHC29 pK19-Lyco)
Mutant: MG1655 *ΔgapA* (pHL621 pK19-lyco)

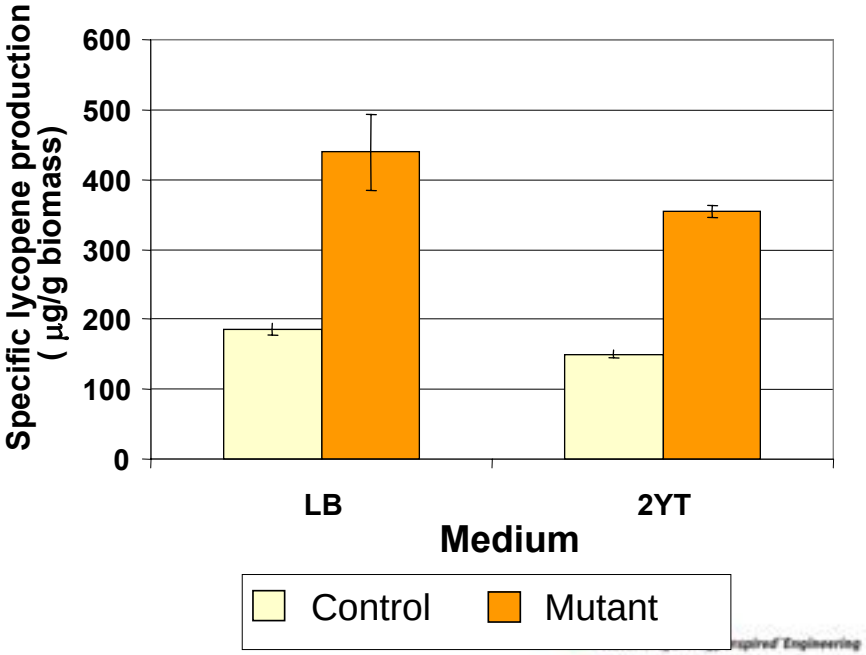
Lycopene Production (Shake Flask)

Lycopene concentration





Specific lycopene production



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

Model Product Experiments

Poly(3-hydroxybutyrate) (PHB)

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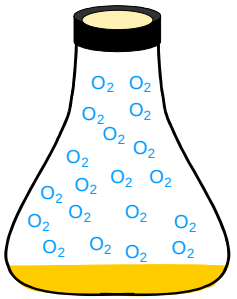
PHB Production (Shake Flasks)

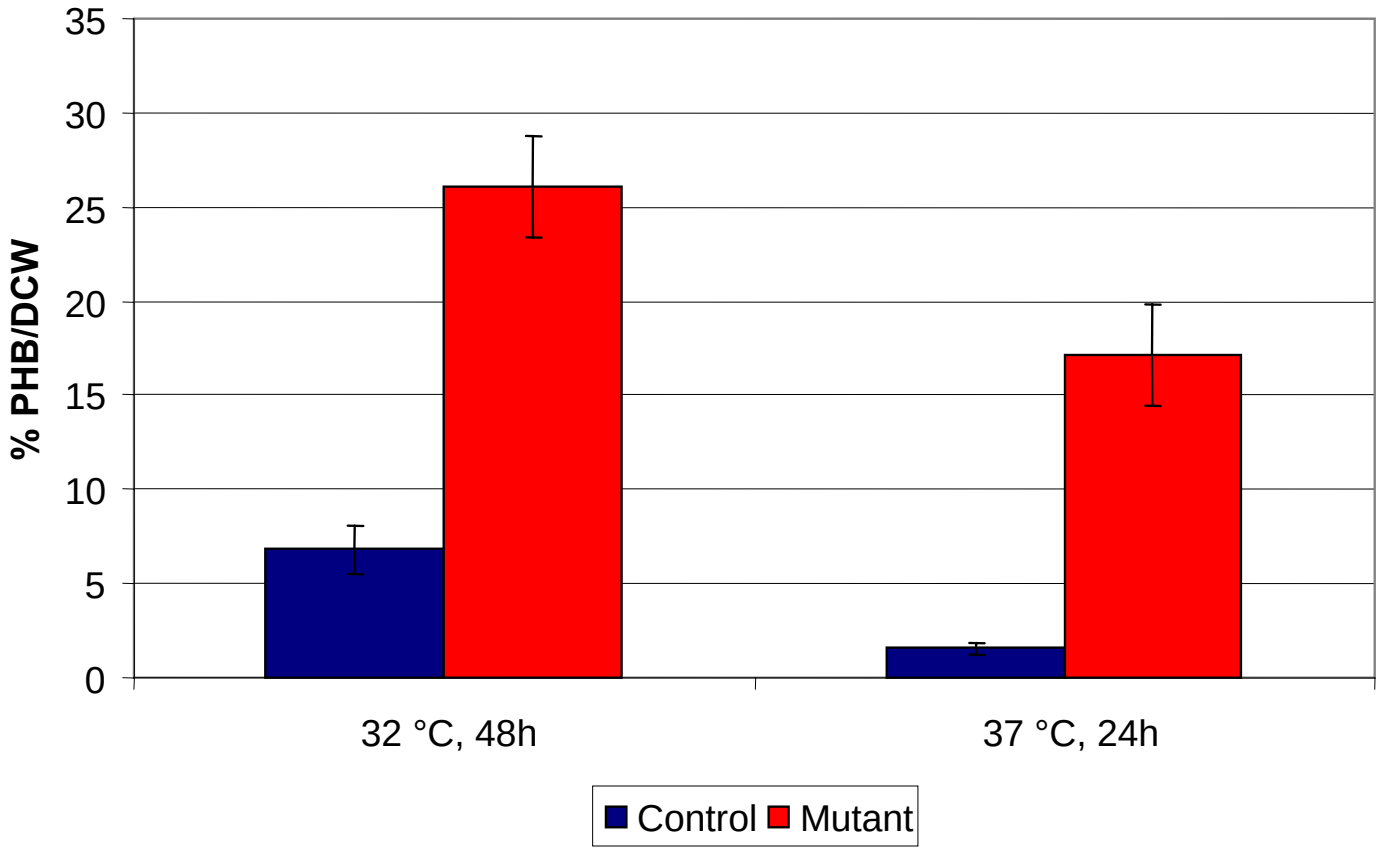
Control: MG1655 (pDHC29, pAeT29)

Mutant: MG1655 *ΔgapA* (pHL621, pAeT29)



pDHC29: cloning vector serve as control
pHL621: pDHC29 carrying a NADPH-dependent GAP
pAeT29 : plasmid carrying the PHB biosynthesis pathway

PHB Production Experiments

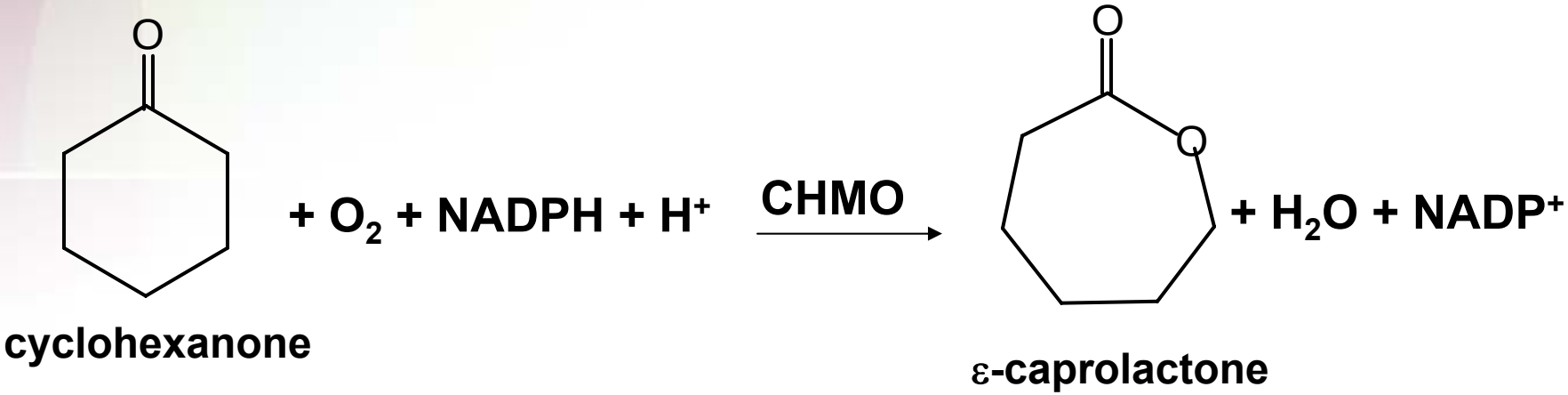


- **Higher final PHB production at the lower temperature**
- **Mutant strain yielded significantly higher PHB than the control strain**

Model Product Experiments

Whole cell single step conversion involving a NADPH-dependent reaction

Whole Cell Single Step Conversion



cyclohexanone

ε-caprolactone

CHMO: cyclohexanone monooxygenase from *Acinetobacter* sp[1].

Cunningham et al. *The Plant Cell* 1994, 6:1107-1121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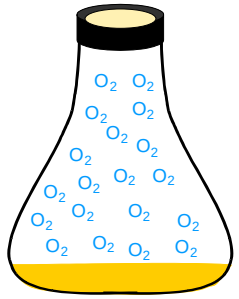
Whole Cell Single Step Conversion

Control: BL21(pDHC29, pMM4)

Mutant: BL21ΔgapA(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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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.**

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Acknowledgments

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