Development of efficient *Escherichia coli* succinate production strains

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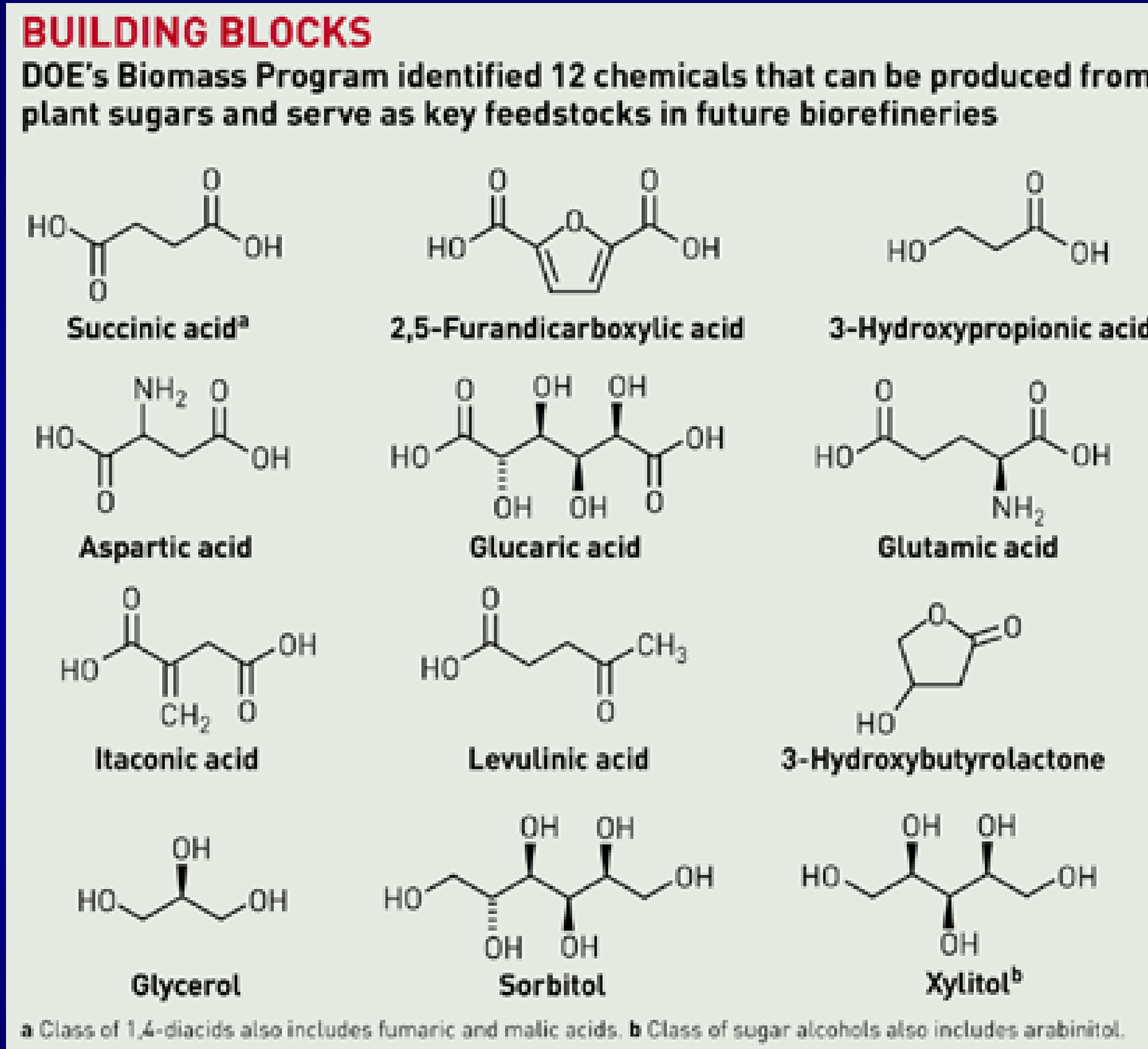


Top Value Added Chemicals from Biomass (2004 DOE report)

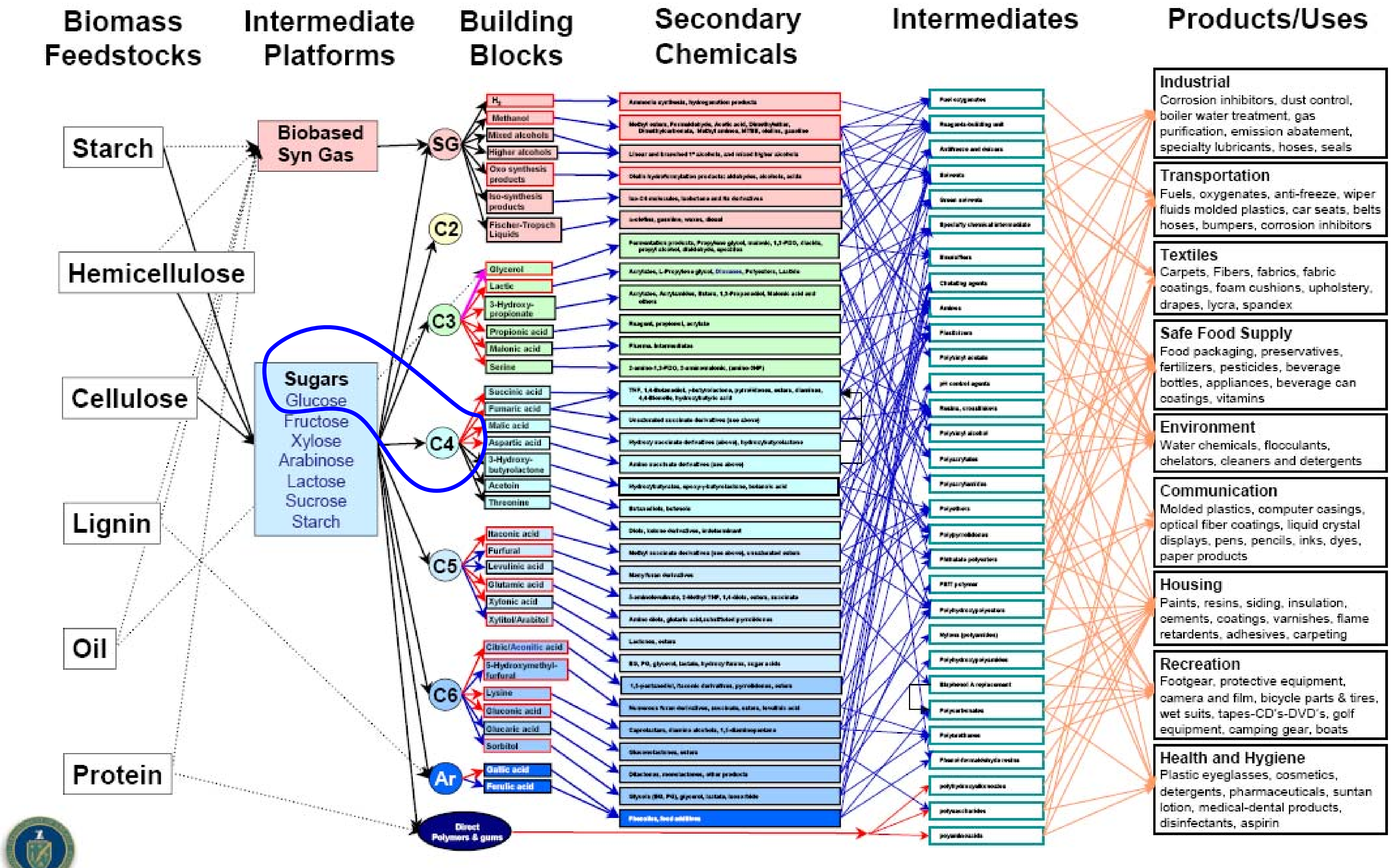
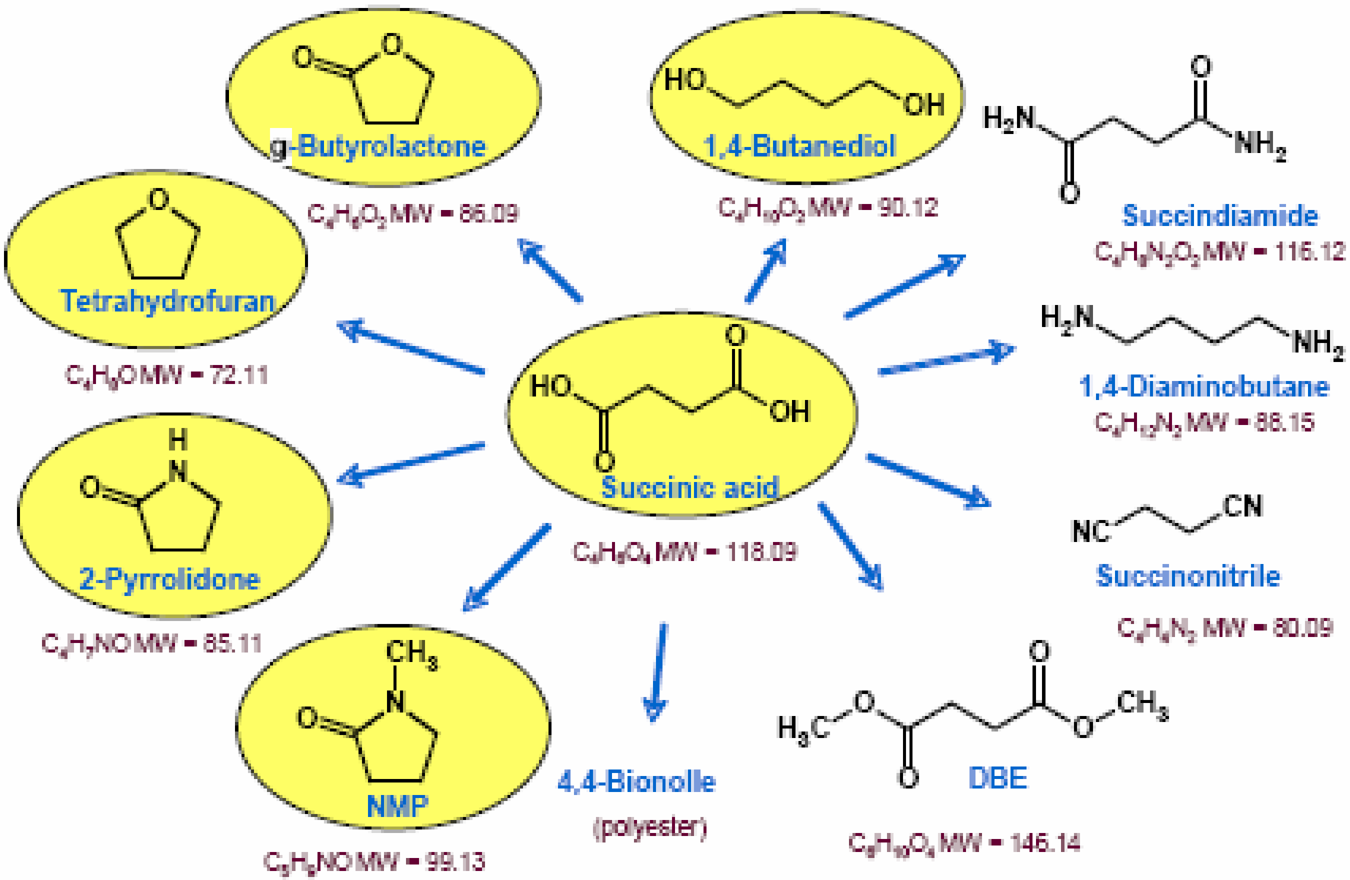


Figure 3 – Analogous Model of a Biobased Product Flow-chart for Biomass Feedstocks

Top Value Added Chemicals from Biomass (2004 DOE report)



Succinic Acid Chemistry to Derivatives

Top Value Added Chemicals from Biomass (2004 DOE report)

Production of succinic acid

<u>Non-recombinant microorganisms :</u>

- *A. succiniproducens*
- *A. succinogenes*
- *E. coli*
  - succinic acid minor fermentation product (~7.8% of total)

<u>Metabolically Engineered *E. coli:*</u>

- Deletion of *ldh*
- overexpression of PEPC/ malic enzyme or PYC
- Deletion of *ldh* and *pfl*
  - NZN111
- Deletion of *ldh/pfl/ptsG*
  - APF111

Current challenges

- **Existing strains have low productivity**
  - Low yield
  - Low production rate (weak strain)
- **Existing strains produce succinate anaerobically**
  - **need two stage fermentation**
- **Product purity – mixed acids fermentation**

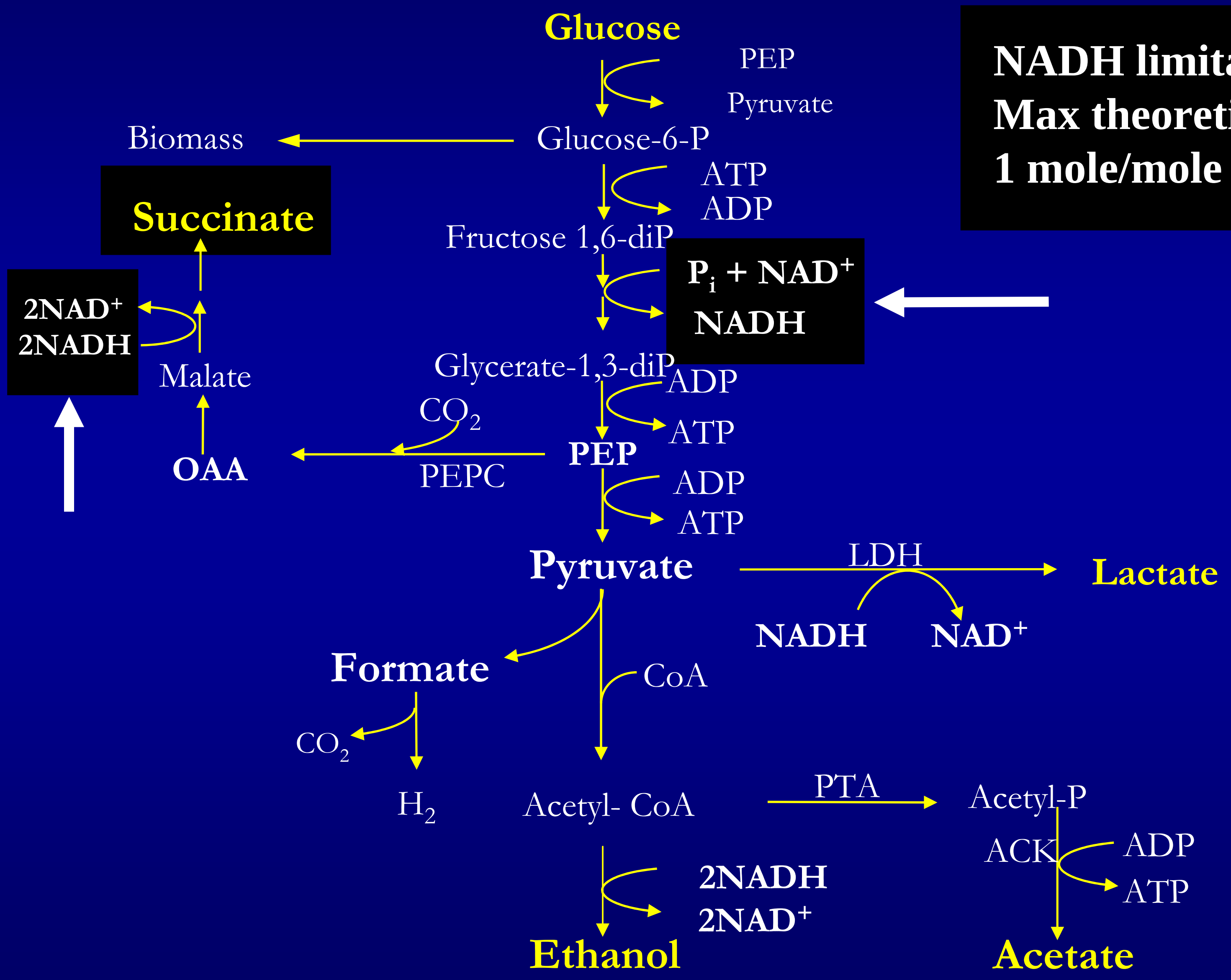
High yield anaerobic succinic acid production system

Design rationale…

High yield anaerobic succinic acid production system

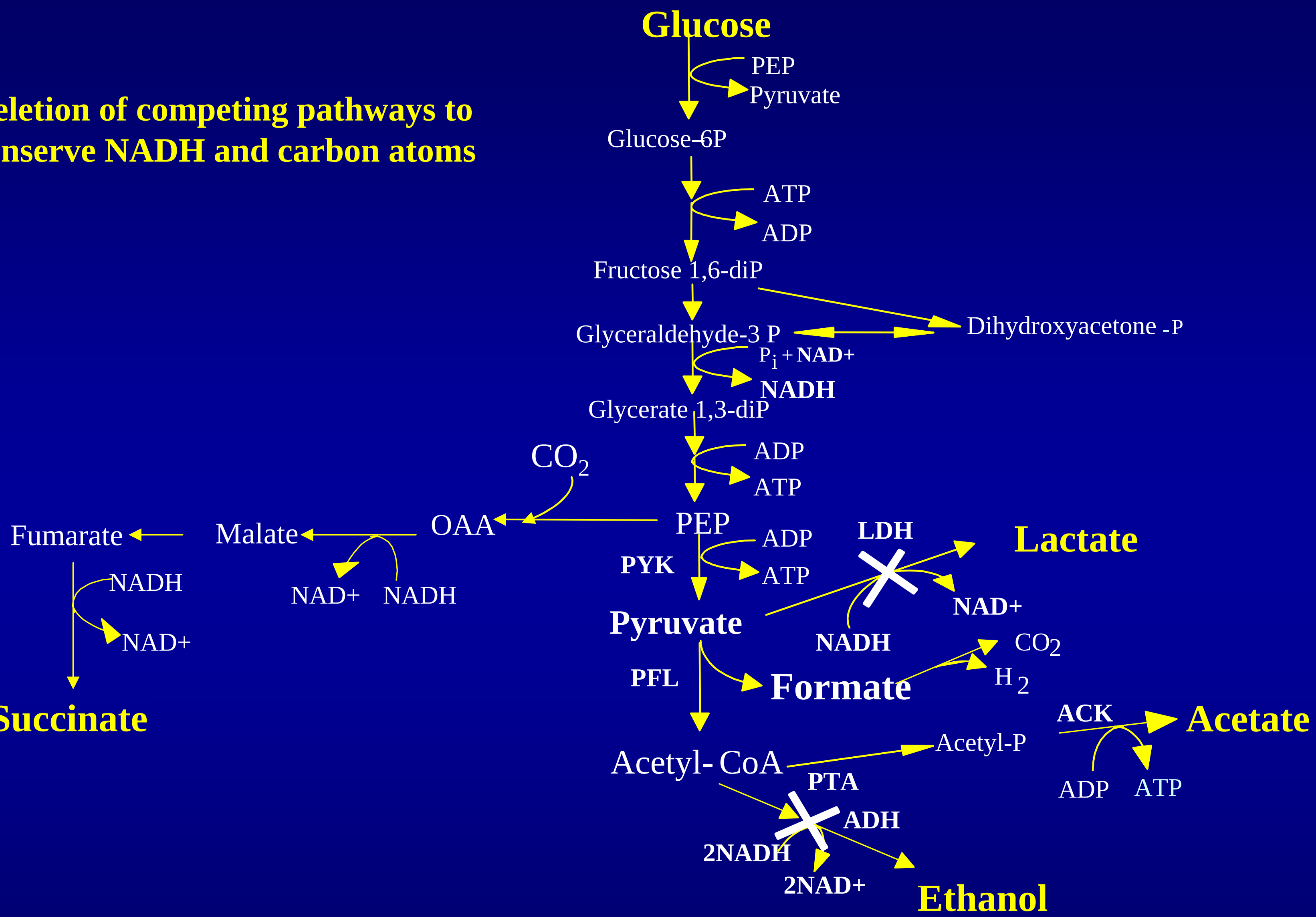
Limitation…

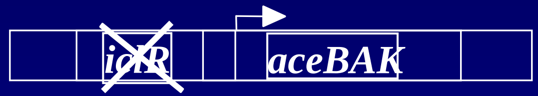
Central anaerobic pathway in *E. coli*



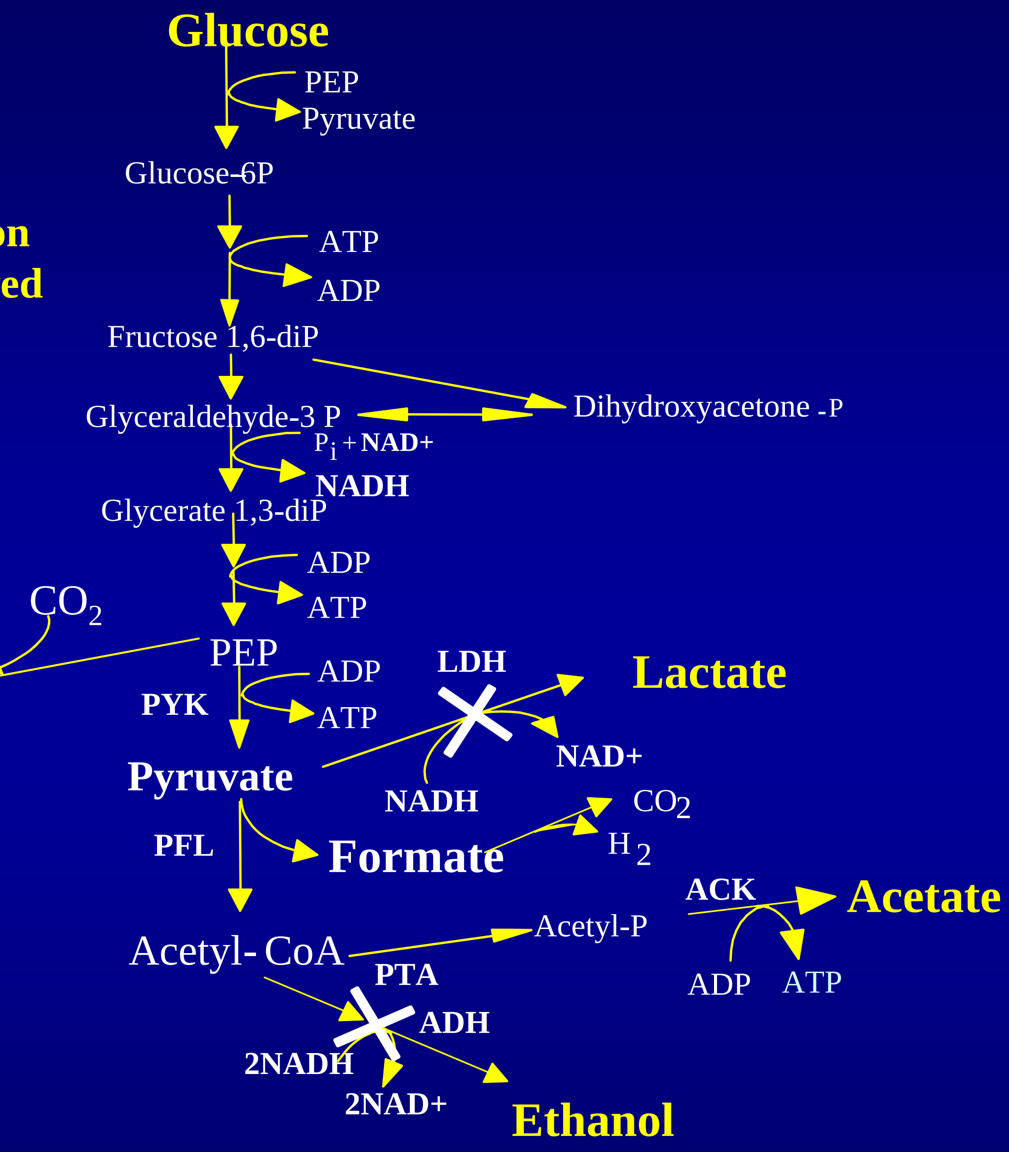
NADH limitation
Max theoretical Yield
1 mole/mole

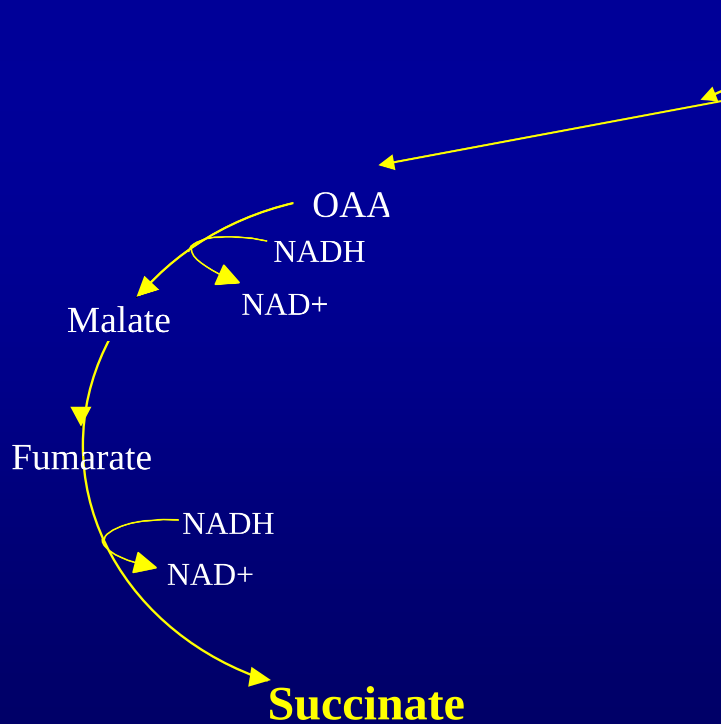
1. **Deletion of competing pathways to conserve NADH and carbon atoms**



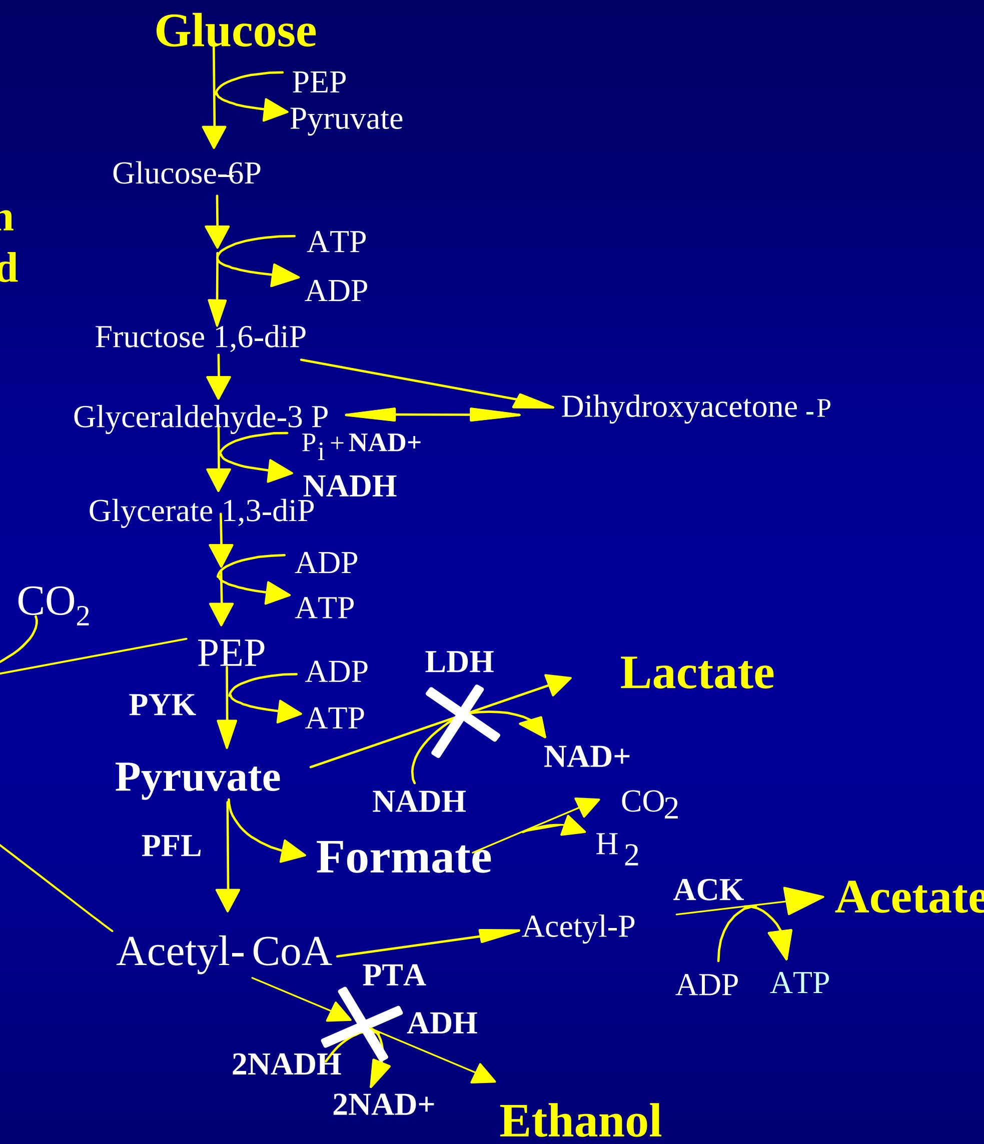


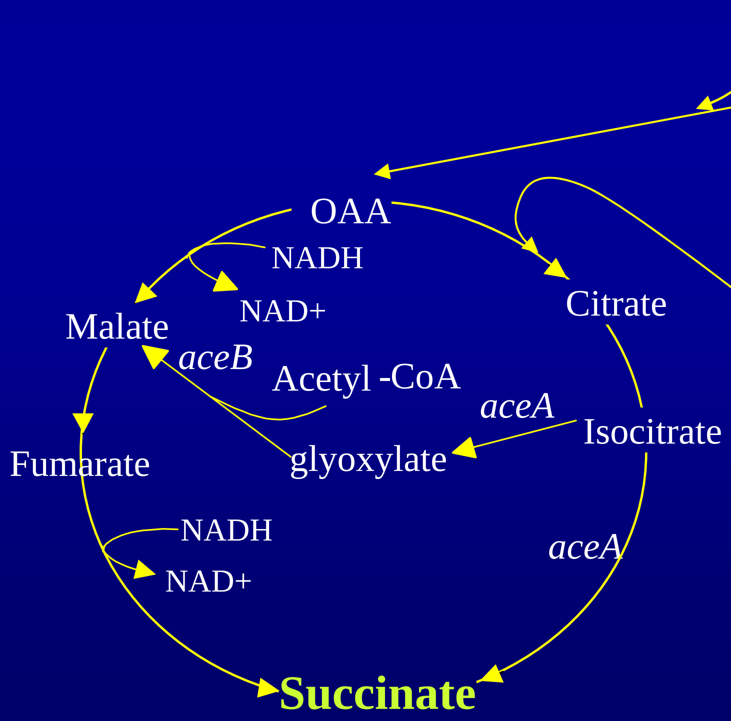
2. **Deletion of *iclR* to relieve the repression of the expression of the enzymes involved in the glyoxylate pathway**





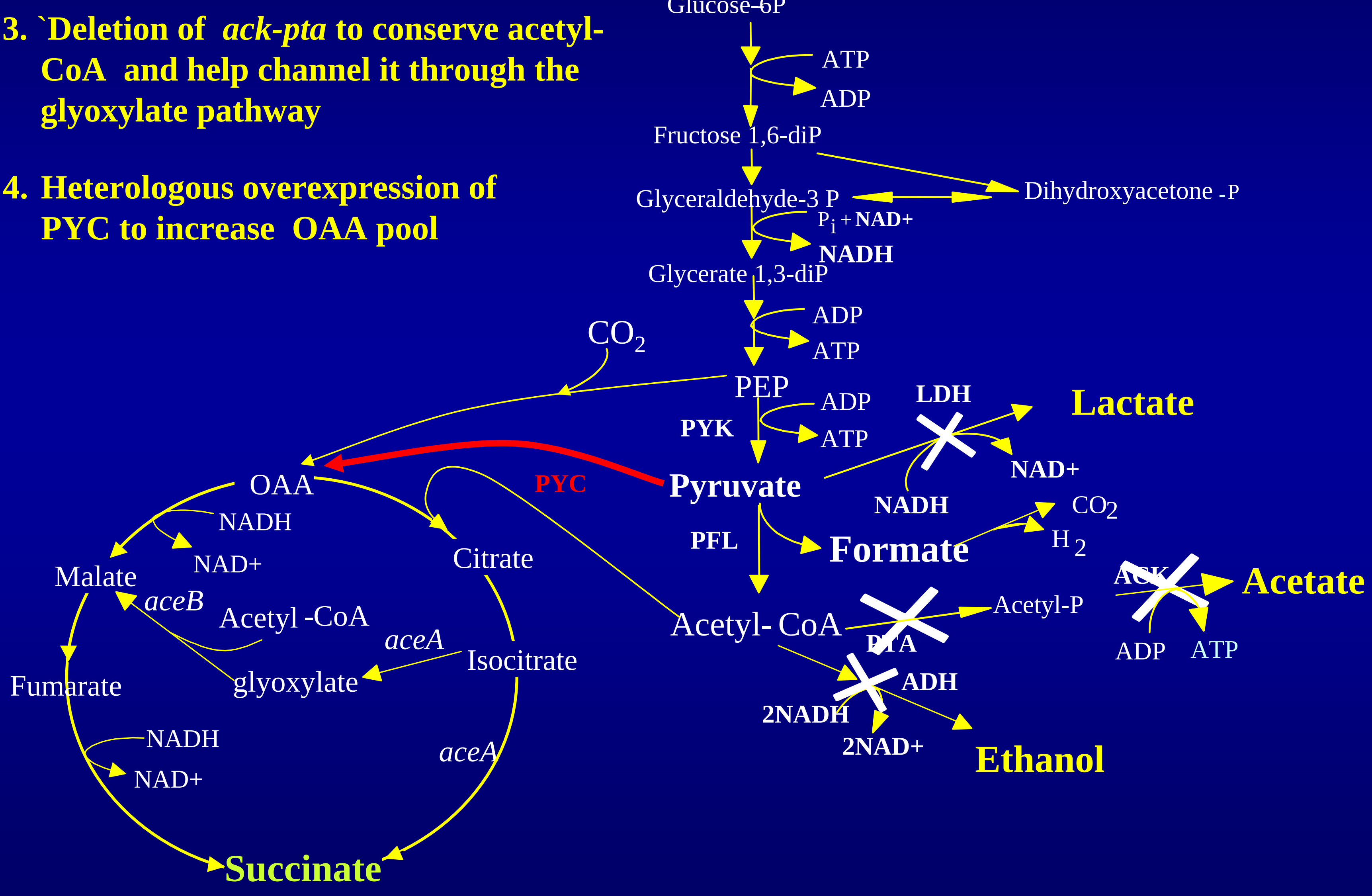
2. `Deletion of *iclR* to relieve the repression of the expression of the enzymes involved in the glyoxylate pathway hence "opening" the acetyl-CoA flow though this pathway



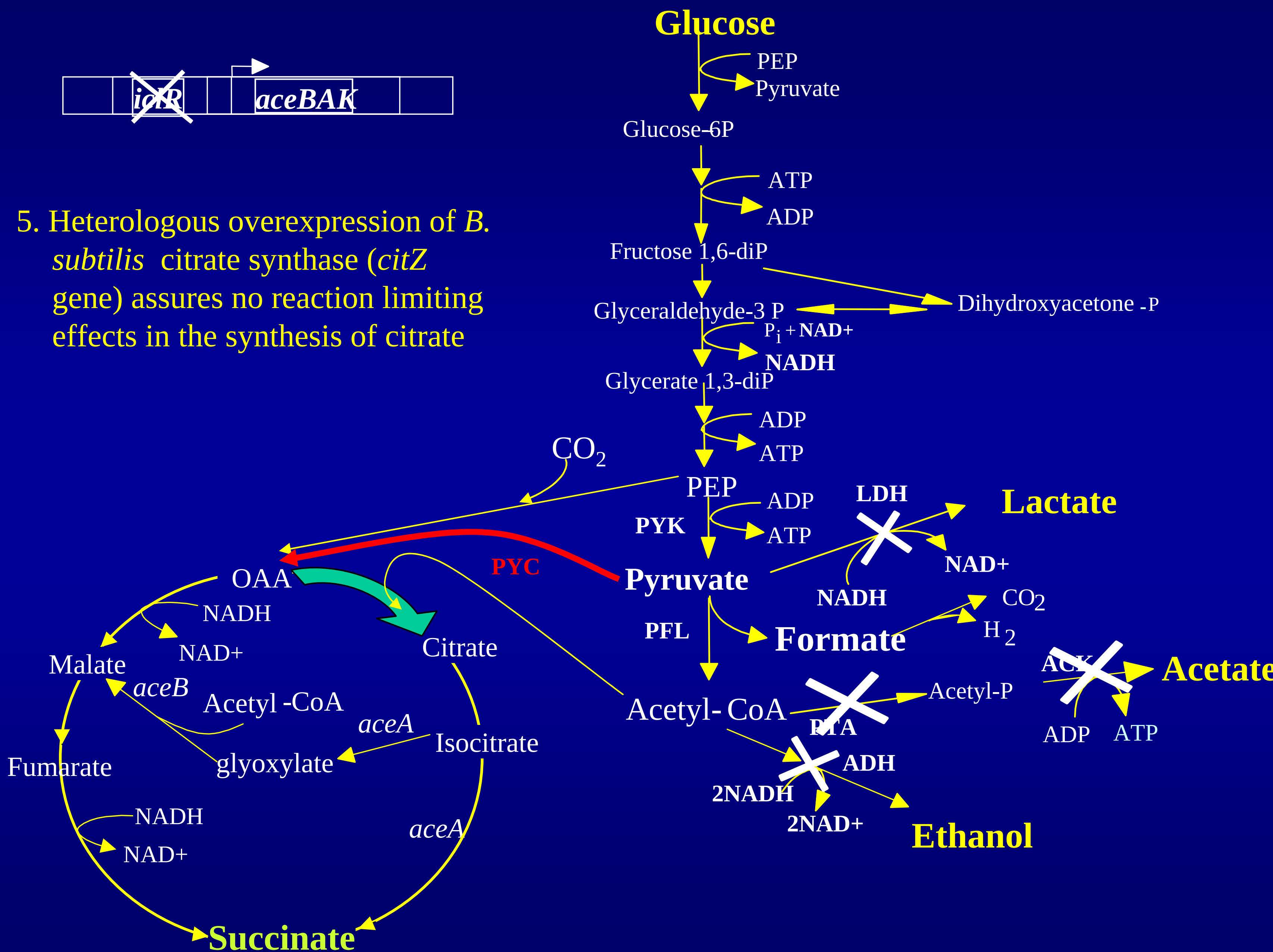


3. `Deletion of *ack-pta* to conserve acetyl-CoA and help channel it through the glyoxylate pathway

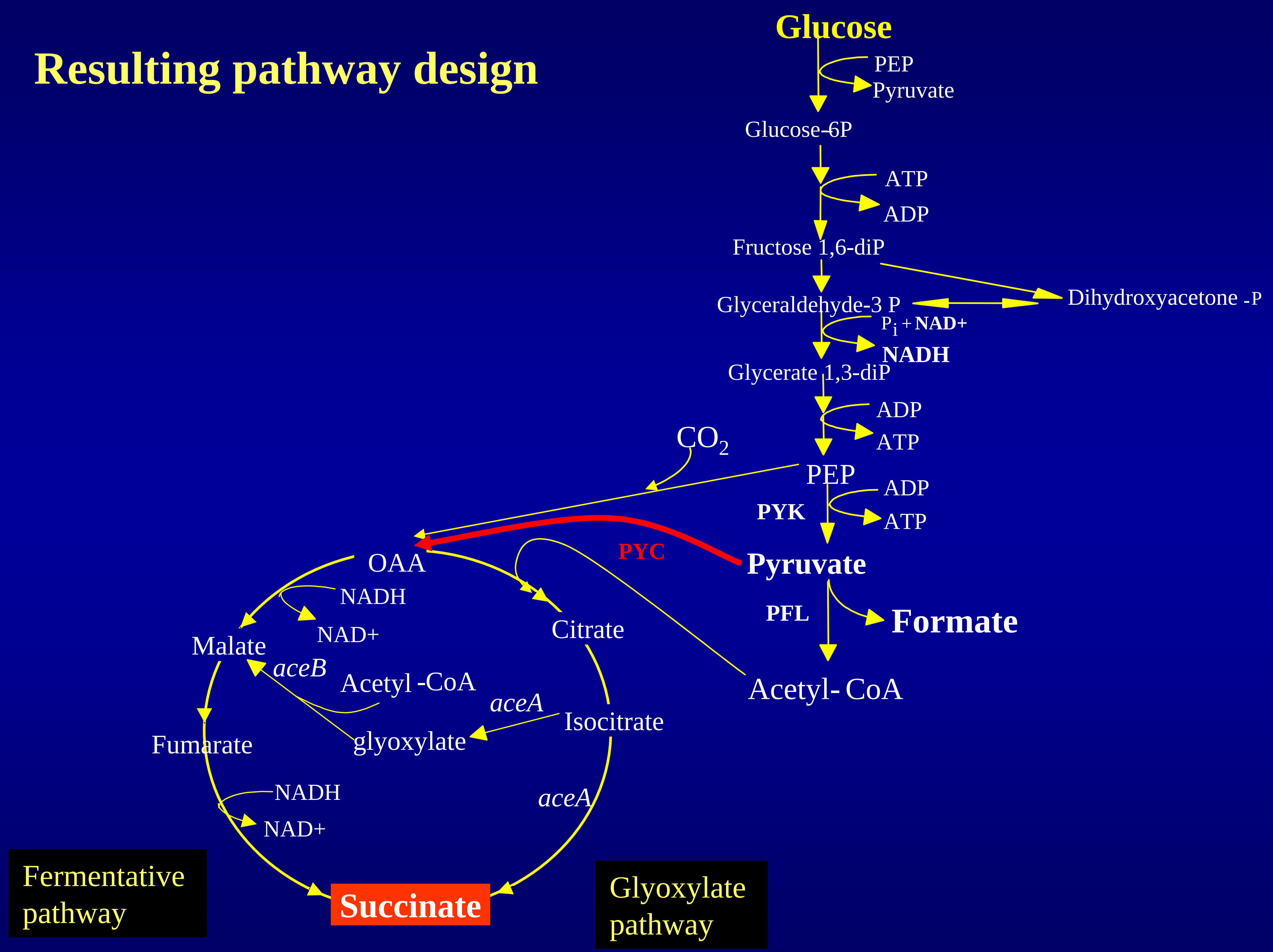
4. Heterologous overexpression of PYC to increase OAA pool



5. Heterologous overexpression of *B. subtilis* citrate synthase (*citZ* gene) assures no reaction limiting effects in the synthesis of citrate



Resulting pathway design

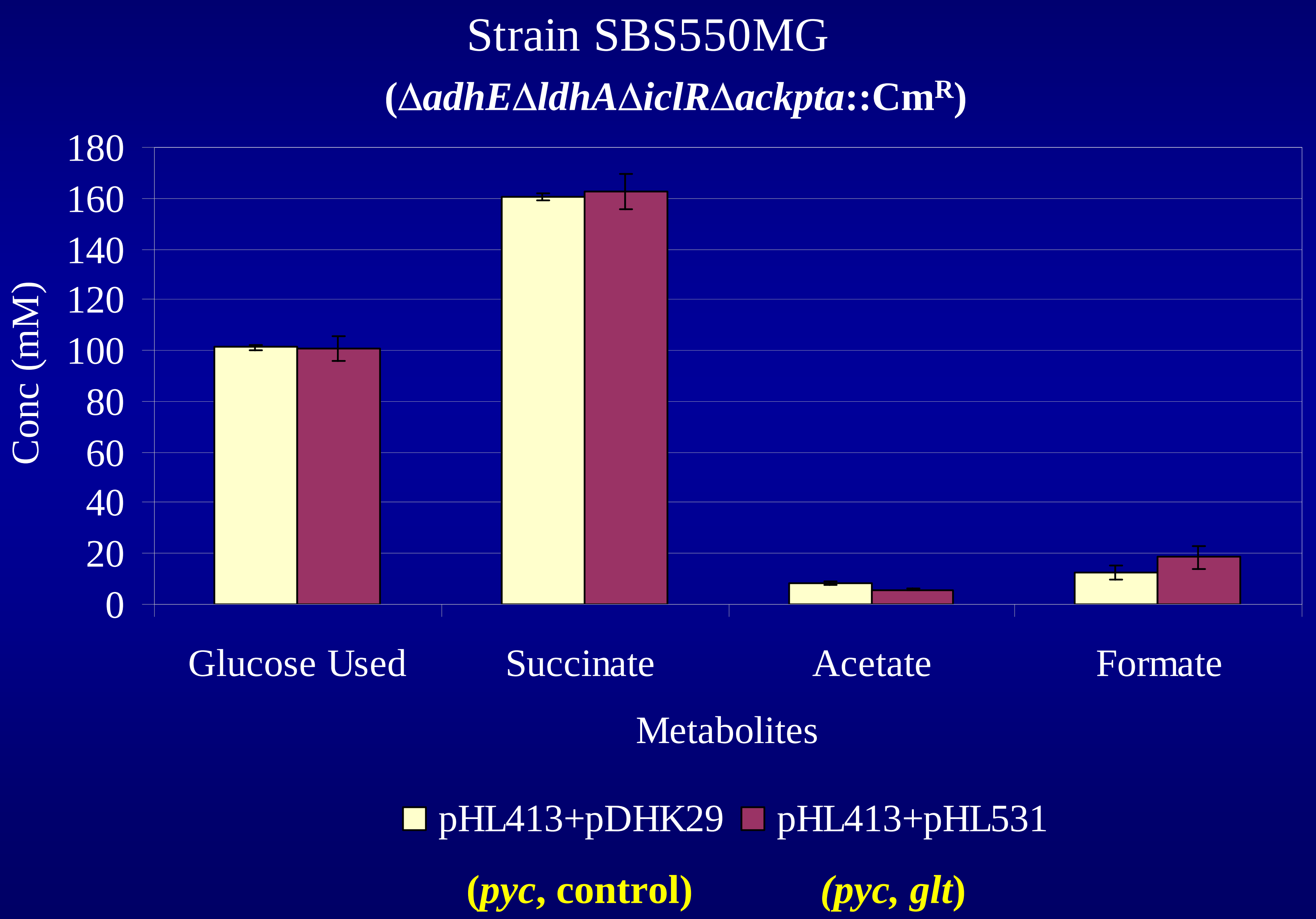


Experimental results

Strain SBS550MG
(Δ*adhE*Δ*ldhA*Δ*iclR*Δ*ackpta*::Cm^R)

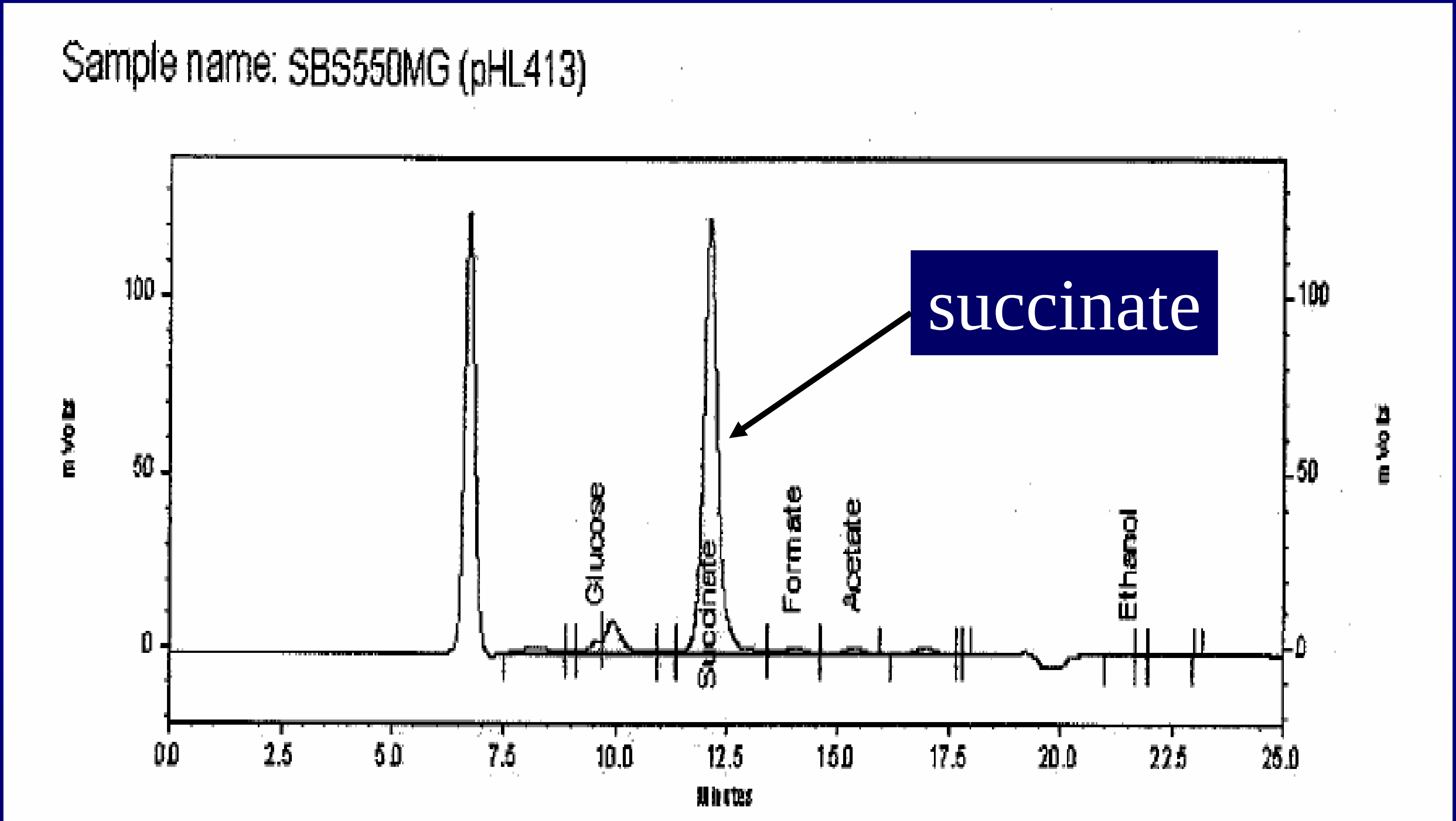
pHL413 - PYC, Ap^R
pHL531 - Citrate Synthase, Km^R

Fermentation Time: 24 hours

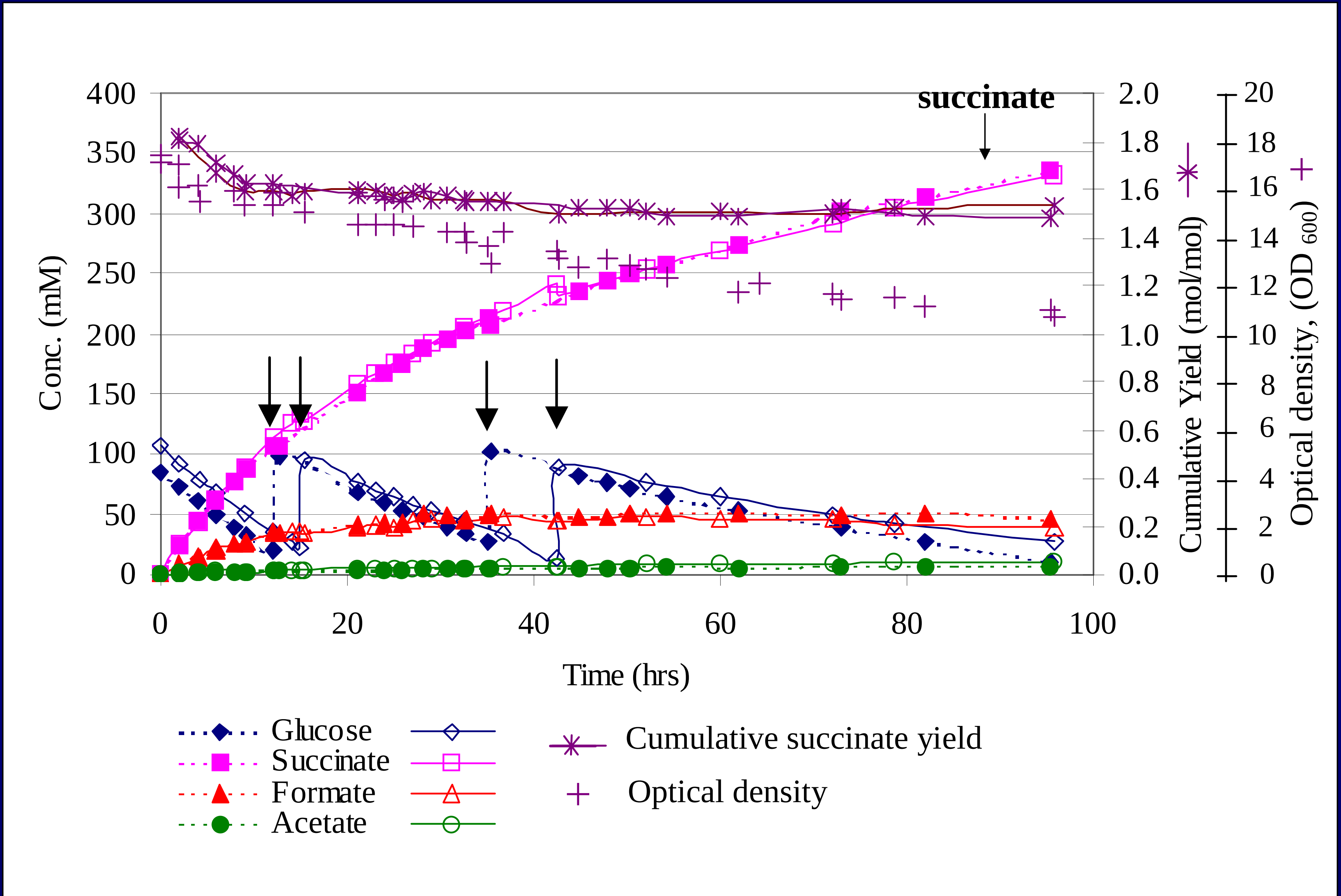


Strain SBS550MG
(Δ*adhE*Δ*ldhA*Δ*iclR*Δ*ackpta*::Cm^R)
180
160
140
120
100
80
60
40
20
0
Conc (mM)
Glucose Used
Succinate
Acetate
Formate
Metabolites
pHL413+pDHK29
pHL413+pHL531
(*pyc*, control)
(*pyc*, *glt*)

Typical HPLC metabolite output

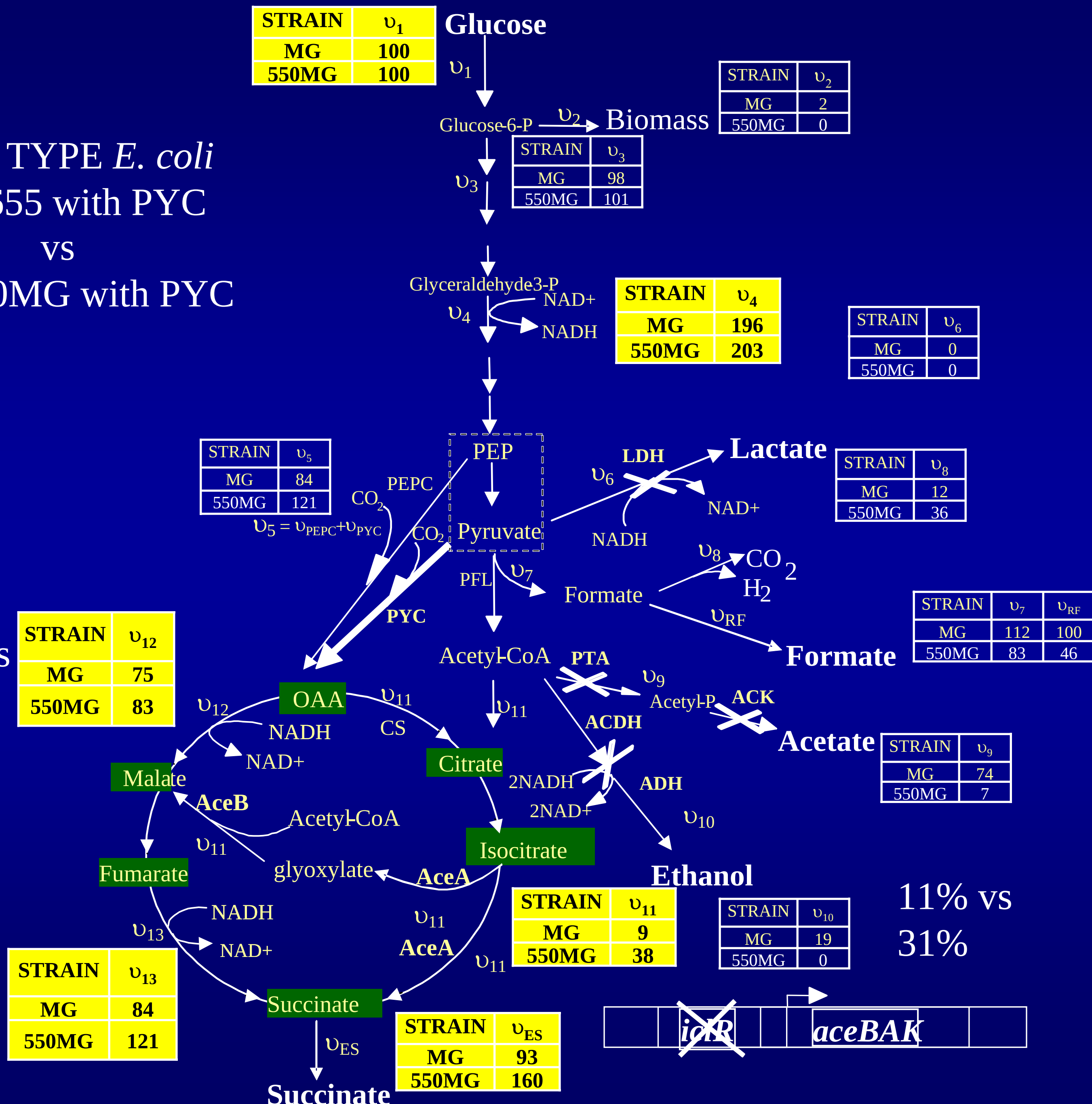


Max usage of biocatalyst - Repeated feeding experiments



(Sanchez *et al*., *Metabolic Engineering*, 2005, 7(3):229-39)

WILD TYPE *E. coli* MG1655 with PYC vs SBS550MG with PYC



STRAIN	υ_1
MG	100
550MG	100

STRAIN	υ_2
MG	2
550MG	0

STRAIN	υ_3
MG	98
550MG	101

STRAIN	υ_4
MG	196
550MG	203

STRAIN	υ_6
MG	0
550MG	0

STRAIN	υ_5
MG	84
550MG	121

STRAIN	υ_8
MG	12
550MG	36

STRAIN	υ_7	υ_{RF}
MG	112	100
550MG	83	46

89% vs 69%

STRAIN	υ_{12}
MG	75
550MG	83

STRAIN	υ_9
MG	74
550MG	7

STRAIN	υ_{11}
MG	9
550MG	38

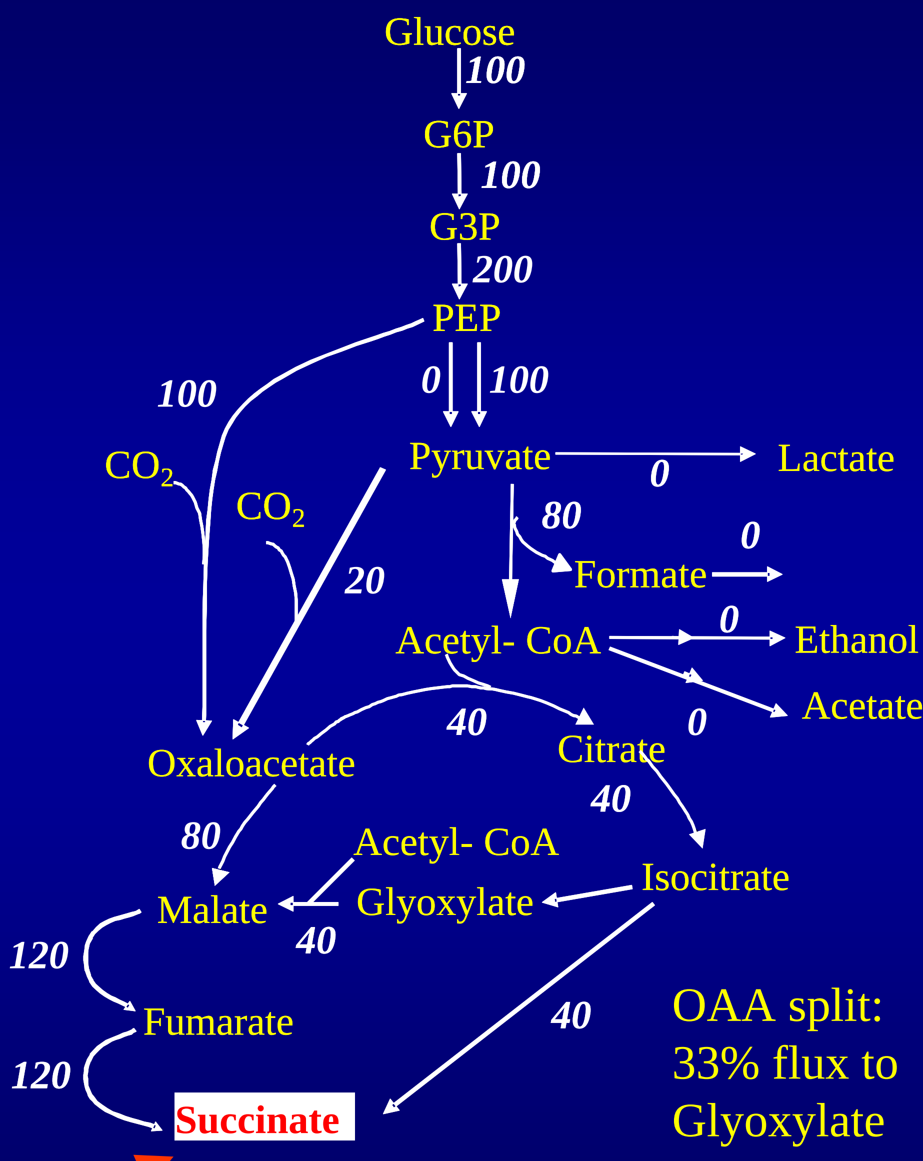
STRAIN	υ_{10}
MG	19
550MG	0

11% vs 31%

STRAIN	υ_{13}
MG	84
550MG	121

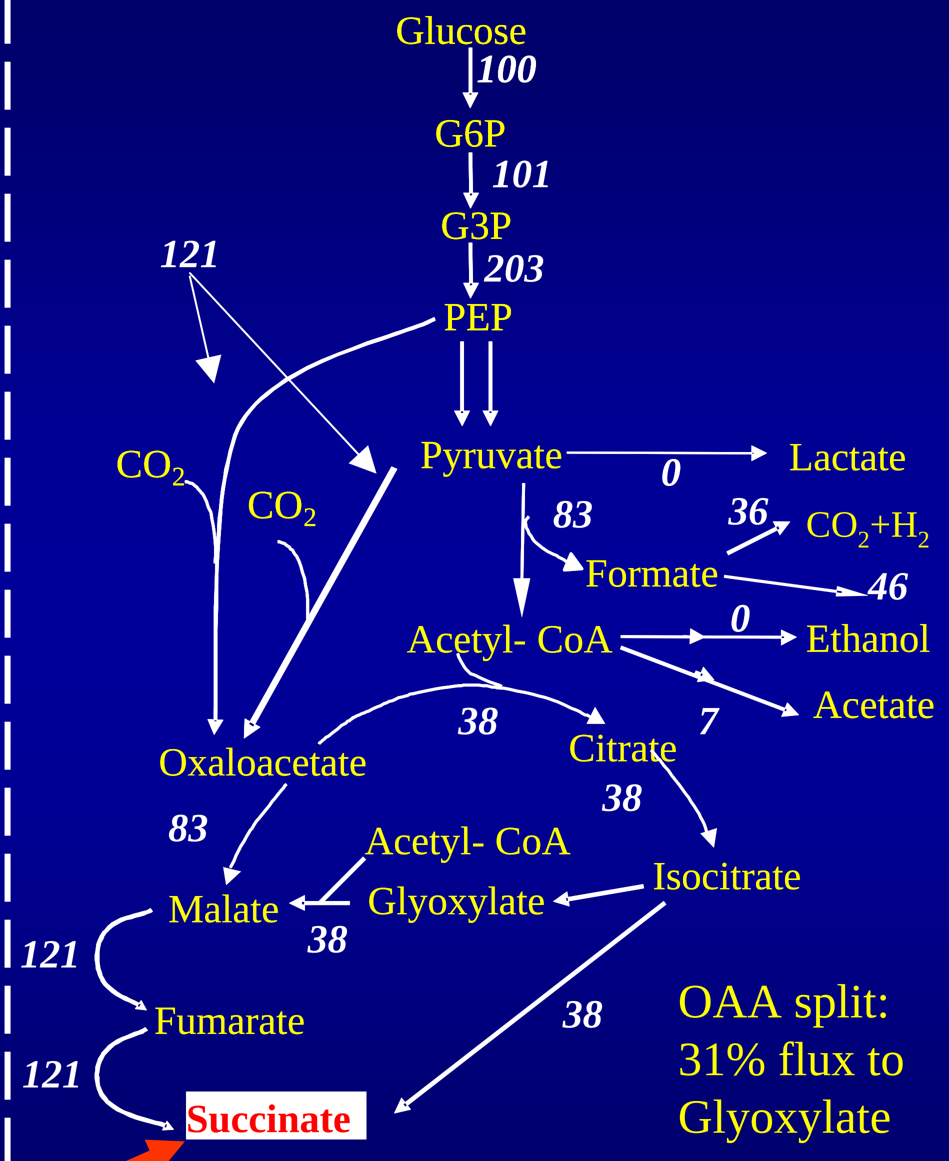
STRAIN	υ_{ES}
MG	93
550MG	160

PATHWAY DESIGN
THEORETICAL OPTIMIZATION



New theoretical yield= 1.6 mol/mol

PATHWAY IMPLEMENTATION
CALCULATED FLUXES FROM EXPERIMENTAL DATA



Experimental yield= 1.6 mol/mol

(Cox *et al.*, *Metabolic Engineering*, Published online (2005)

Summary (anaerobic)

- We have successfully designed and implemented a very robust dual-route succinate production system
- This system converts glucose to succinate at fast rates and high yields with minimal by-products
- The yield* is very close to the maximum theoretical value
- The NADH requirement is reduced relative to the sole fermentative pathway, from two moles of NADH per mole of succinate to ~ 1.25 therefore maximizing succinate production

* during production phase

Aerobic succinate production system

Reconstructing the TCA cycle and glyoxylate bypass

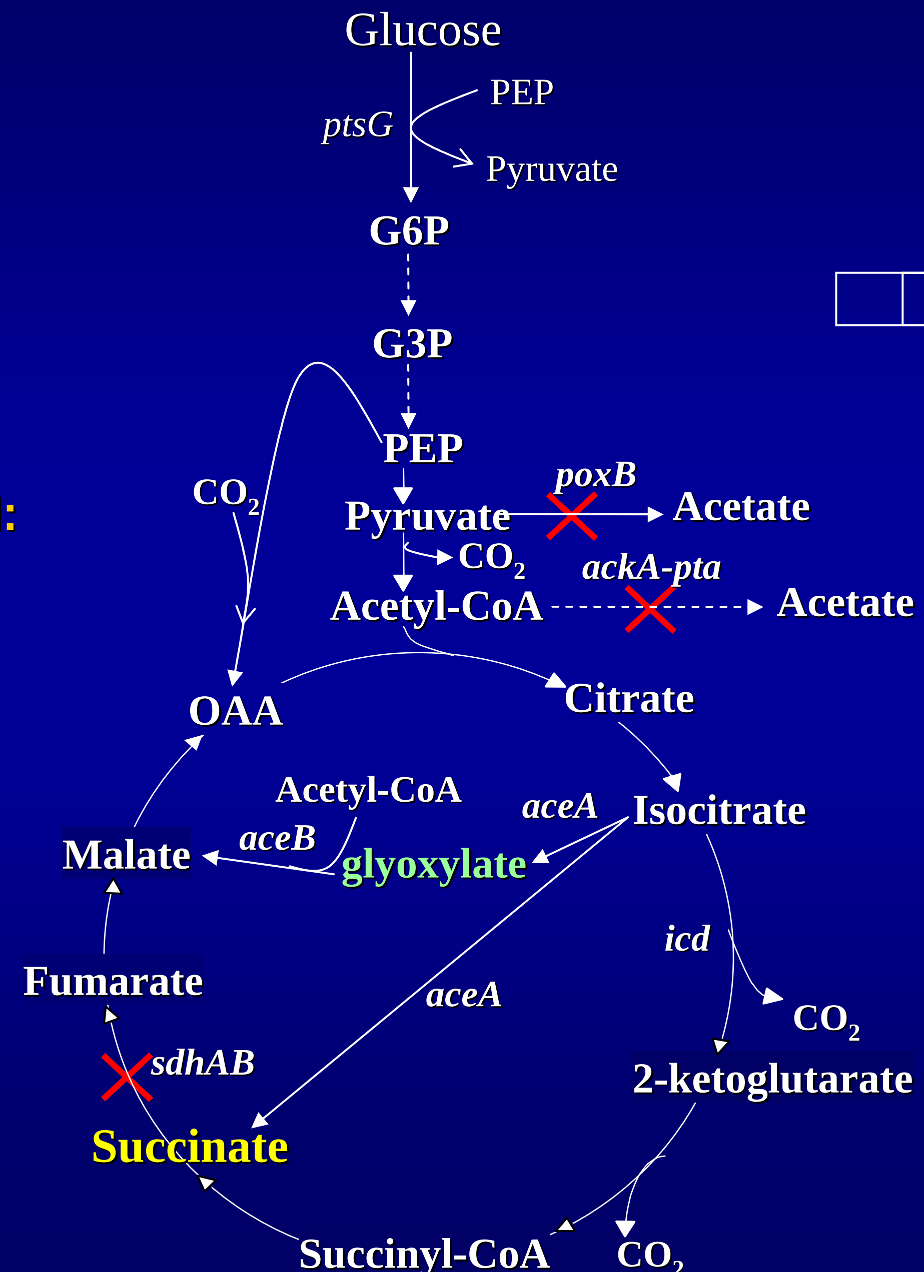
DNA Techniques:

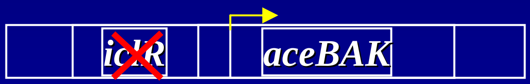
λ Red recombinase

P1 phage transduction

Verified by genomic PCR

Max. theoretical yield:
1.0 (mol succinate/ mol glucose)





sdhAB - 2

iclR - 5

poxB - 6

ackA-pta - 7

Two routes engineered for succinate production

Strain HL2765k

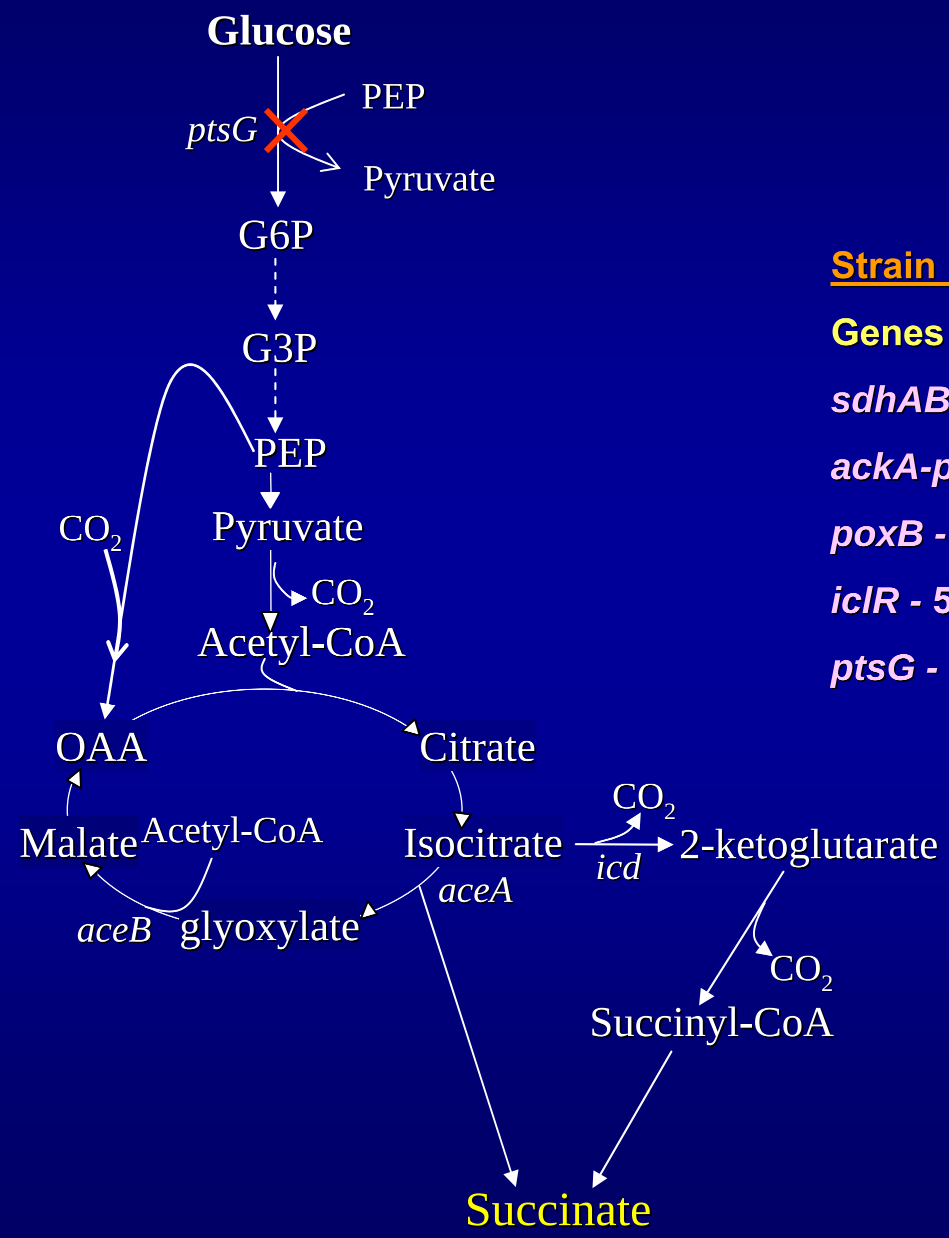
Genes inactivated:

***sdhAB* - 2**

***ackA-pta* - 7**

***poxB* - 6**

***iclR* - 5**



Strain HL27659k

Genes inactivated:

***sdhAB* - 2**

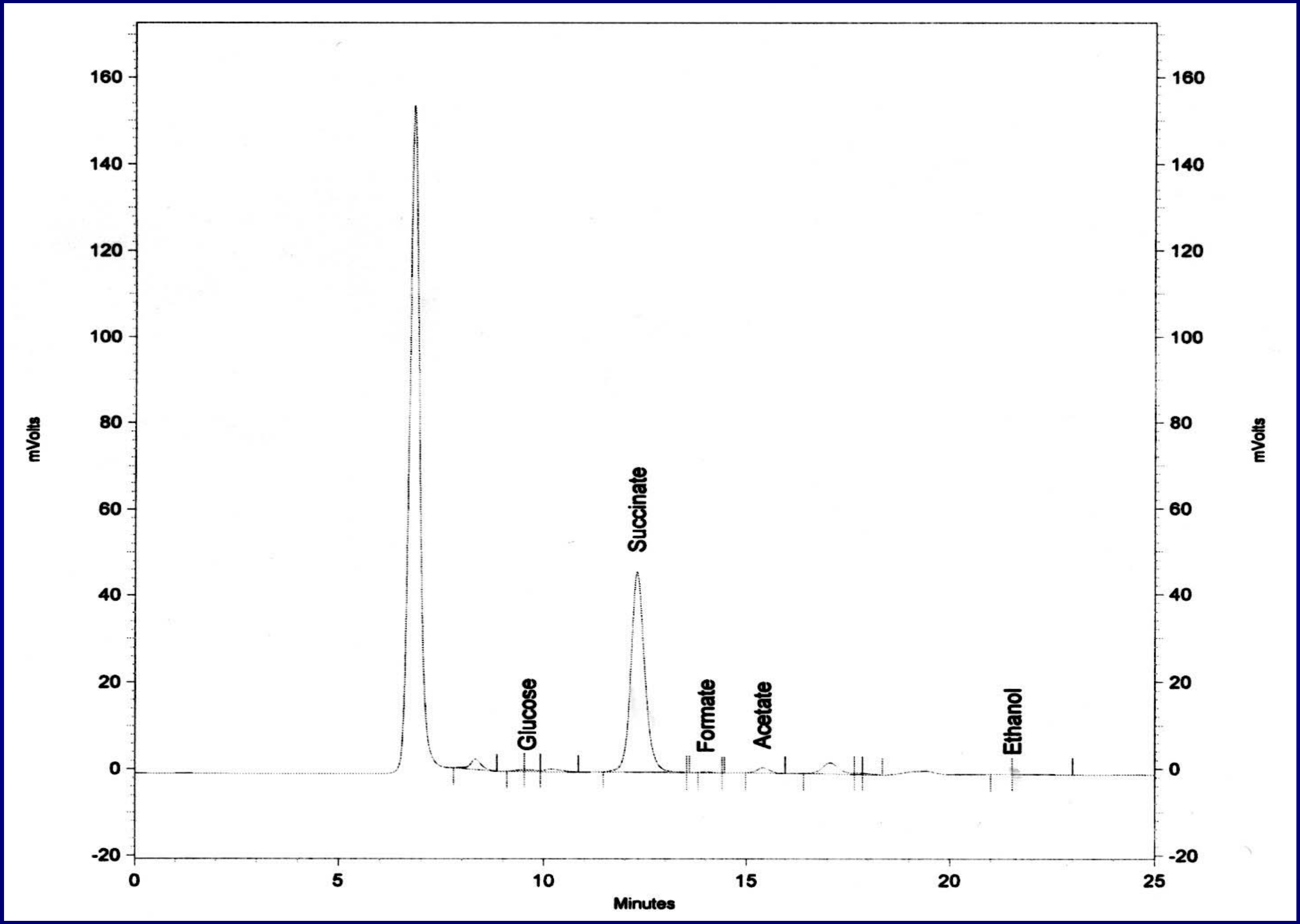
***ackA-pta* - 7**

***poxB* - 6**

***iclR* - 5**

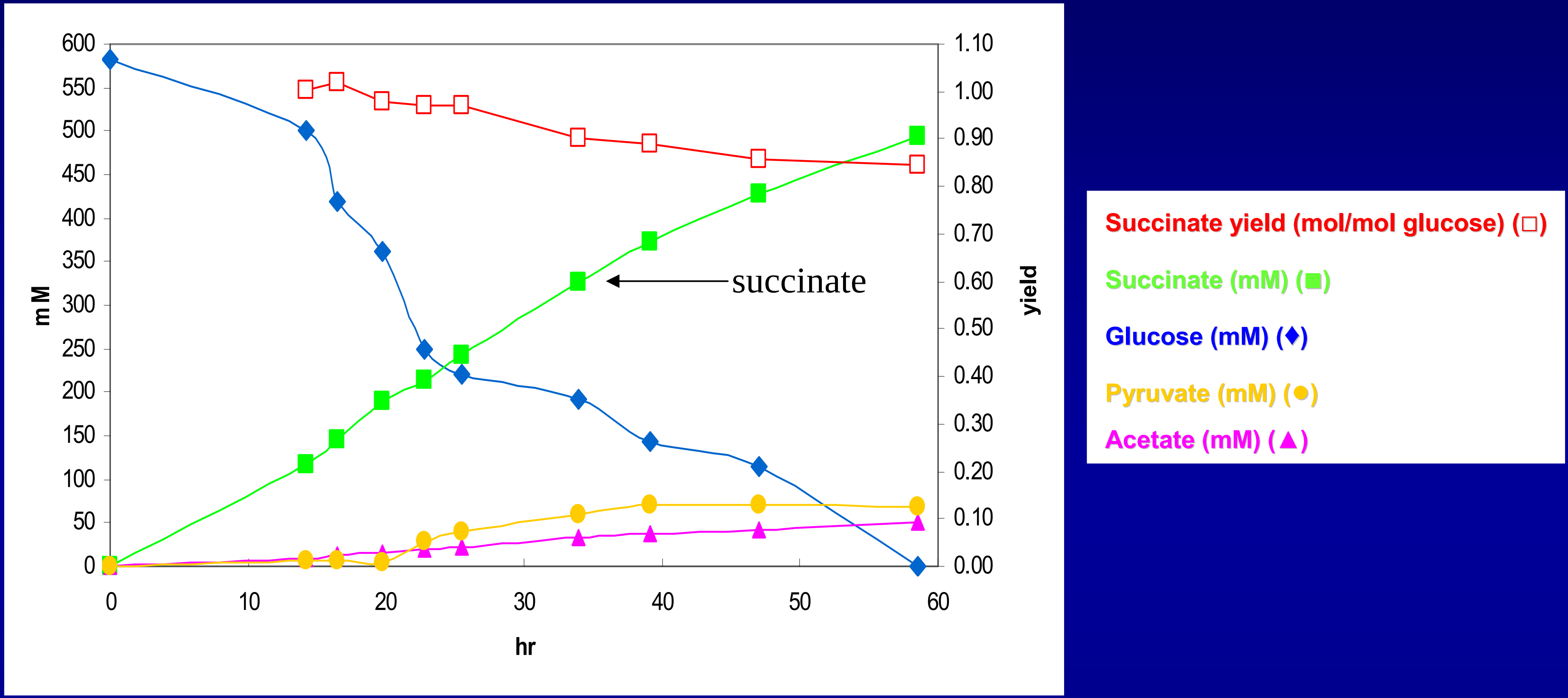
***ptsG* - 9**

HPLC metabolite output for strain HL27659k(pKK313)



Strain HL27659k(pKK313) is the aerobic succinate production system

Fed batch culture of strain HL27659k(pKK313)



- 58.25 g/l (493.63 mM) succinate produced in 59 hrs
- Average succinate yield was 0.94 ± 0.07 mol/mol glucose
- Average productivity was 1.08 ± 0.06 g/l-hr
- Average specific productivity was 89.77 ± 3.40 mg/g-hr.

(Lin *et al*., *Biotechnol. Bioeng*., 2005, 20;90(6):775-9)

Summary (aerobic)

- We have successfully designed and implemented a very robust dual-route aerobic succinate production system
- This system converts glucose to succinate at fast rates and high yields with minimal by-products
- The synthesis of succinate through these two pathways does not require any NADH

Hybrid succinate production systems

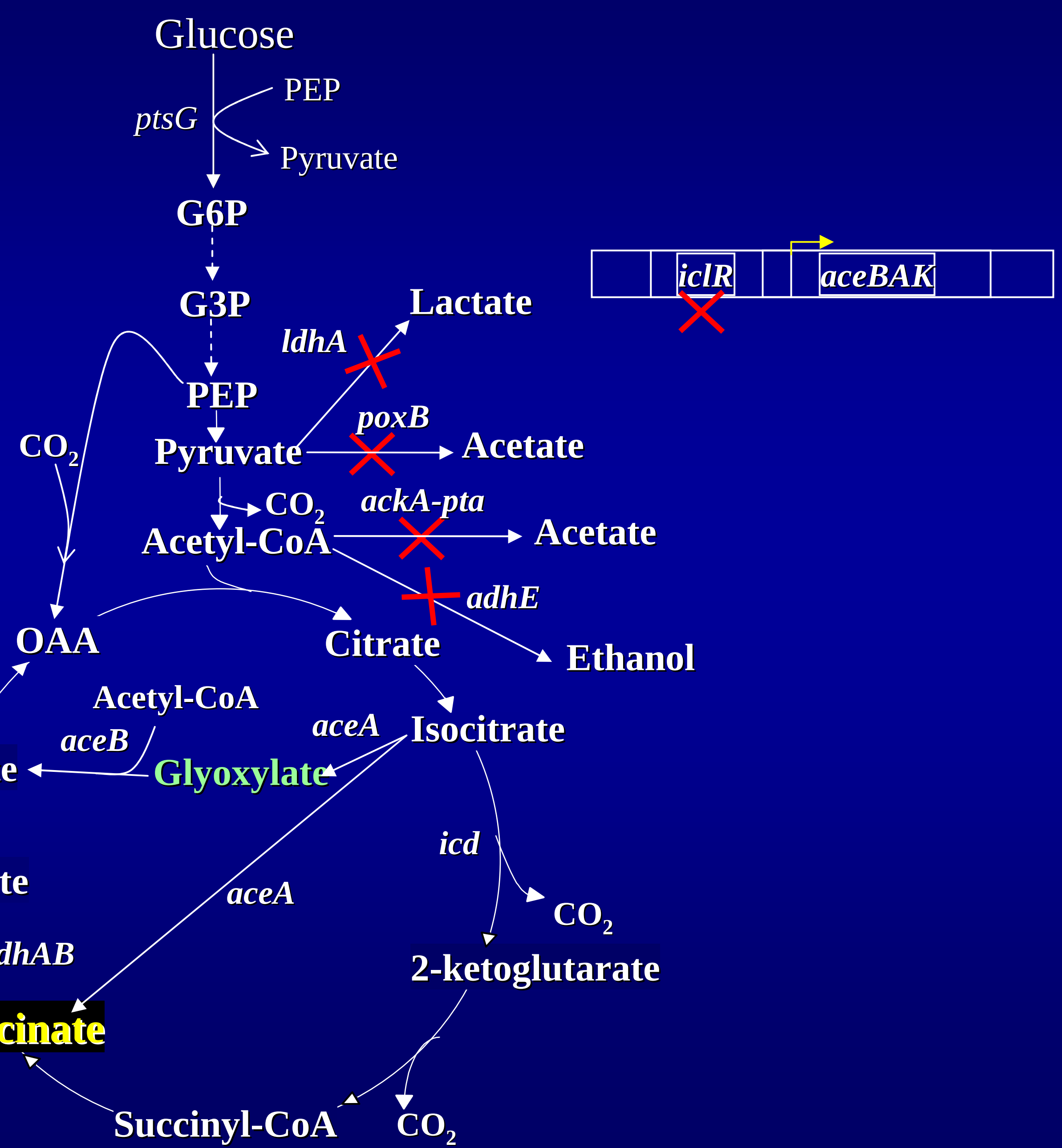
Construction of a hybrid succinate production system

DNA Techniques:

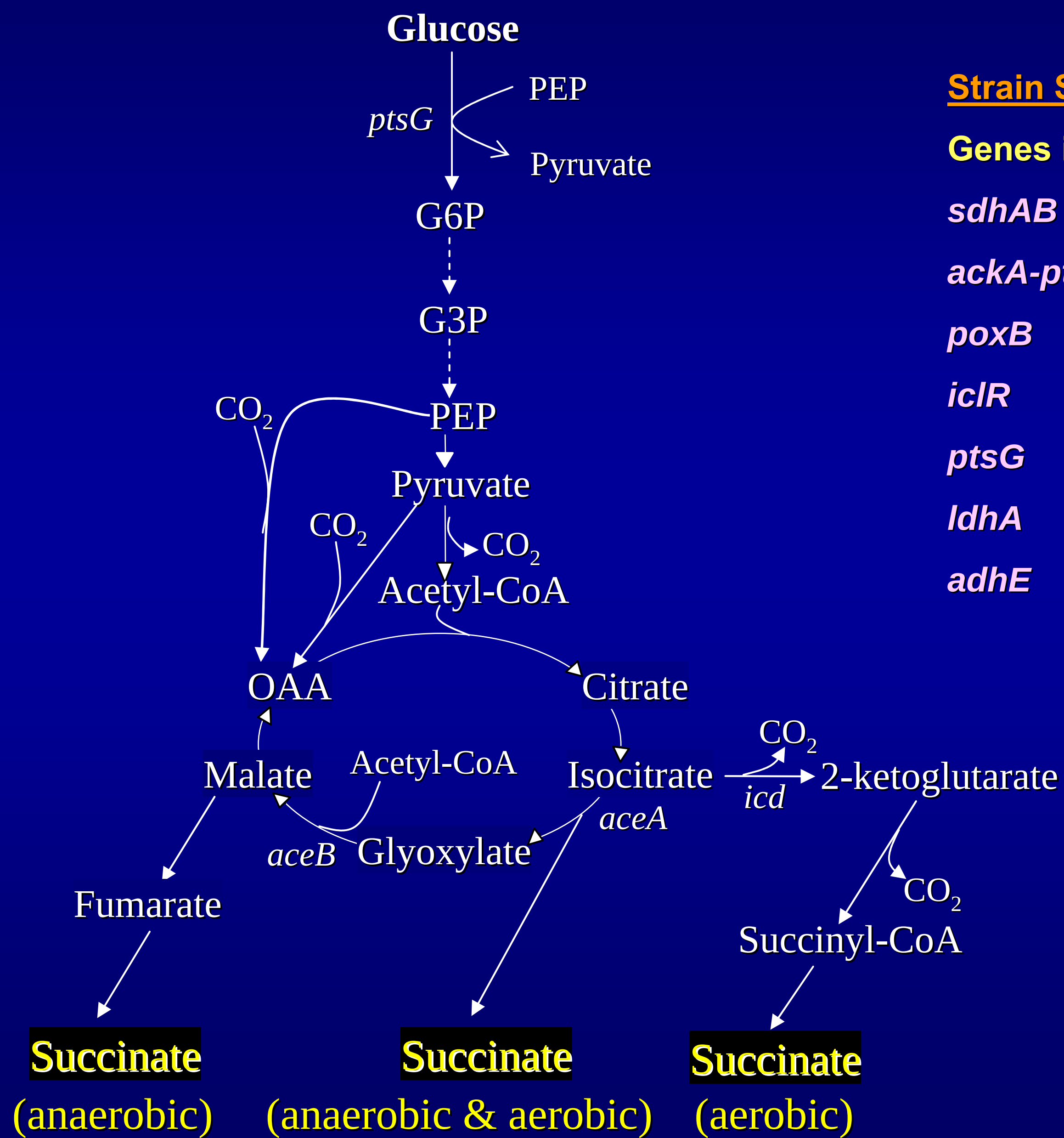
λ Red recombinase

P1 phage transduction

Verified by genomic PCR



Three routes engineered for succinate production



Strain SBS552

Genes inactivated:

- ***sdhAB***
- ***ackA-pta***
- ***poxB***
- ***iclR***
- ***ptsG***
- ***ldhA***
- ***adhE***

Overall Summary

- We have developed:
  - an efficient aerobic succinate production system with a molar yield of close to 1
  - a high yield anaerobic succinate production system with a molar yield of close to 1.6
  - A hybrid system that is capable of producing succinate under aerobic and anaerobic conditions
- Same technology can be used to produce other C-4 compounds, such as fumarate and malate

Acknowledgments

Collaborator:	**George N. Bennett**
Graduate Students:	**Ailen M. Sanchez** **Henry Lin** **Irene Martínez** **Susana Berrios-Rivera**

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