Metabolic Engineering of *Escherichia coli* W3110 for Redox Neutral & Oxidized Products

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Agricultural Opportunity - BioRefinery

Cargill Dow Dedicates PLA Refinery in April 2002

Biomass Feedstock

– Trees
– Grasses
– Agricultural Crops
– Agricultural Residues
– Animal Wastes
– Municipal Solid Waste

Conversion Processes

- Enzymatic Fermentation
- Gas/liquid Fermentation
- Acid Hydrolysis/Fermentation
- Gasification
- Combustion
- Co-firing

USES

Renewable Fuels:
– Ethanol
– Bio-Diesel

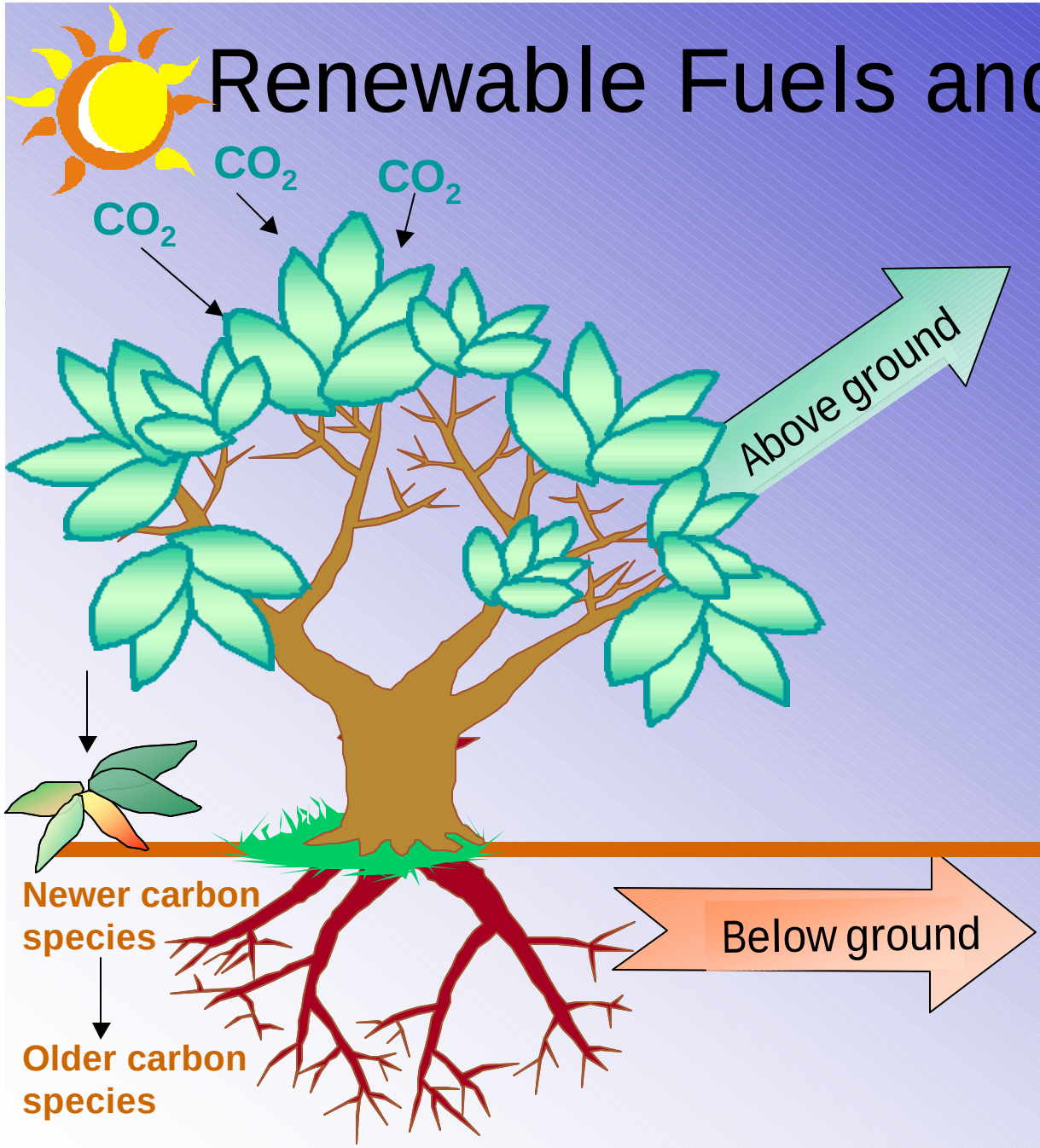
Renewable Power:
– Electricity
– Heat or CHP

Renewable Chemicals
– Plastics
– Solvents
– Chemical Intermediates
– Phenolics
– Adhesives
– Furfural
– Fatty acids
– Acetic Acid
– Carbon black
– Paints
– Dyes, Pigments, and Ink
– Detergents
– Etc.

Food and Feed and Fiber

DOE, 2002

Renewable Fuels and Chemicals:



- **Displacement of oil**
  - **Commodity chemicals**
    - **polylactic acid**
    - **3-HP, 1-3 PD**
    - **solvents**
    - **acids**
  - **Fuels**
    - **ethanol**
    - **biodiesel**
    - **power**
  - **Rural Employment**

- **Carbon Sequestration of in soil (short term)**

DOE, 2002

What are the limitation of Metabolic Engineering in *E. coli*?

1. Can we combine the beneficial features of aerobic and anaerobic metabolism?

- Aerobic: High growth rate, low NADH, external electron acceptor (O_2)
- Anaerobic: High glycolytic flux, low CO_2 production, low cell yield, high product yield

2. As an example, glucose to acetate (pyruvate)

Overview of Metabolism

Aerobic, + oxygen

Glucose, $C_6H_{12}O_6$

Cell Mass
50% of Carbon

CO_2 + 36 ATP (max)
50% of carbon

Anaerobic, - oxygen

Glucose, $C_6 H_{12} O_6$

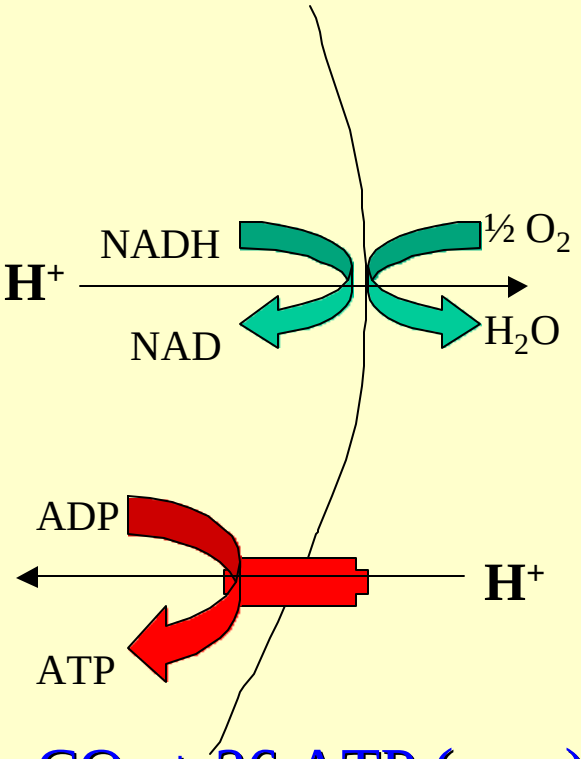
Cell Mass
5% of Carbon

Fermentation Products + 2 ATP
95% of Carbon
(most commodity products)

Overview of the Problem

Aerobic, + oxygen

Glucose, $C_6H_{12}O_6$



Glucose

2 Pyruvate

TCA

CO_2

+

Cell Mass

CO_2 + 36 ATP (max)

50% of carbon as cell mass

50% of carbon as $C0_2$

Anaerobic, - oxygen

Glucose, $C_6 H_{12} O_6$

NAD

NADH

Glycolysis

2 Pyruvate + 2 ATP

NADH

NAD

(TCA incomplete)

2 Lactate, Succinate

or

2 Ethanol + 2 CO_2

Fermentation Products + 2 ATP

5% of carbon as cell mass

95% of carbon as product

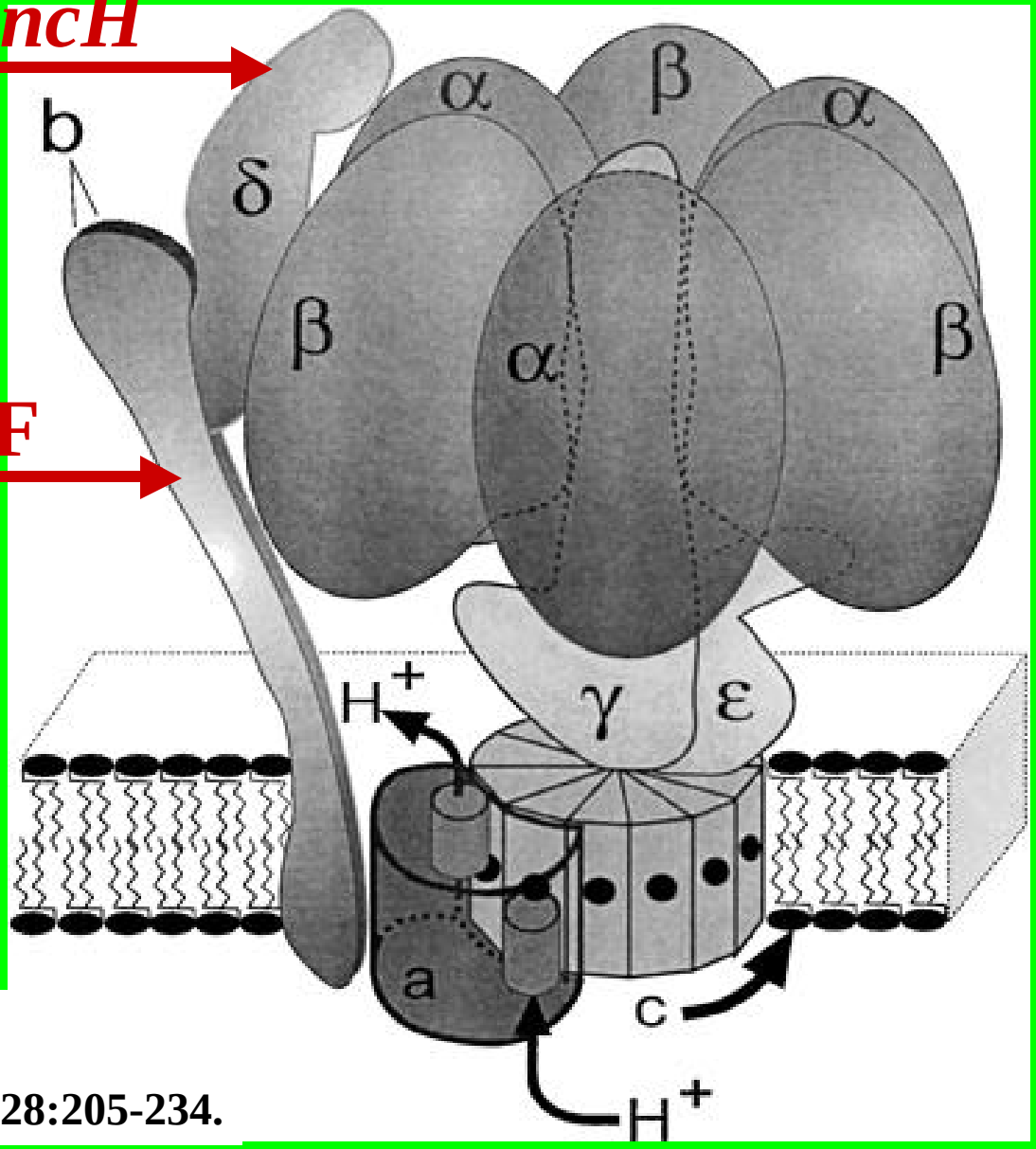
Three Problem Areas

1. Cells →Too many – reduce ATP

2. Volatiles → Too much carbon lost
   – interrupt TCA cycle, inactivate ADH

3. Fermentation products → potential sink
   – inactivate pathways

"Just turn me loose!"

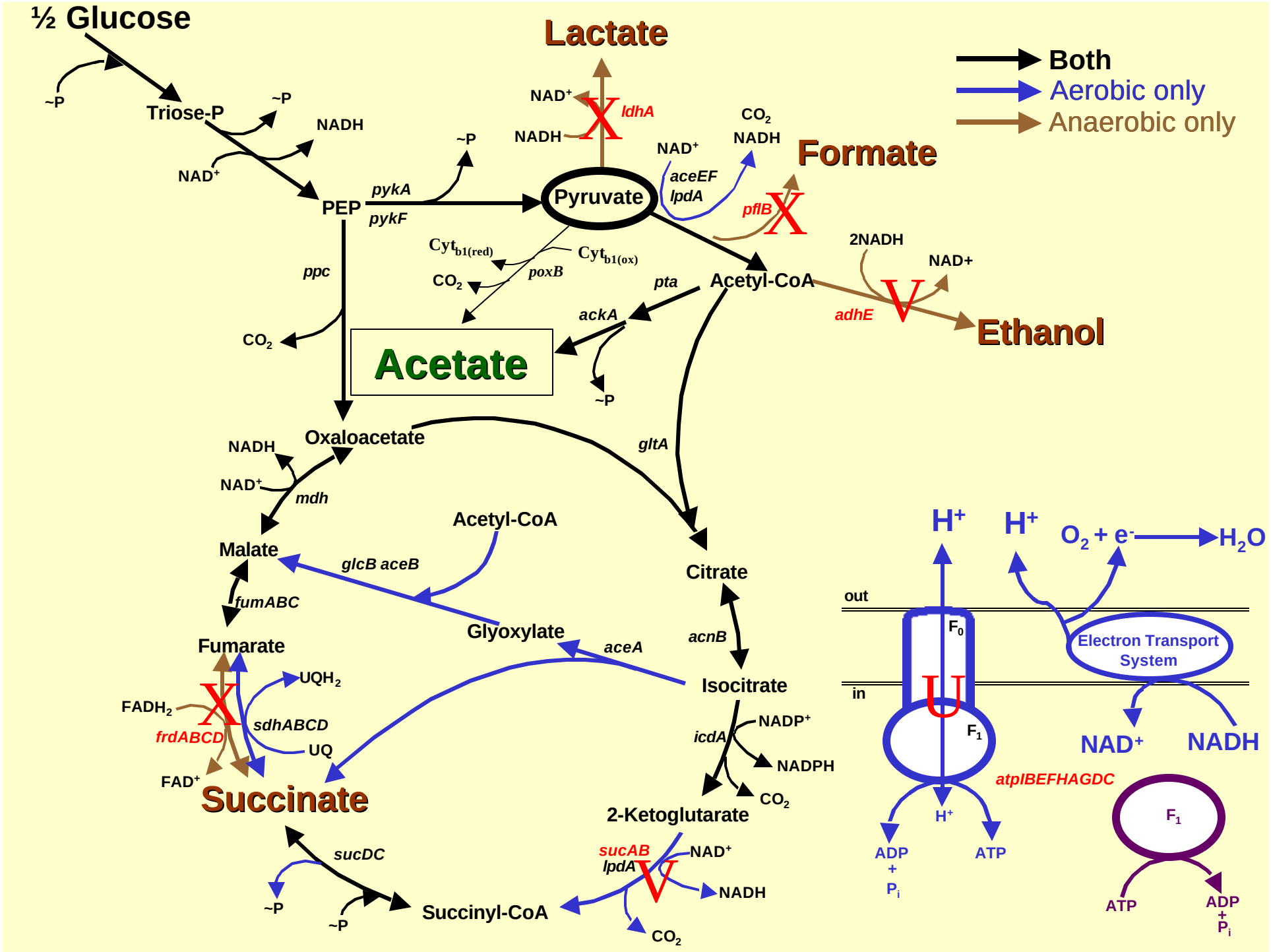


Oxidative Phos negative

Gratuitous consumption of ATP by F_1ATPase

Nakamoto et al., 1999

Ann. Rev Biophys. Biomol. Struct. 28:205-234.


½ Glucose
~P
Triose-P
~P
NADH
NAD+
PEP
pykA
pykF
~P
Pyruvate
Lactate
NAD+
NADH
ldhA
CO2
NADH
NAD+
aceEF
lpdA
Formate
pflB
Both
Aerobic only
Anaerobic only
Cytb1(red)
Cytb1(ox)
poxB
CO2
ppc
CO2
pta
ackA
Acetyl-CoA
2NADH
NAD+
adhE
Ethanol
Acetate
~P
Oxaloacetate
gltA
NADH
NAD+
mdh
Acetyl-CoA
Malate
glcB aceB
Citrate
fumABC
Glyoxylate
acnB
aceA
Fumarate
Isocitrate
UQH2
FADH2
sdhABCD
frdABCD
UQ
FAD+
Succinate
icdA
NADP+
NADPH
CO2
2-Ketoglutarate
sucDC
~P
~P
Succinyl-CoA
sucAB
lpdA
NAD+
NADH
CO2
H+
H+
O2 + e-
H2O
out
in
F0
F1
Electron Transport System
NAD+
NADH
atpIBEFHAGDC
H+
ADP + Pi
ATP
F1
ATP
ADP + Pi

Strain Construction

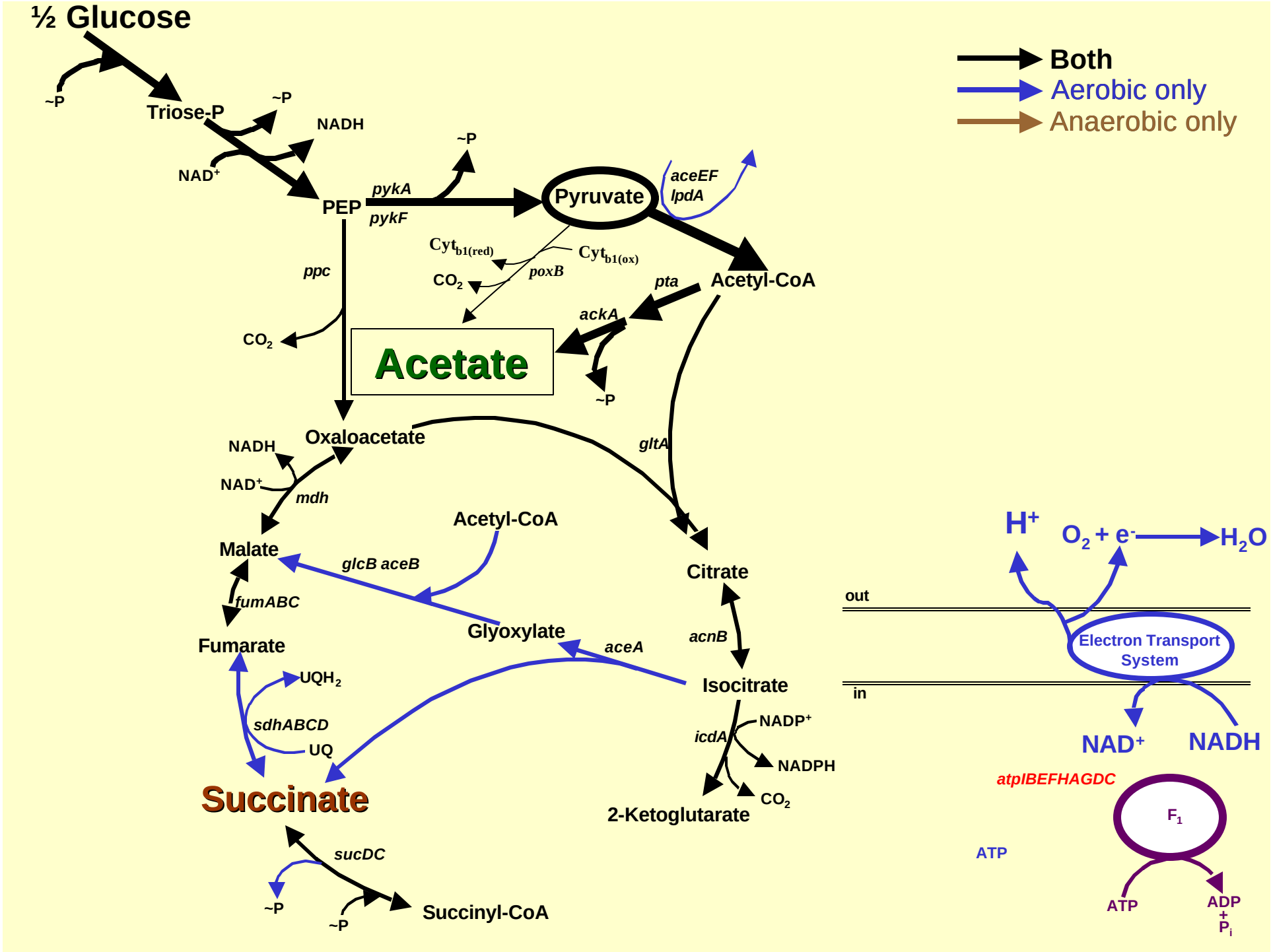
1. Homologous recombination, confirmed by PCR and sequence, one mutation per strain

2. Phage P1 to combine mutations into single strain

3. Flanking FRT sites and FLP recombinase to remove antibiotic markers used for selection

4. Fusaric acid selection to create deletions from Tn10 insertions

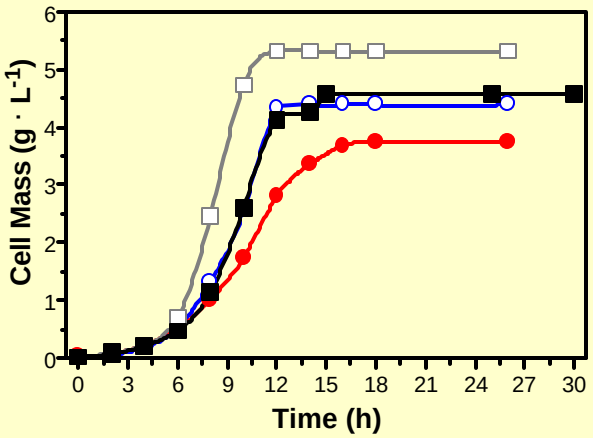
5. PCR cloning and sequencing to confirm each step

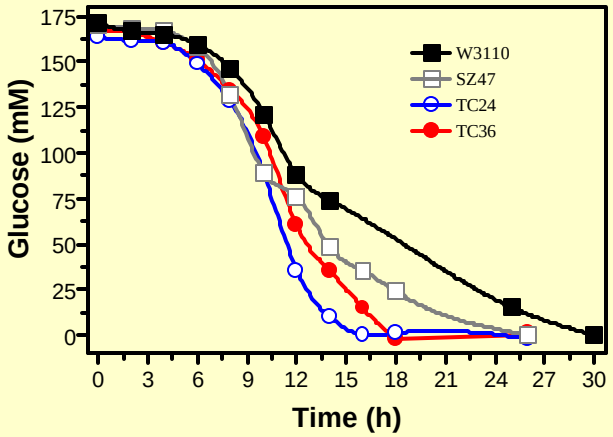

½ Glucose
~P
Triose-P
~P
NADH
NAD+
PEP
pykA
pykF
~P
Pyruvate
aceEF
lpdA
Cyt b1(red)
Cyt b1(ox)
poxB
CO2
ppc
CO2
pta
Acetyl-CoA
ackA
Acetate
~P
Oxaloacetate
gltA
NADH
NAD+
mdh
Acetyl-CoA
Malate
glcB aceB
Citrate
fumABC
Glyoxylate
acnB
Fumarate
aceA
UQH2
Isocitrate
sdhABCD
UQ
icdA
NADP+
NADPH
CO2
Succinate
2-Ketoglutarate
sucDC
~P
~P
Succinyl-CoA
Both
Aerobic only
Anaerobic only
H+
O2 + e-
H2O
out
Electron Transport System
in
NAD+
NADH
atpIBEFHAGDC
F1
ATP
ATP
ADP + Pi

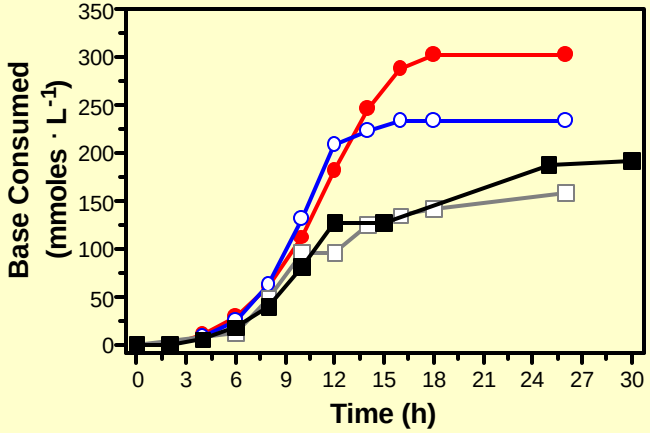
Fermentation Conditions:

- mineral salts, 37^0C
- 3 % glucose
- starting OD_{550} = 0.1
- 1% inoculum
- 10 L initial volume
- agitation set @ 450 rpm
- pH controlled @ 7.00 with 45% w/w KOH (11.4 M)
- DO controlled @ 5% of air saturation by adjusting the ratio of air, O_2 and N_2

Gas flow 1 L/min (0.1vvm)







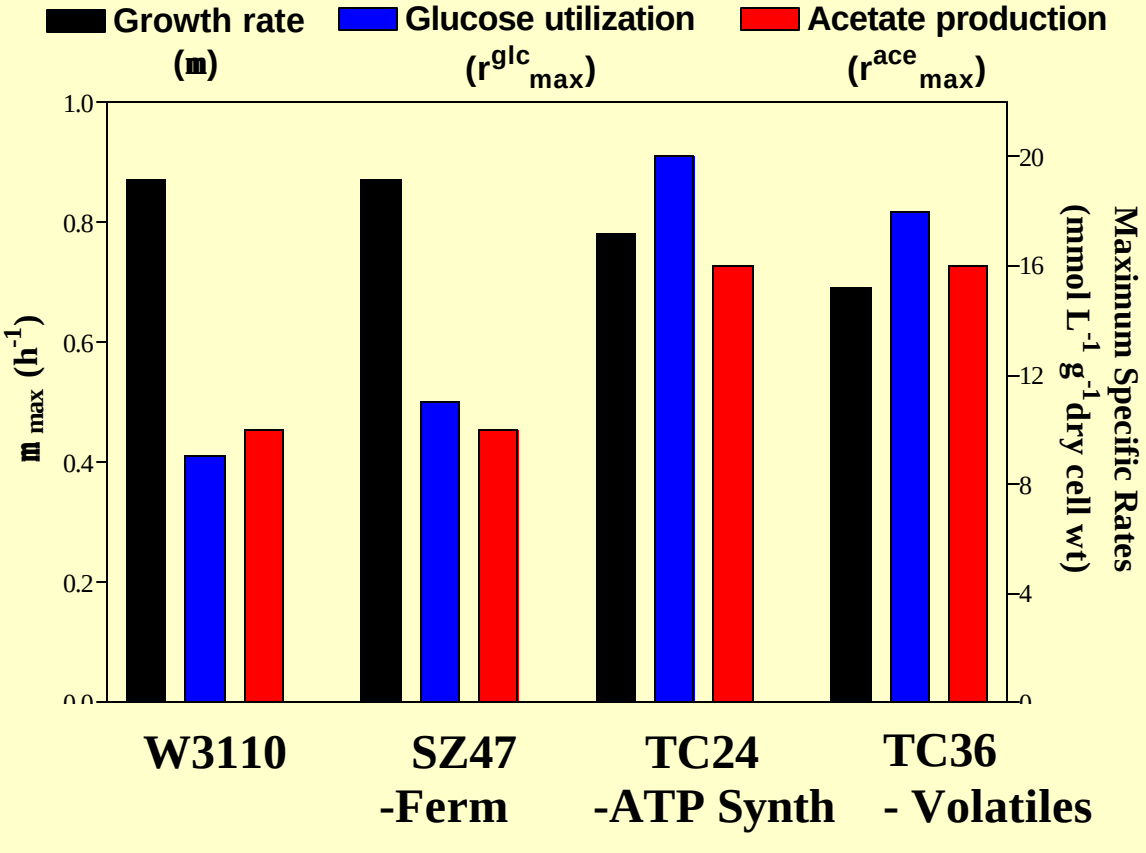
Strain W3110 (wild type) ~30 ATP/glu

Strain SZ47 (D*focA-pflB* D*frdBC* D*ldhA*) ~30 ATP/glu

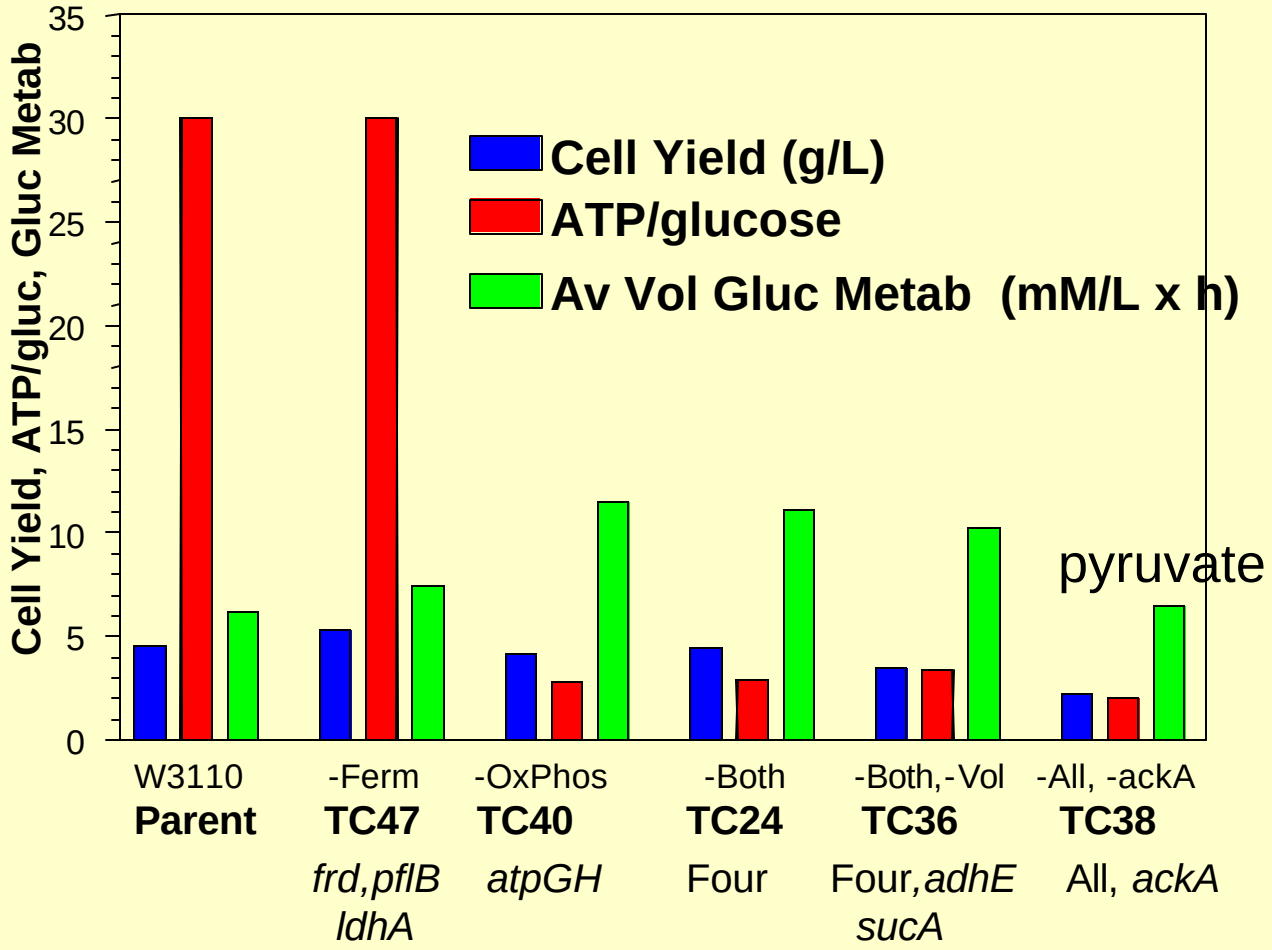
Strain TC24(D*focA-pflB* D*frdBC* D*ldhA* D*atpFH*) ~3ATP

Strain TC36 (D*focA-pflB* D*frdBC* D*ldhA* D*atpFH* D*adhE* D*sucA*) ~3.8 ATP/glucose

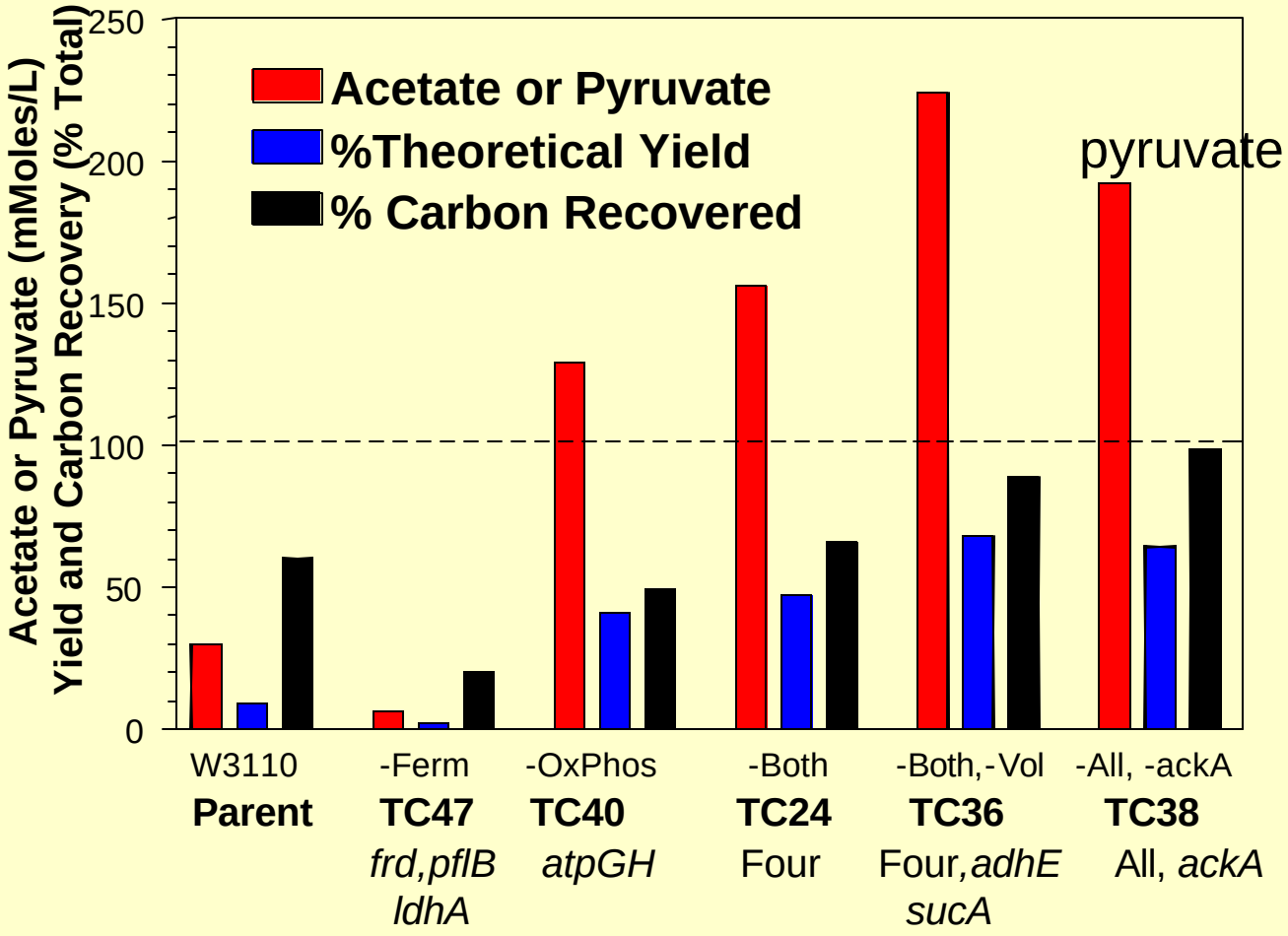
Comparison of Maximum Specific Rates

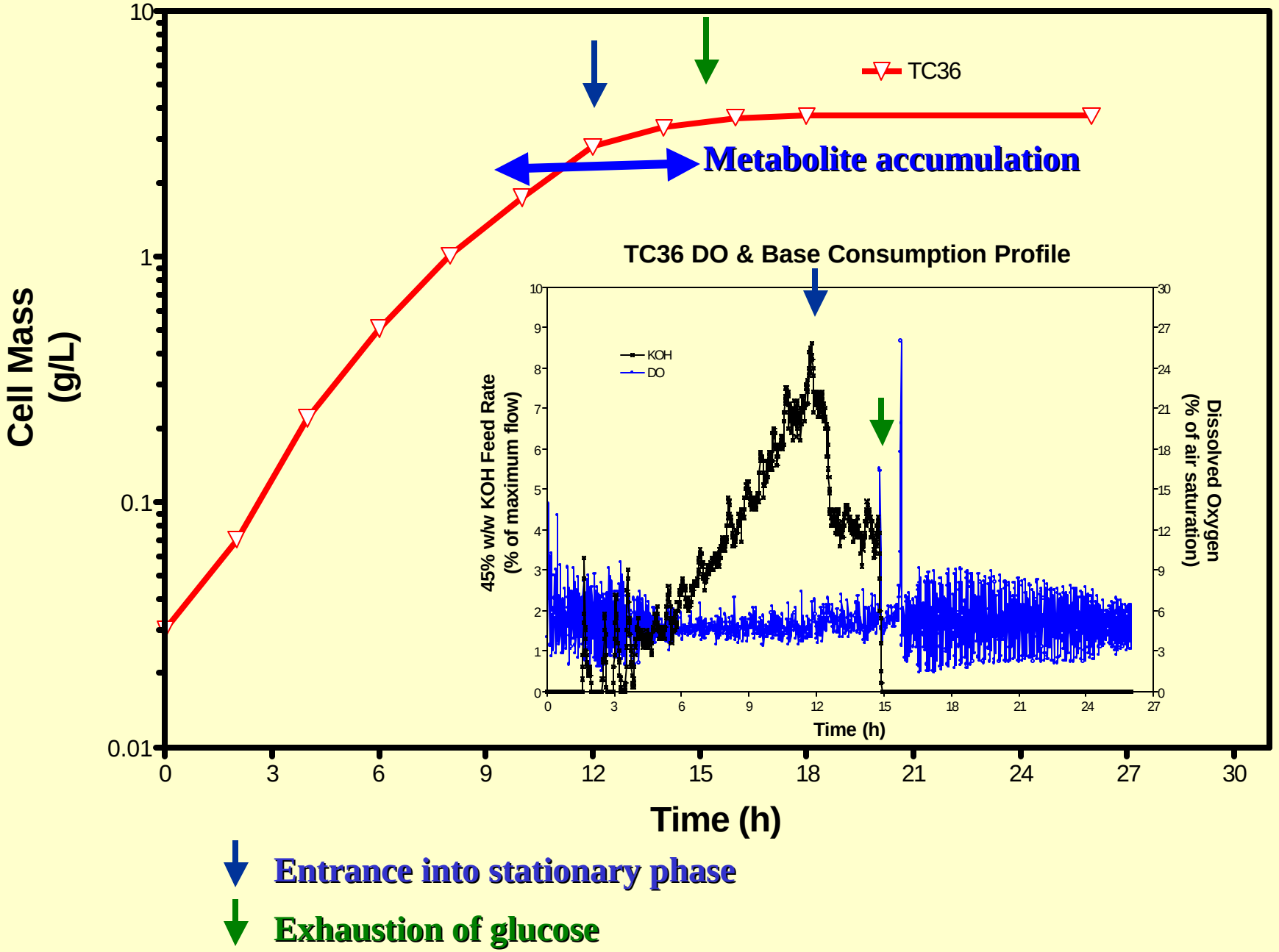


Comparison of Strains



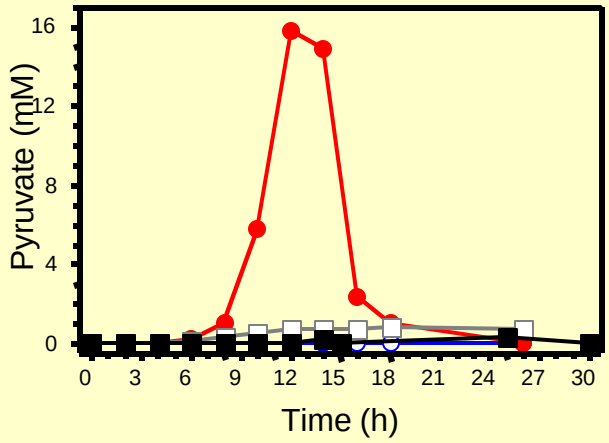
Comparison of Strains

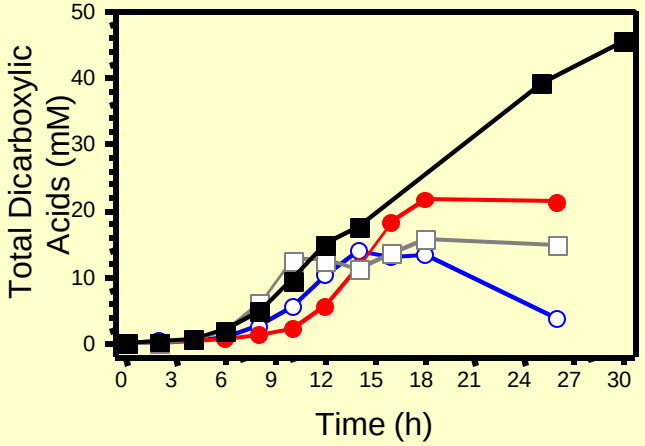


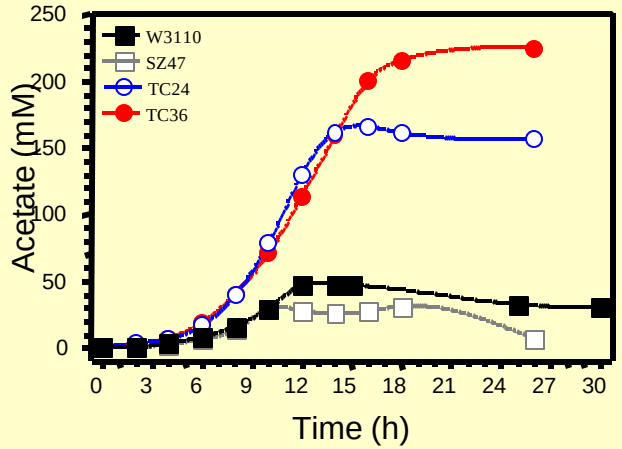


Entrance into stationary phase

Exhaustion of glucose







➔ **Accumulation of pyruvate indicates that glucose uptake & glycolysis may not be limiting acetate production.**

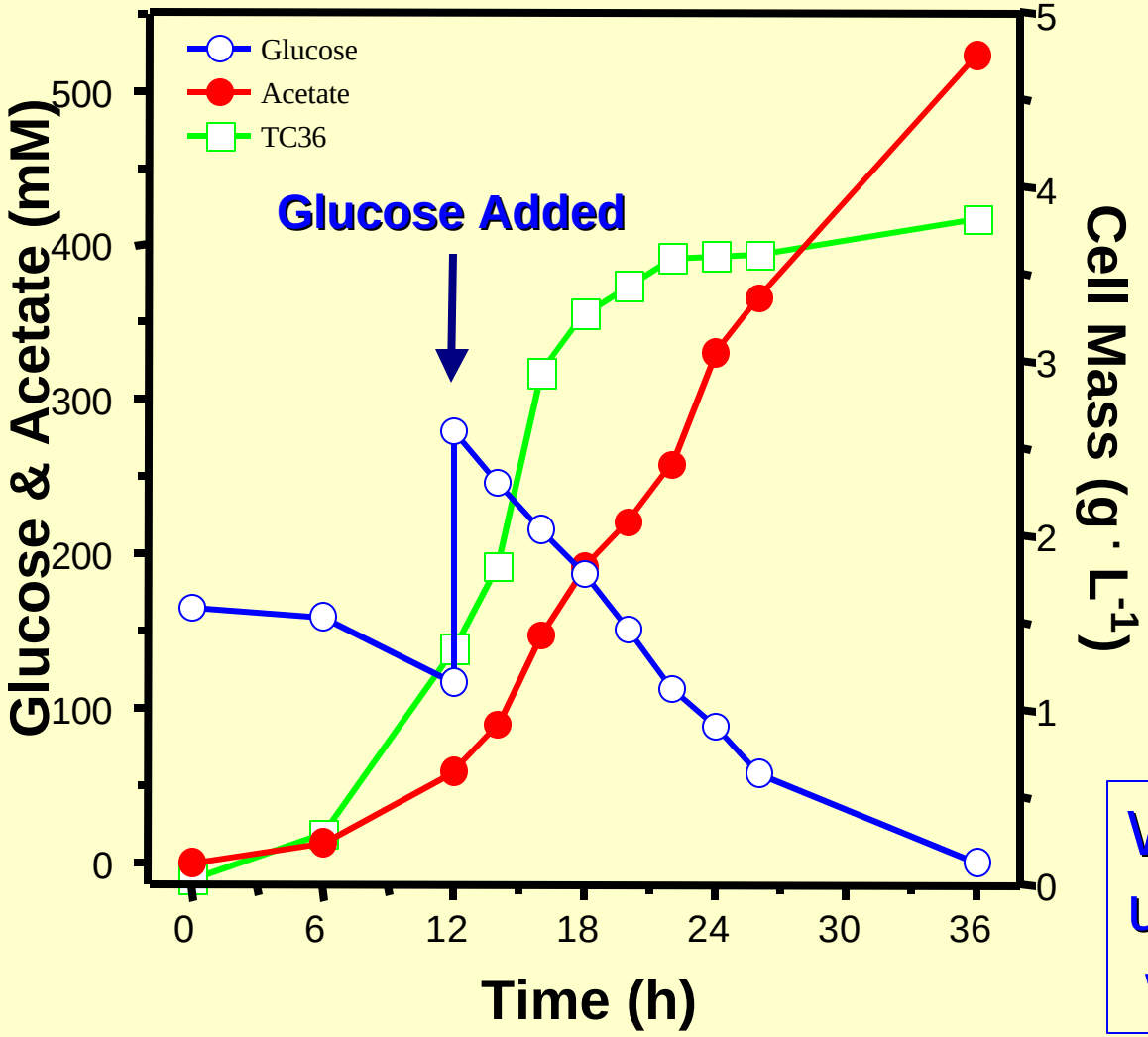
➔ **W3110 accumulated ~ 3 fold higher concentrations of dicarboxylic acids than the engineered strains.**

➔ **In general, accumulation of dicarboxylic acids was correlated with entry into stationary phase.**

➔ **D*atpFH* resulted in ~25 fold increase in final acetate concentration/yield.**

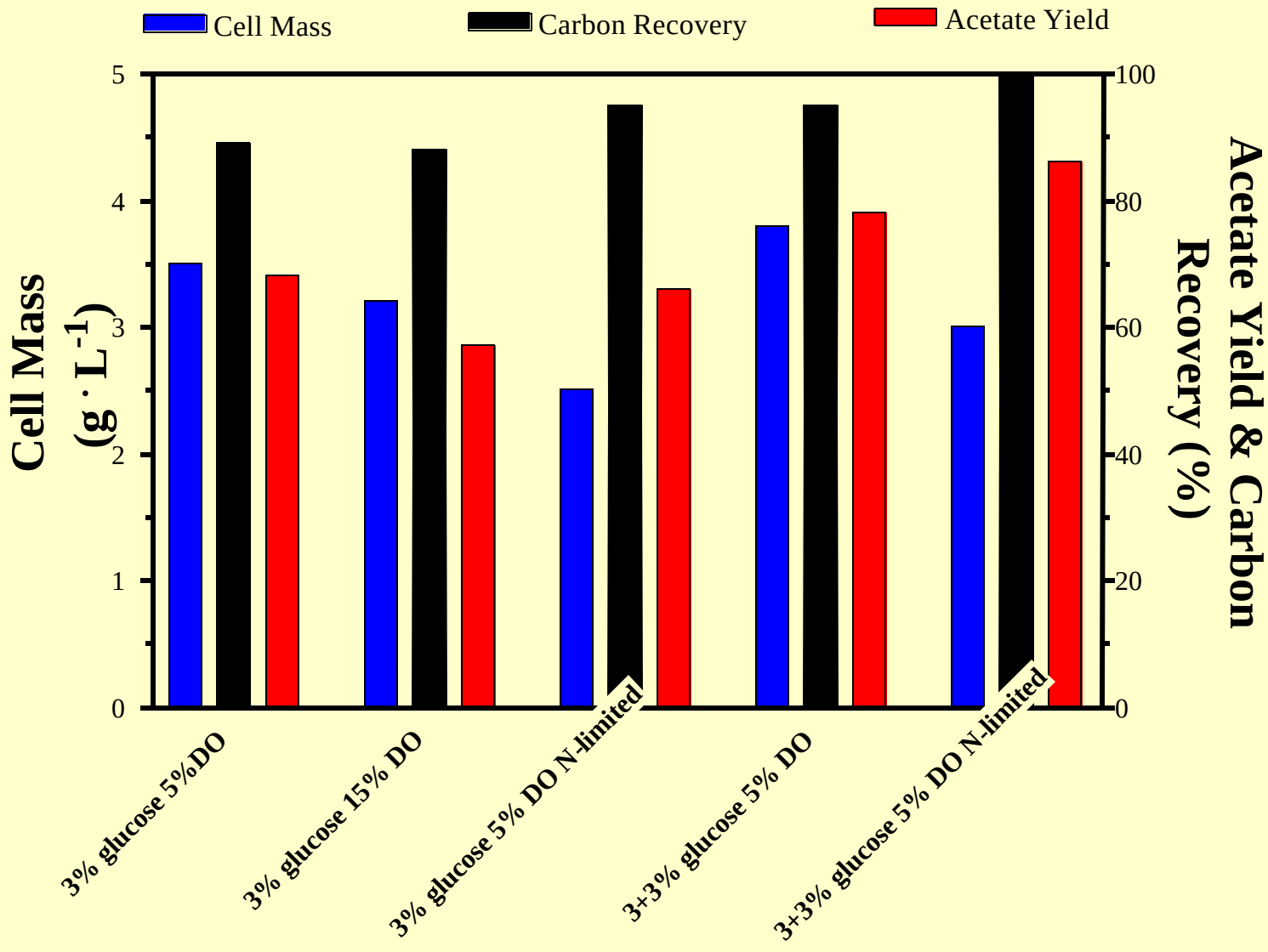
➔ **D*adhE* D*sucA* resulted in a further 1.4 fold increase in acetate concentration.**

Fermentation of 3% + 3% Glucose to Acetate



With glucose excess, up to 900 mM acetate was produced.

Acetate Production Can Be Improved By Altering The Process Conditions (TC36)



Conclusions

- ***E. coli*** **can be engineered for efficient production of redox neutral & oxidized products (ace, pyr).**
- **)*atpFH* increased glycolytic flux by over 50%, acetate concentration and yield by 3-fold.**
- **)*adhE*)*sucA* resulted in an additional increase in acetate concentration and yield, and improved carbon balance.**
- **The increase in glycolytic flux observed for TC24 & TC36 was attributed to the)*atpFH* which reduced ATP production and provided gratuitous hydrolysis of excess ATP.**
- **Max acetate yields of 86% of theoretical; 10% of substrate carbon converted into biocatalyst**
- **Product stream relatively pure after cell removal**