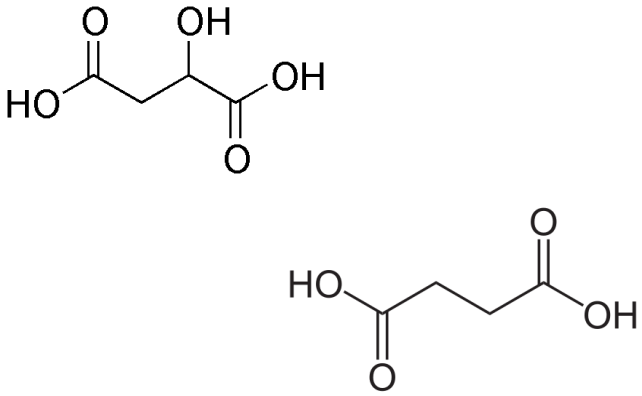
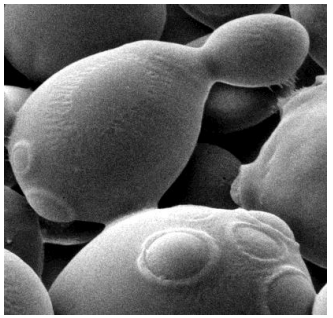
Engineering of carboxylation in *S. cerevisiae:*
PEPCK and malic enzyme

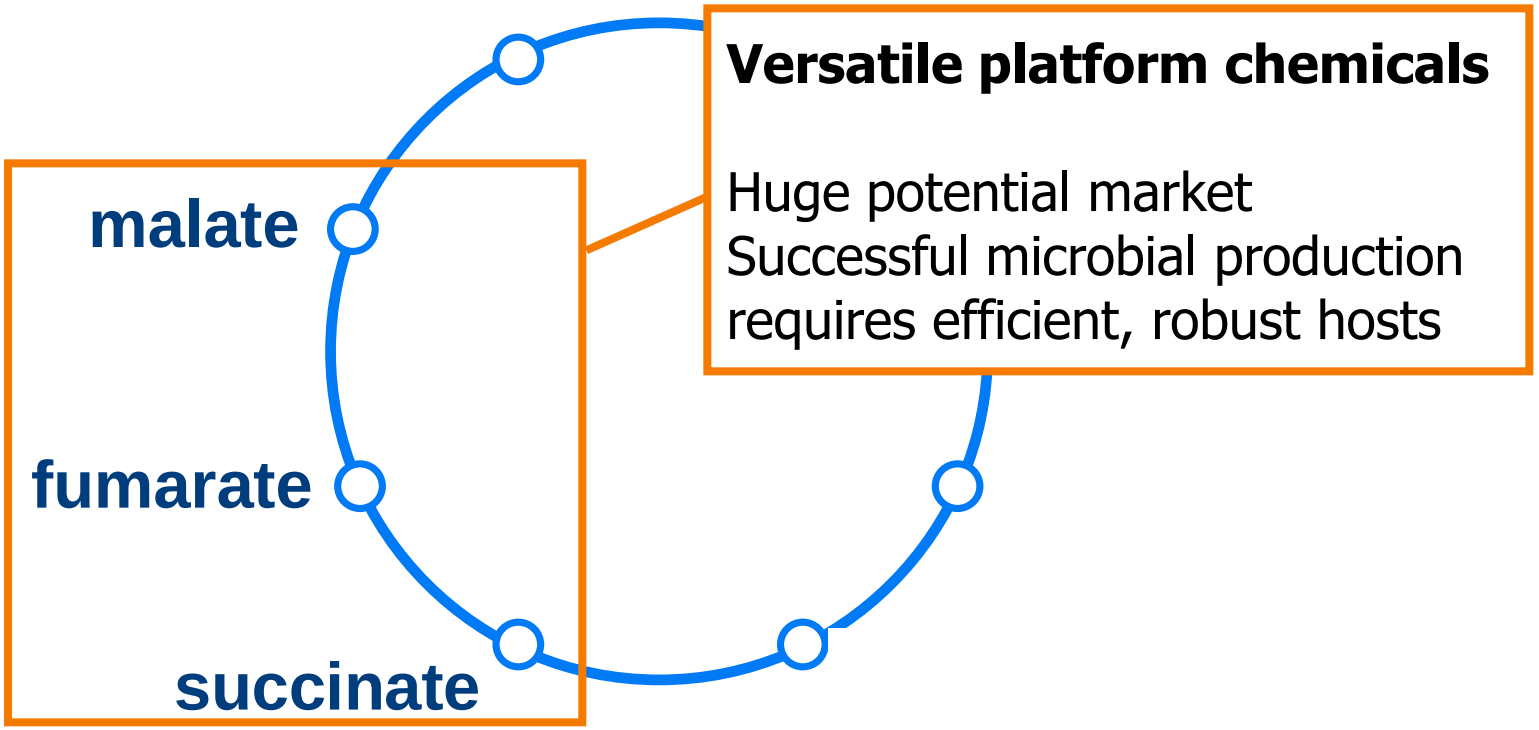


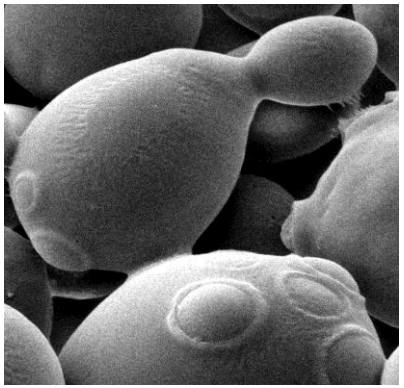
Ton van Maris

Industrial Microbiology - Delft University of Technology
Kluyver Centre for Genomics of Industrial Fermentation
Delft, The Netherlands



1,4-dicarboxylic acids and *Saccharomyces cerevisiae*

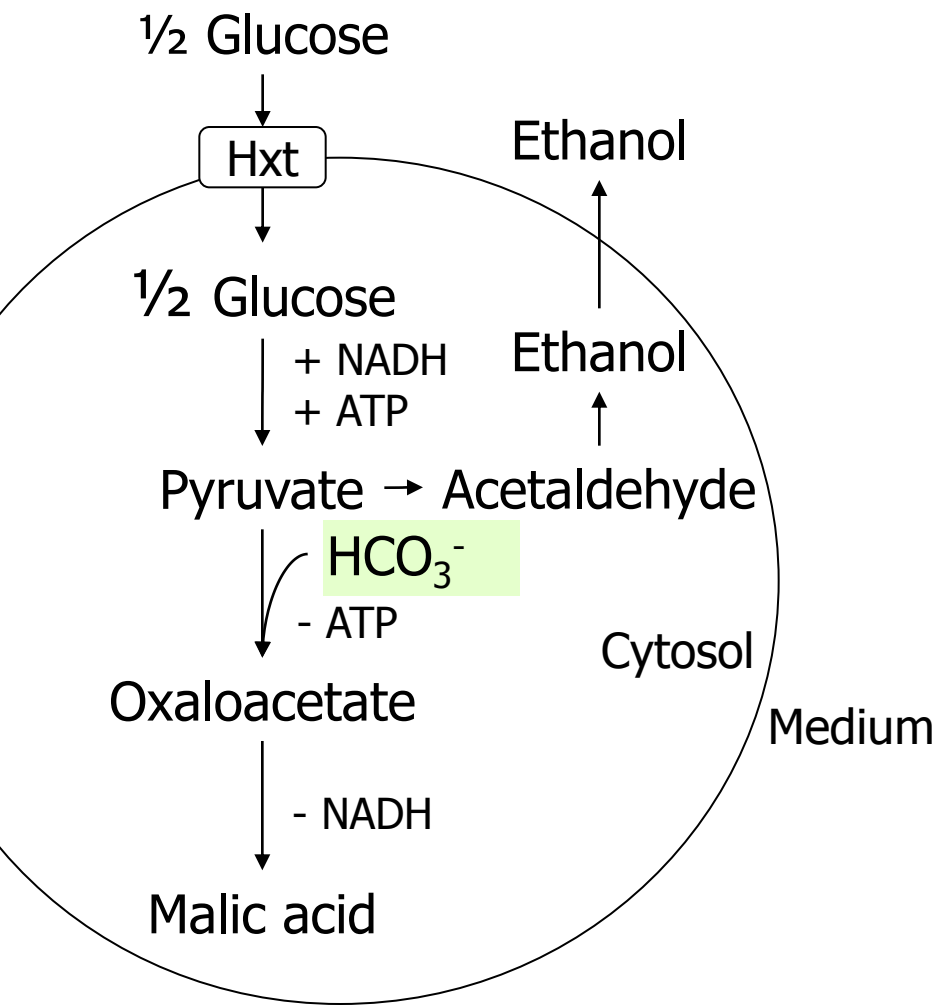




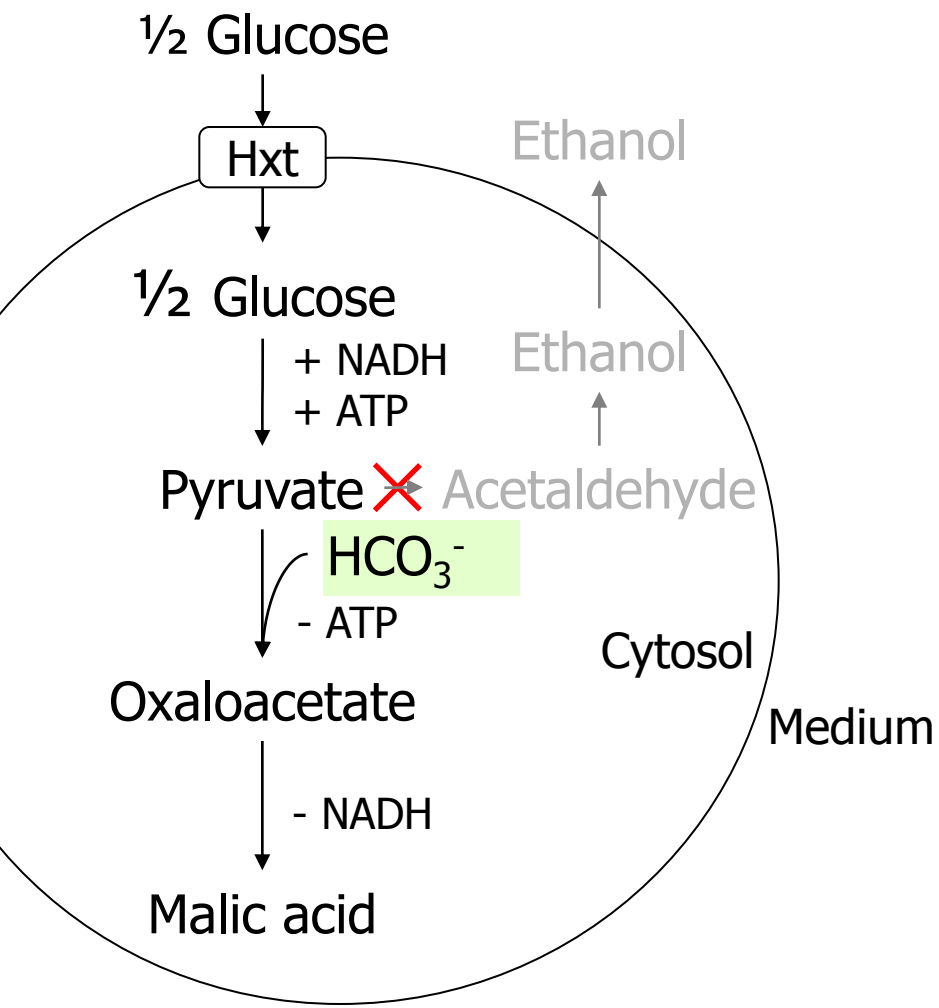
- Ultimate goal: high-yield production of free acids
- Very good tolerance to acidic conditions
- GRAS status for several applications

→ Wild-type strains produce only traces of C_4 acids

Rerouting metabolism for malate production

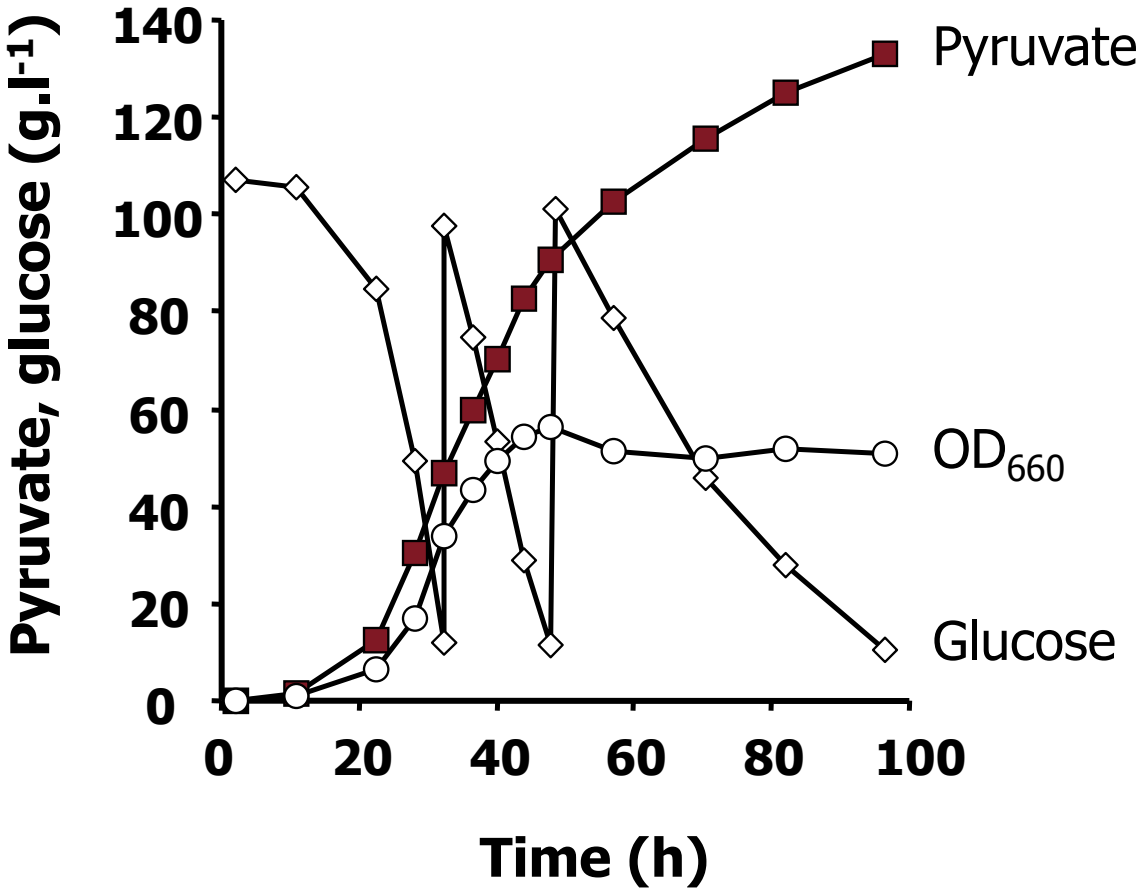


Rerouting metabolism for malate production



An ethanol-negative, pyruvate producing yeast platform

C_2-independent, glucose-tolerant *pdc1,5,6* strain of *S. cerevisiae* obtained by evolutionary engineering ("TAM")



[pyruvate] = 135 $g.l^{-1}$

$Y_{pyr,\ gluc}$ = 0.55 $g.g^{-1}$

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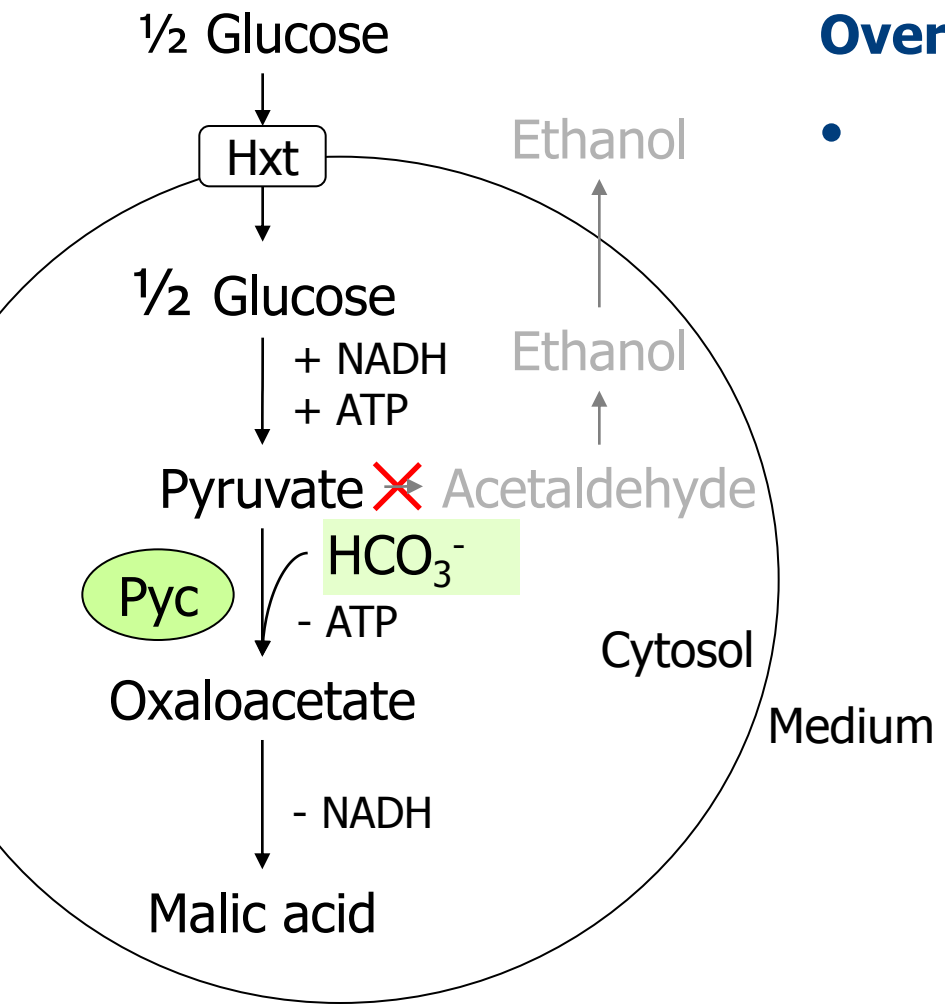
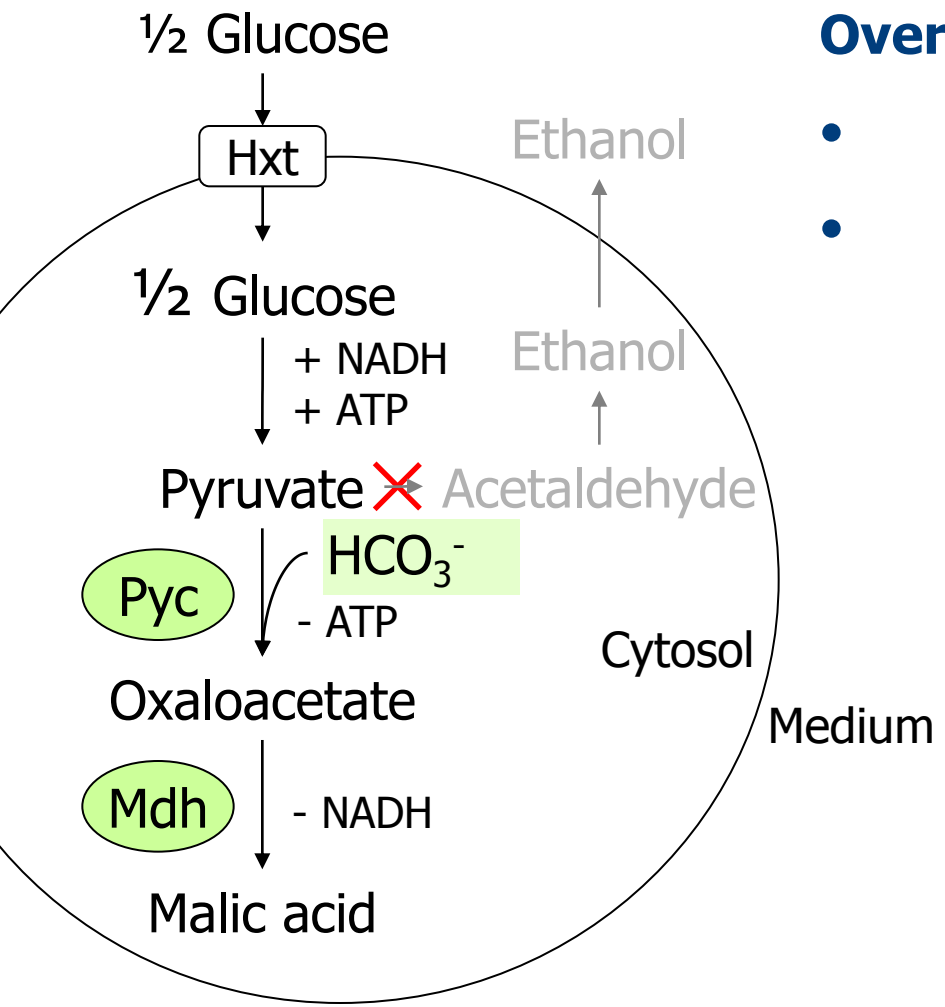
Rerouting metabolism for malate production



Overexpressed genes:

- **pyruvate carboxylase**

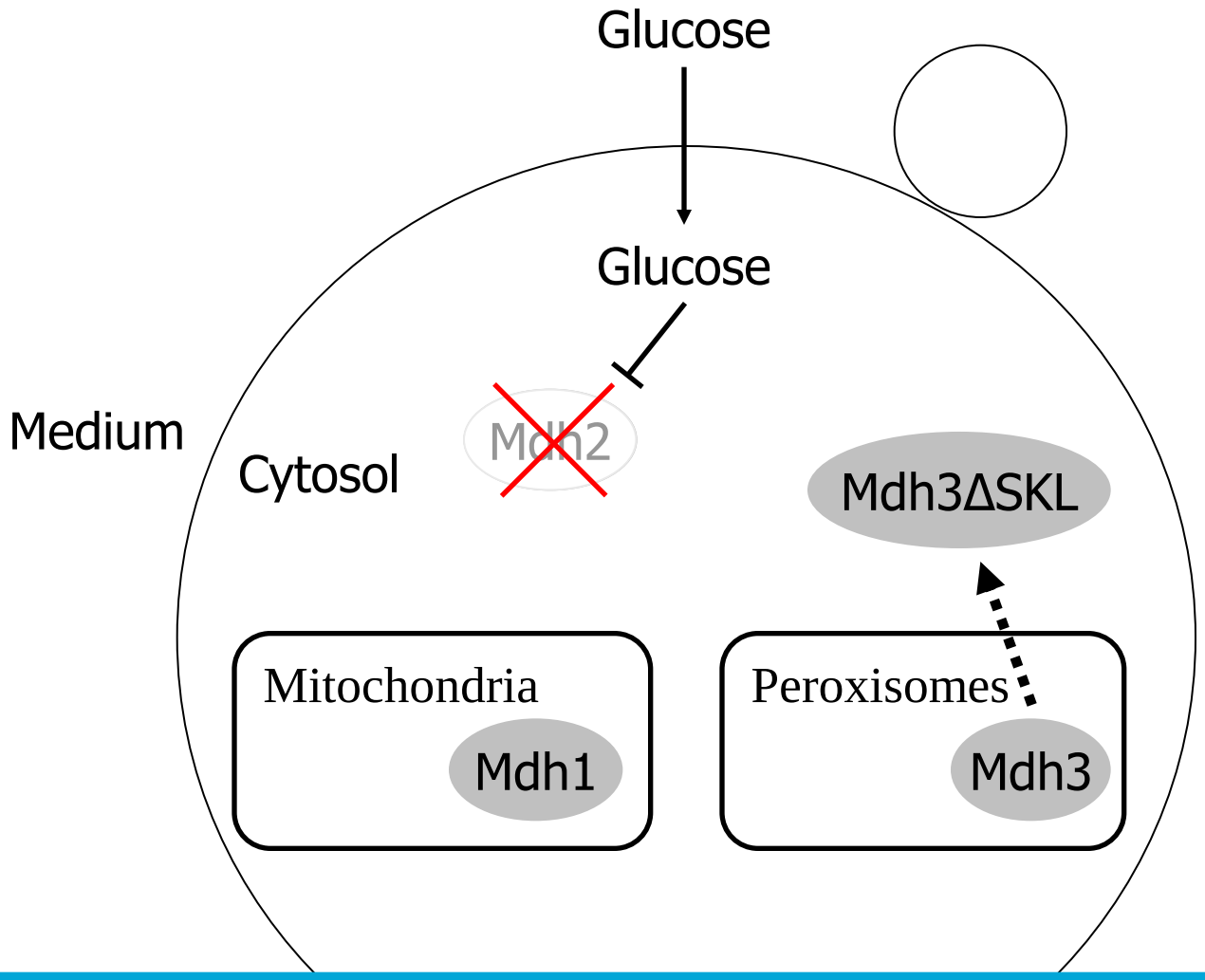
Rerouting metabolism for malate production



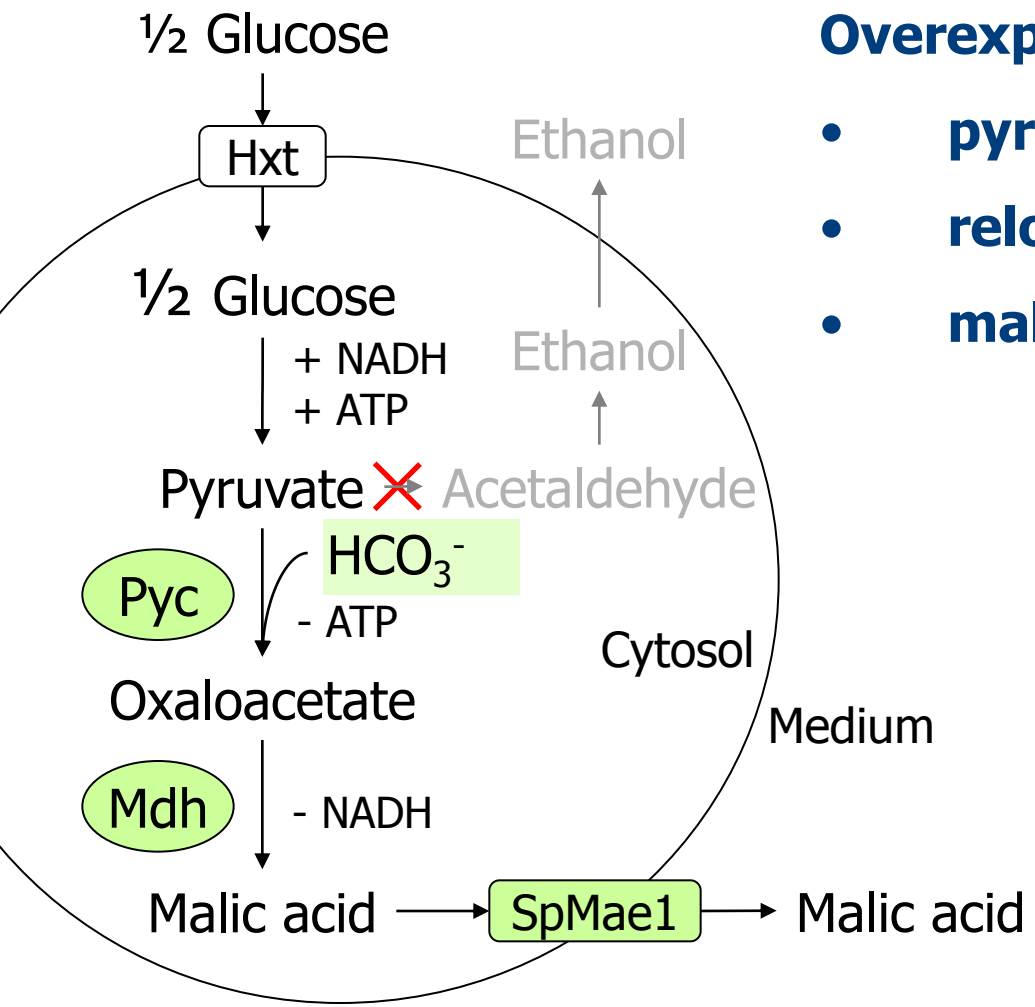
Overexpressed genes:

- **pyruvate carboxylase**
- **relocated malate dehydrogenase**

Rerouting metabolism for malate production



Rerouting metabolism for malate production



Overexpressed genes:

- **pyruvate carboxylase**
- **relocated malate dehydrogenase**
- **malate transporter from *S. pombe***

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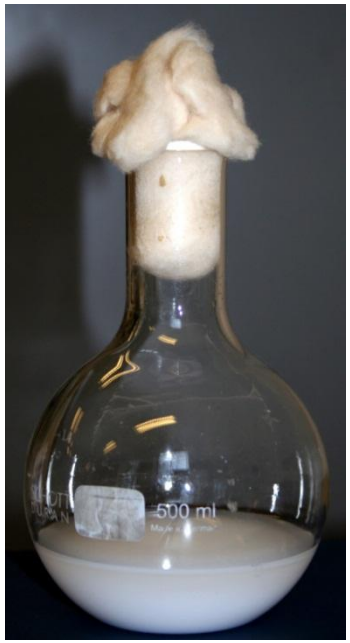
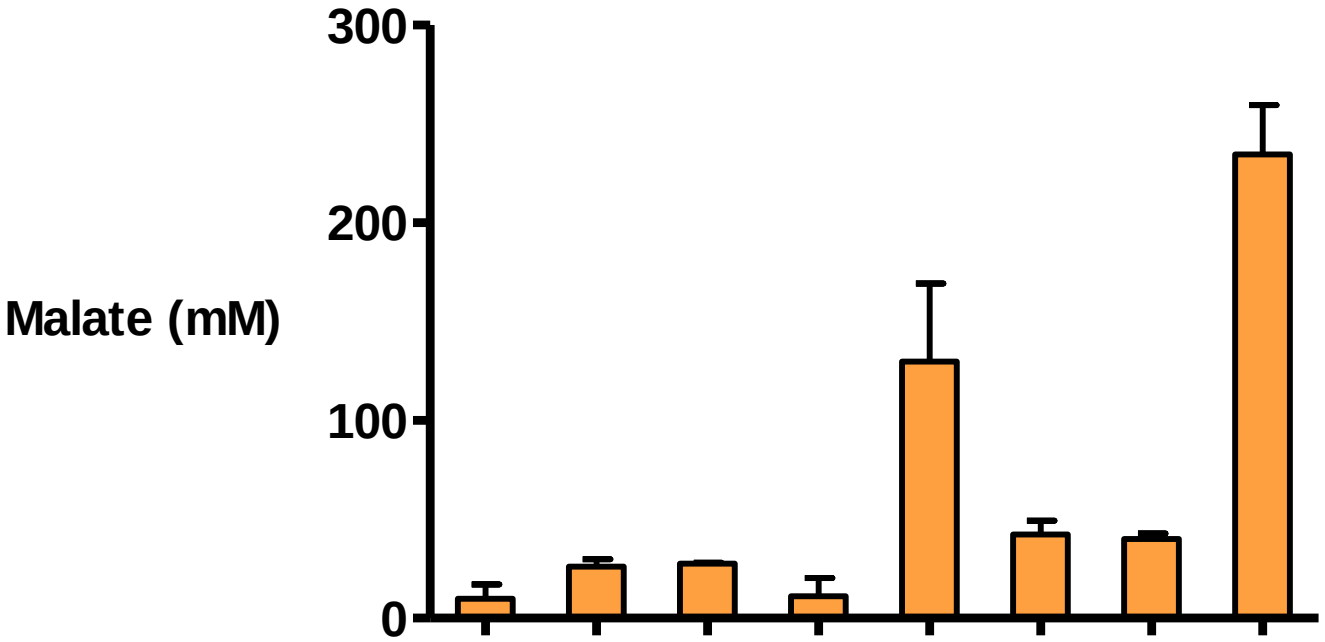
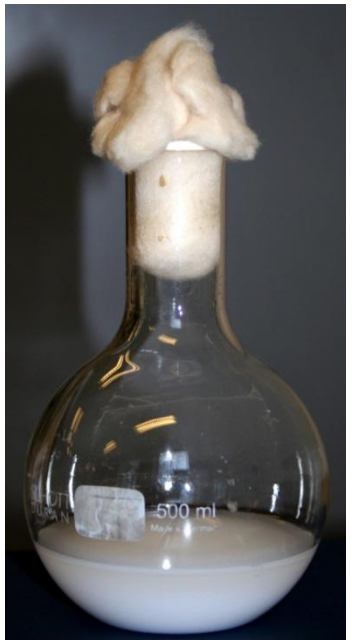
Rerouting metabolism for malate production



Rerouting metabolism for malate production





Malate transporter	—	+	—	—	+	—	+	+	*SpMAE1*
Malate dehydrogenase	—	—	+	—	+	+	—	+	*MDH3ΔSKL*
Pyruvate carboxylase	—	—	—	+	—	+	+	+	*PYC2*

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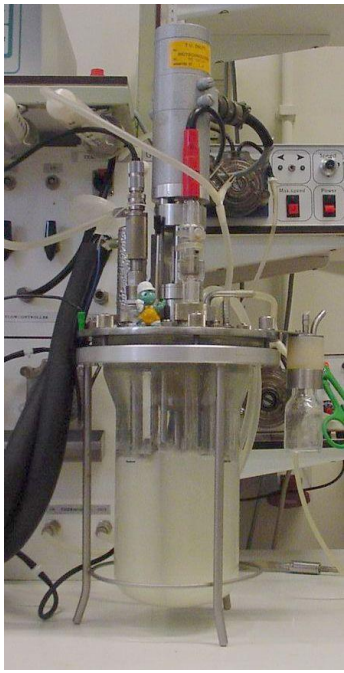
Transfer to fermenters and (some) optimization



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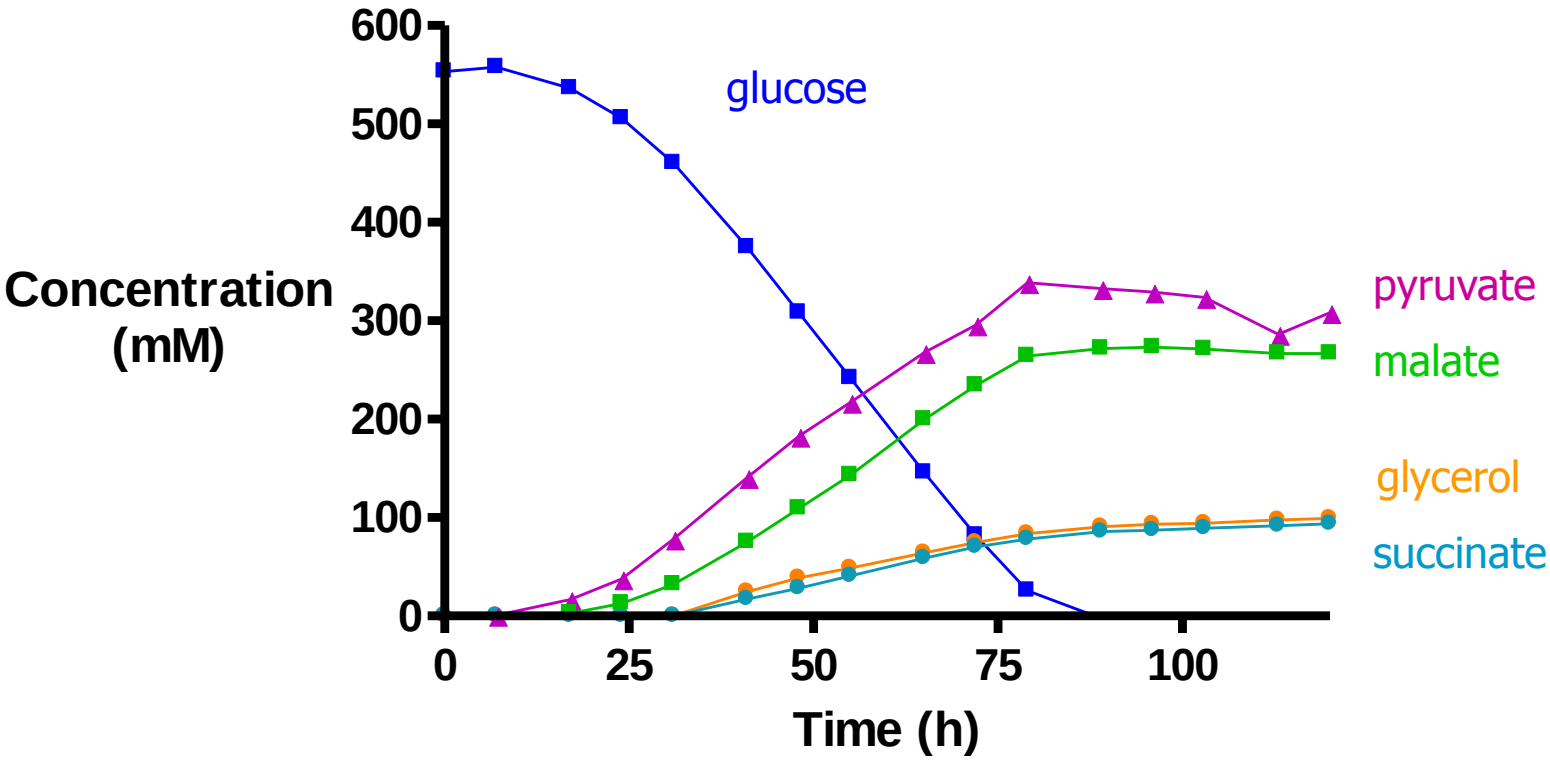
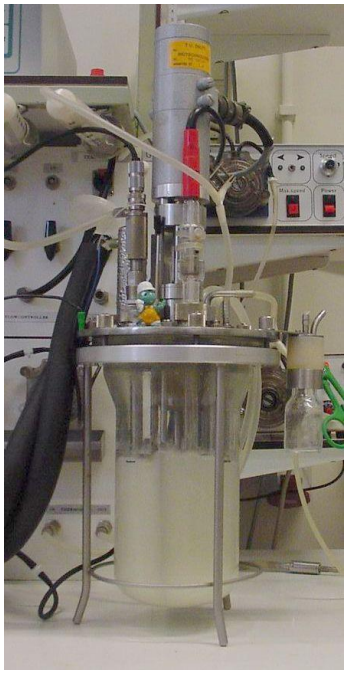
Transfer to fermenters and (some) optimization





Air with 3% O_2 and 15% CO_2, pH 6.8 & 10 mM $CaCl_2$

Conversion of 100 ± 1 g l^{-1} glucose to 36 ± 1 g l^{-1} malate (Y_{SP} of 0.48 ± 0.01 mol mol^{-1})

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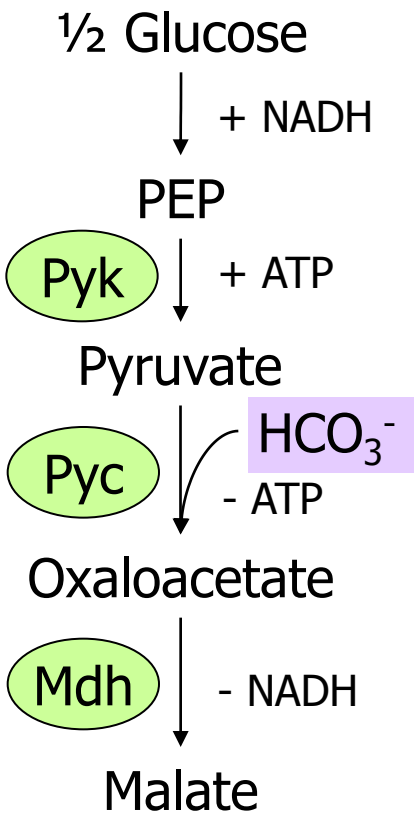
Alternative C3→C4 carboxylating reactions



ATP neutral

Rintze Zelle *et al*. 2010
Appl. Environ. Microbiol. **76**:5383

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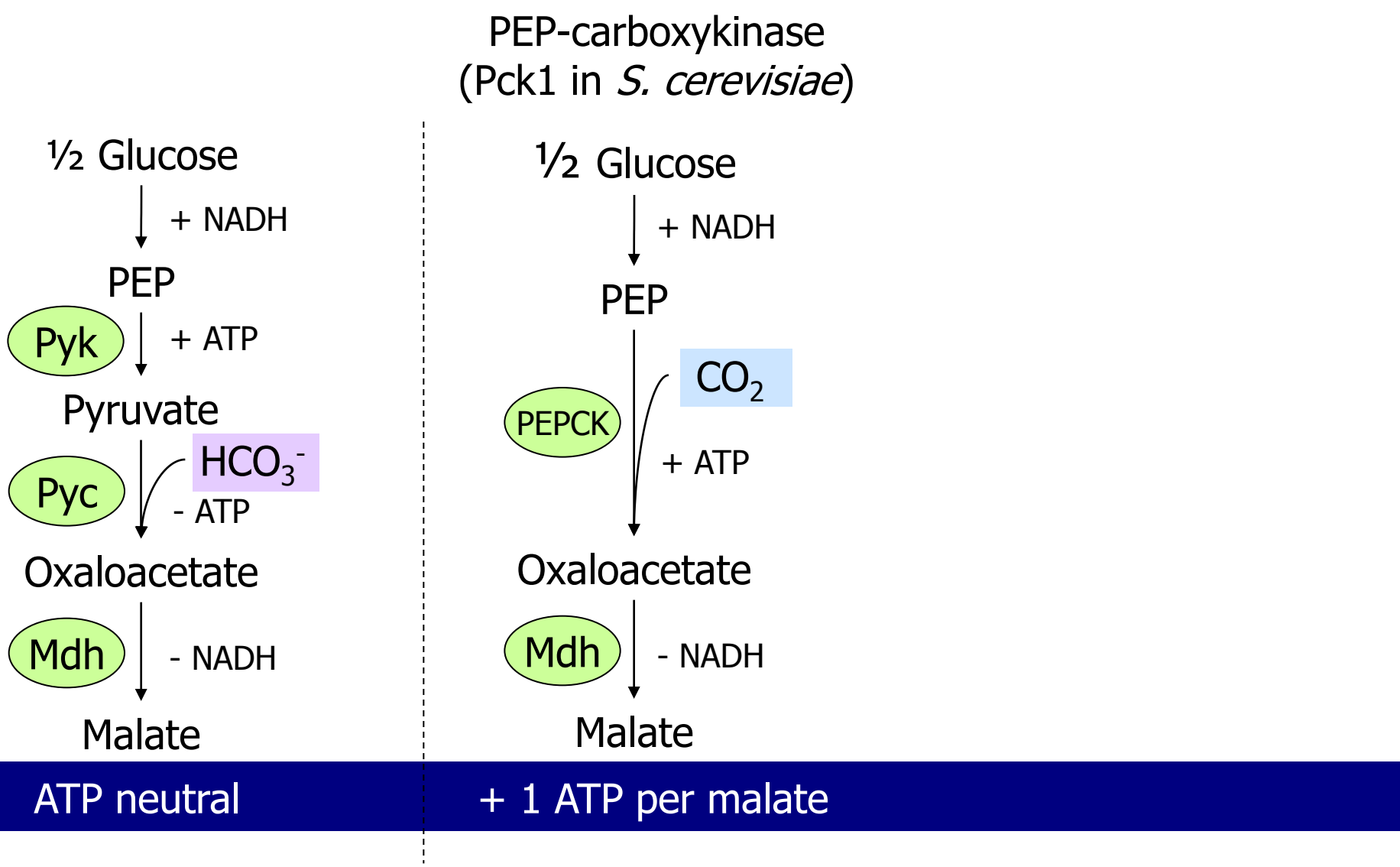
Alternative C3→C4 carboxylating reactions



Rintze Zelle *et al*. 2010
Appl. Environ. Microbiol. **76**:5383

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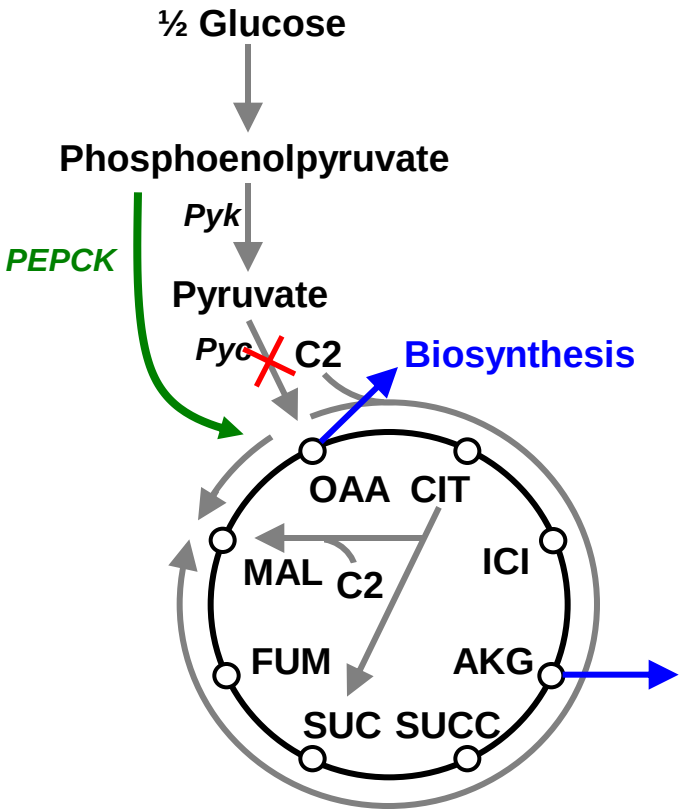
Phospho-enol-pyruvate carboxykinase (PEPCK)

PEP + CO_2 + ADP → oxaloacetate + ATP



***S. cerevisiae* Pck1**:

- decarboxylating role is essential in gluconeogenesis (reaction marked 'irreversible' in some models)
- cannot replace pyruvate carboxylase (Pyc1, Pyc2) in anaplerotic C3→C4 carboxylation under standard conditions
- strong glucose repression and glucose catabolite inactivation
- microorganisms that use PEPCK for carboxylation grow at high pCO_2

Rintze Zelle *et al*. 2010
Appl. Environ. Microbiol. **76**:5383

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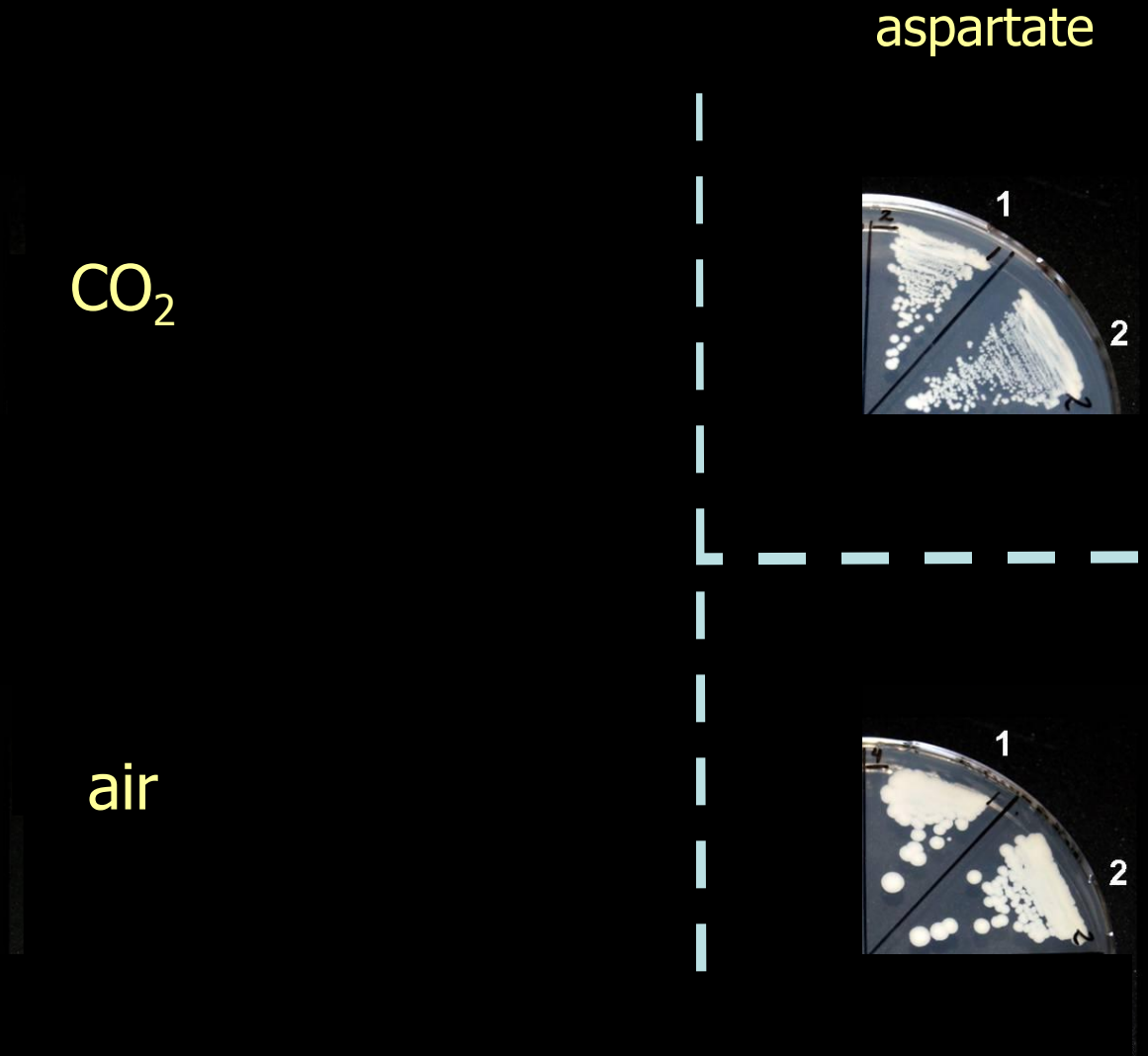
A. succinogenes PEPCK cannot complement an *S. cerevisiae pyc1,2Δ* mutant

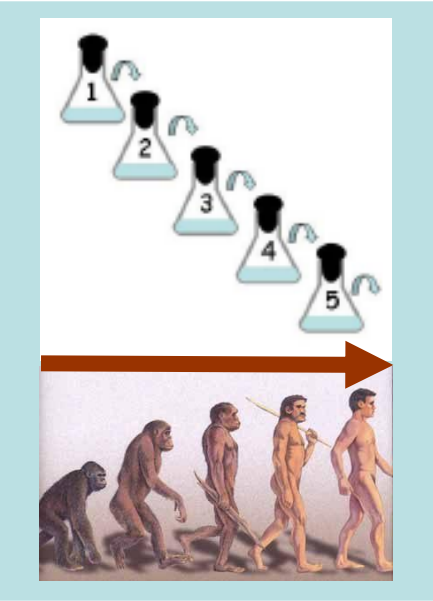
PEPCK activity in cell extracts: ca. 0.4 µmol min^{-1} $(mg\ protein)^{-1}$



Strains
1 CEN.PK 113-7D (reference)
2 IMY002 (Pyc^-, AsPEPCK)

Rintze Zelle *et al.* 2010
Appl. Environ. Microbiol. **76**:5383

Adaptive evolution of *pyc1,2Δ* strain expressing *A. succinogenes* PCK gene



- Precultivation in CO_2-sparged chemostats with aspartate, switch to medium without aspartate

- Before complete washout stop glucose feed, continue as batch culture

- CO_2-dependent growth observed after several days of incubation (2 independent experiments)

- Single-cell lines isolated and characterized

Rintze Zelle *et al*. 2010
Appl. Environ. Microbiol. **76**:5383

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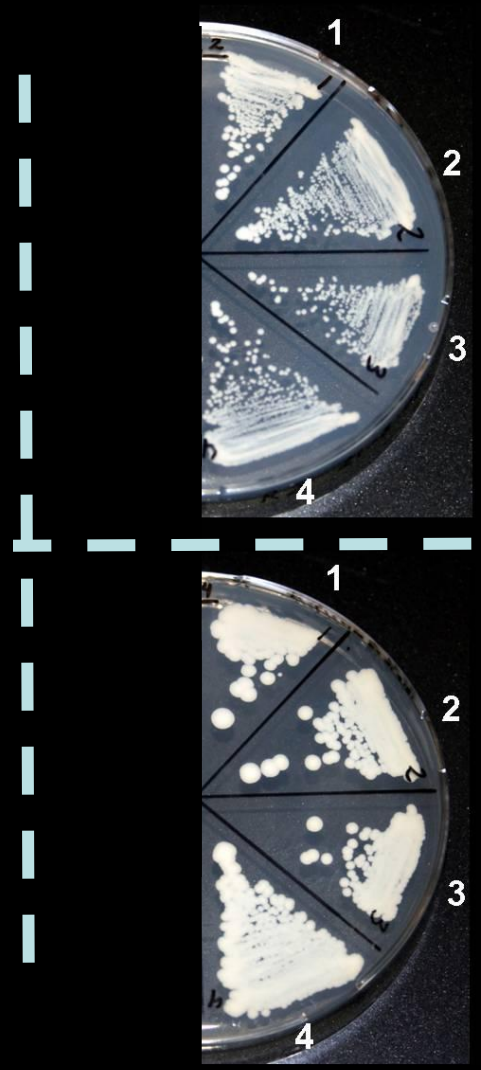
CO_2-dependent complementation of an evolved *S. cerevisiae pyc1,2Δ* strain by *A. succinogenes* PEPCK

aspartate

CO_2

air



Strains
1 CEN.PK 113-7D
2 IMY002 (Pyc$^-$, AsPEPCK)
3 IMY050 (Pyc$^-$, AsPEPCK, evolved)
4 IMY051 (Pyc$^-$, AsPEPCK, evolved)

Rintze Zelle *et al.* 2010
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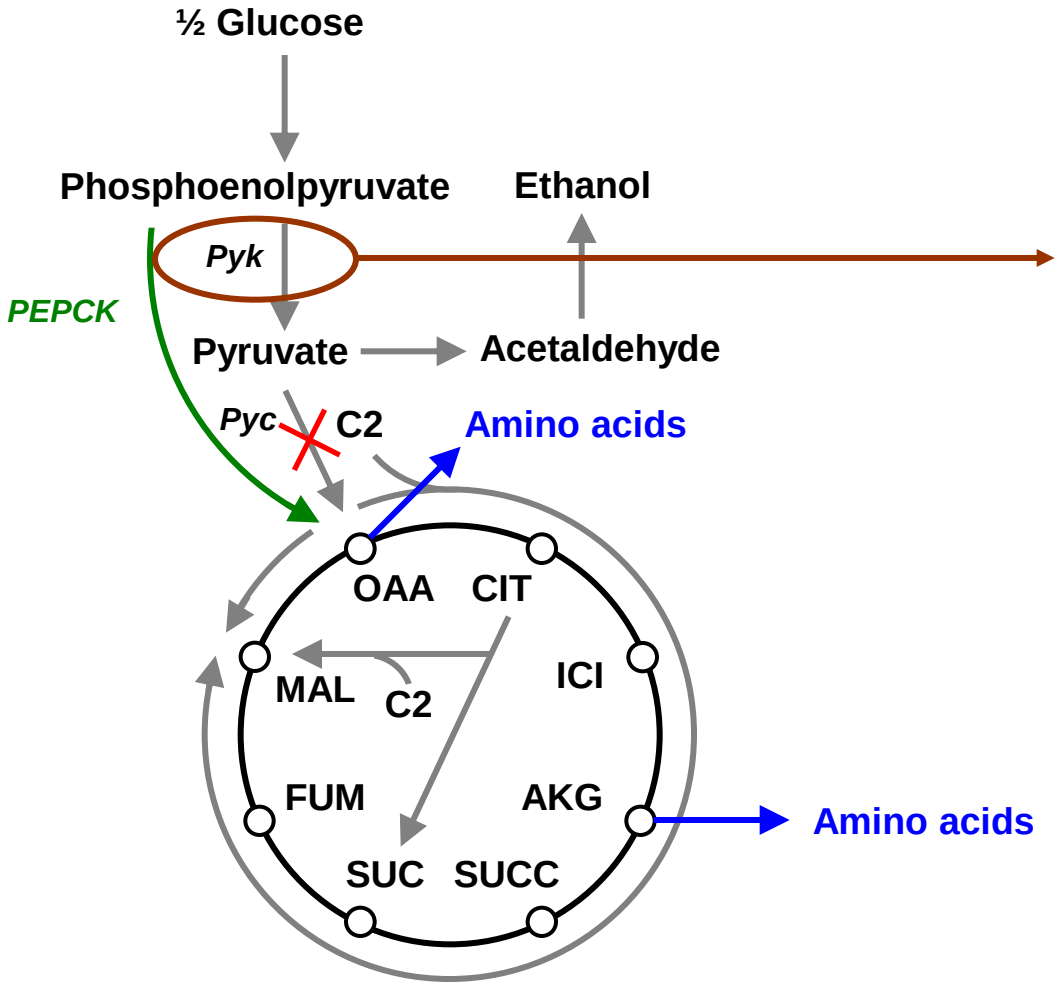
A hunch (very genomics…)



Hypothesis:
High pyruvate kinase activities compete with PEPCK for pyruvate

Strain	Enzyme activity (μmol·min^{-1}·mg protein^{-1})	
	PEPCK	Pyruvate kinase
IMY002 (non-evolved)	0.4 ± 0.1	8.5 ± 0.6
IMY050 (evolved #1)	0.9 ± 0.2	4.1 ± 0.4
IMY051 (evolved #2)	0.7 ± 0.3	5.1 ± 0.4

Rintze Zelle *et al*. 2010
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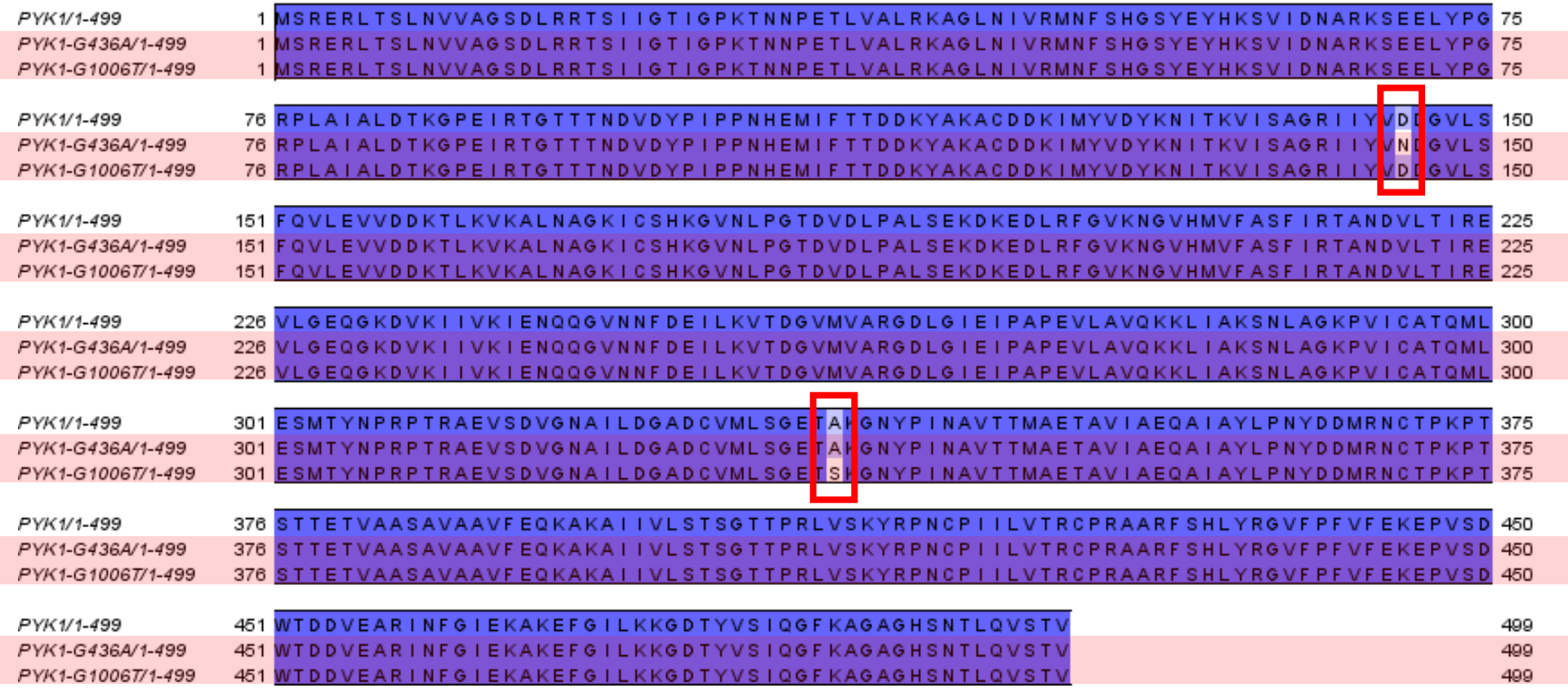
PYK1 point mutations in two independent evolved strains



Rintze Zelle *et al*. 2010
Appl. Environ. Microbiol. **76**:5383

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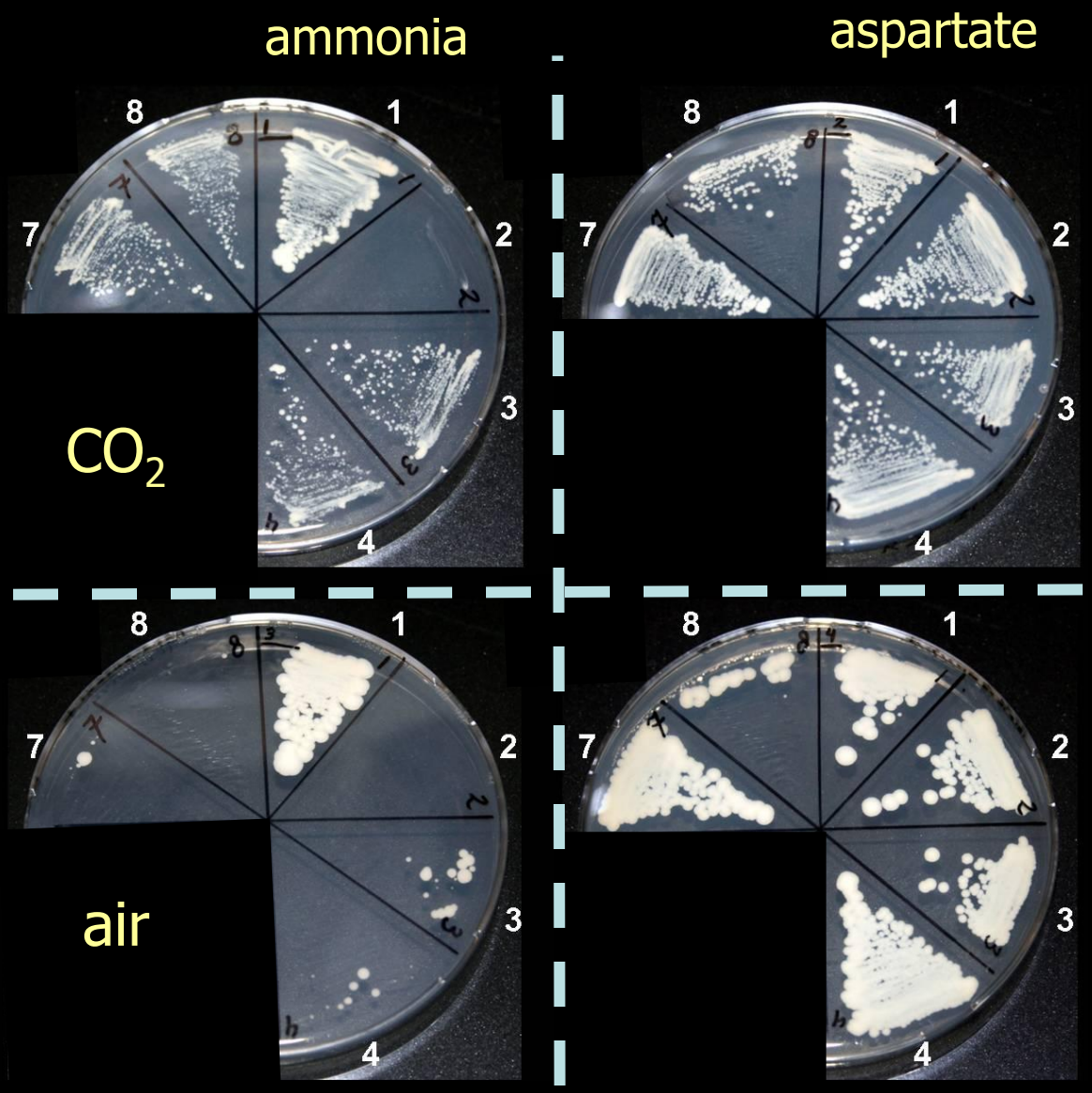
A. succinogenes PEPCK complements non-evolved *pyc1,2Δ* mutants carrying *pyk1* point mutations



Strains

1 CEN.PK 113-7D
2 IMY002 (Pyc$^-$, AsPEPCK)
3 IMY050 (Pyc$^-$, AsPEPCK, evolved)
4 IMY051 (Pyc$^-$, AsPEPCK, evolved)

7 IMY014 (Pyc$^-$, AsPEPCK, G436A)
8 IMY015 (Pyc$^-$, AsPEPCK, G1006T)

PEPCK as C3→C4 carboxylating enzyme in *S. cerevisiae*

Carboxylating function requires high CO_2 and increased intracellular PEP concentrations (inferred from pyruvate kinase expp)

Evolved strains grow anaerobically at 0.14 ± 0.01 h^{-1} (wild type: 0.30 h^{-1})

Estimated carboxylating flux (MFA): 0.3 mmol g^{-1} h^{-1}
Malate production in pyruvate-carboxylase-based strain: 2 mmol g^{-1} h^{-1}

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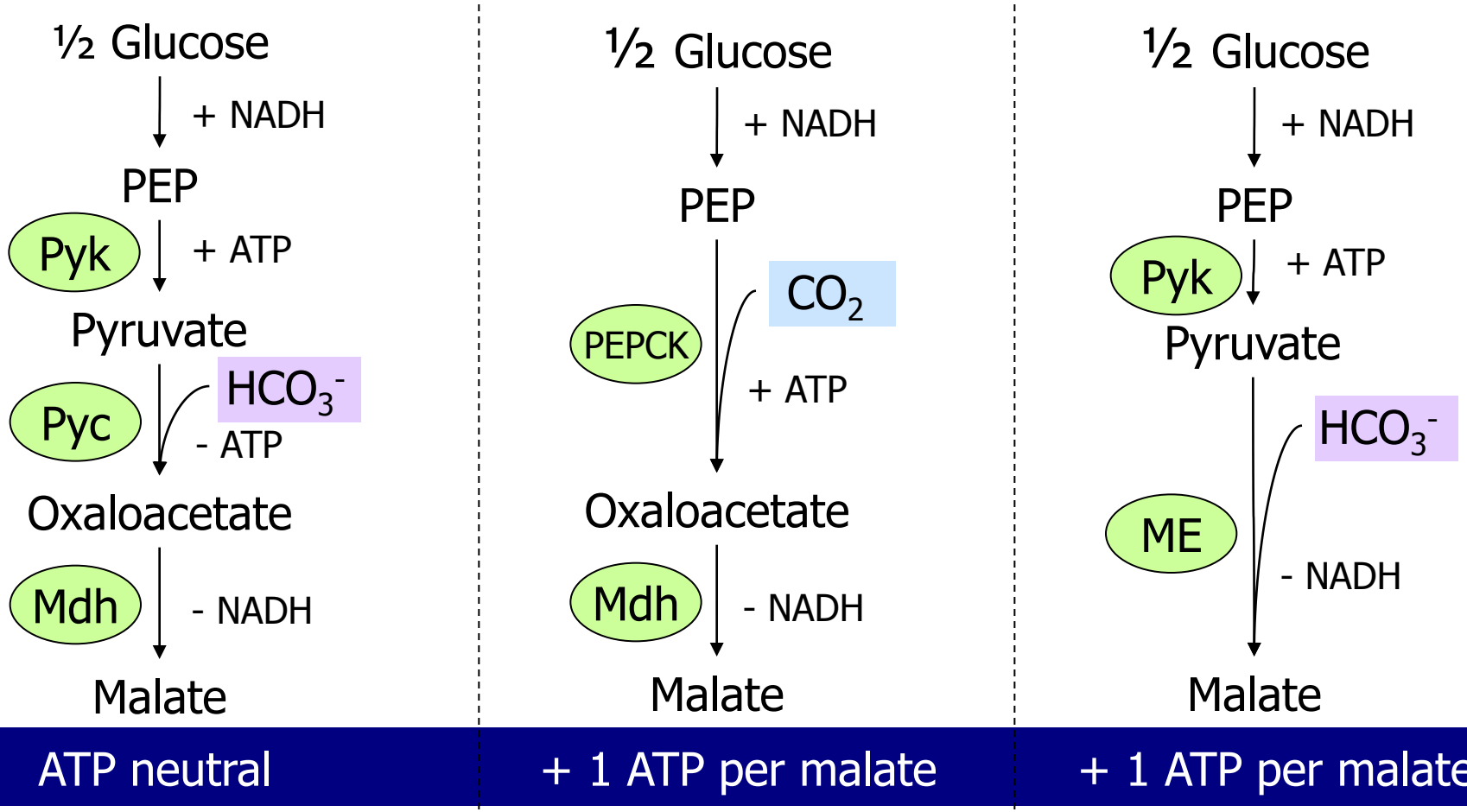
Alternative C3→C4 carboxylating reactions



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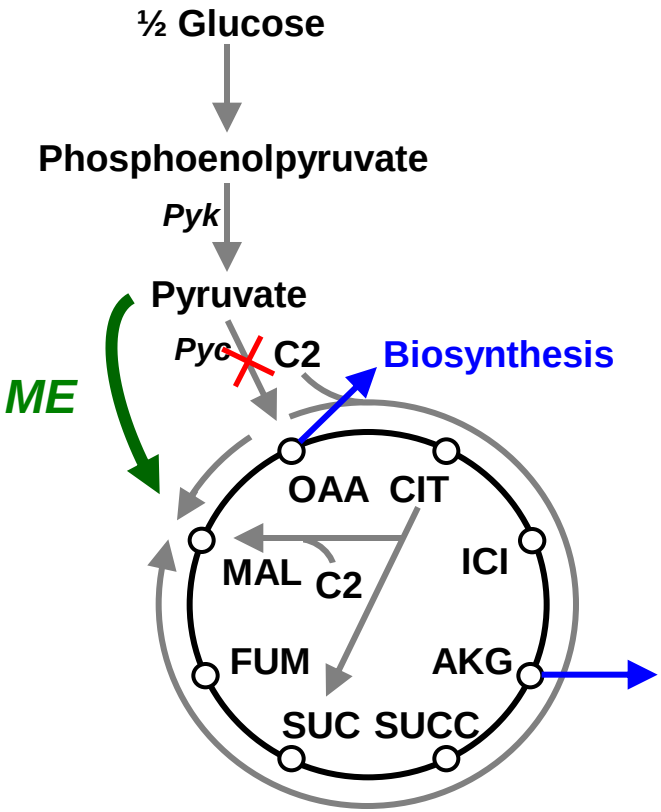
Malic enzyme

Pyruvate + NAD(P)H + H^+ + HCO_3^- → malate + $NAD(P)^+$



S. cerevisiae Mae1:

- *decarboxylating* role implicated in mitochondrial NADPH, pyruvate production (reaction marked 'irreversible' in some models)
- cannot replace pyruvate carboxylase (Pyc1, Pyc2) in anaplerotic C3→C4 carboxylation under standard conditions
- located in mitochondrial matrix
- dual cofactor specificity (NAD/NADP)

Rintze Zelle *et al*. submitted
Appl. Environ. Microbiol.

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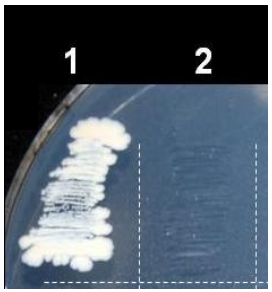
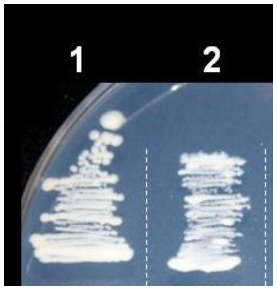
Investigating a possible carboxylating role of *E. coli* malic enzyme (sfcA) in *S. cerevisiae*

- pyruvate carboxylase-negative mutant (*pyc1,2Δ*)
- deletion in *PDC2* gene (regulator of pyruvate decarboxylase expression, leads to increased pyruvate levels)
- growth under CO_2 atmosphere

N source ammonia



N source aspartate



Strain	Malic enzyme activity (μmol·min^{-1}·mg protein^{-1})	
	NAD	NADP
CEN.PK113-7D (reference)	0.04 ± 0.00	0.03 ± 0.00
IMY016 (*pyc1Δ pyc2Δ pdc2Δ sfcA*↑)	1.0 ± 0.1	0.03 ± 0.01

Rintze Zelle *et al*. 2010
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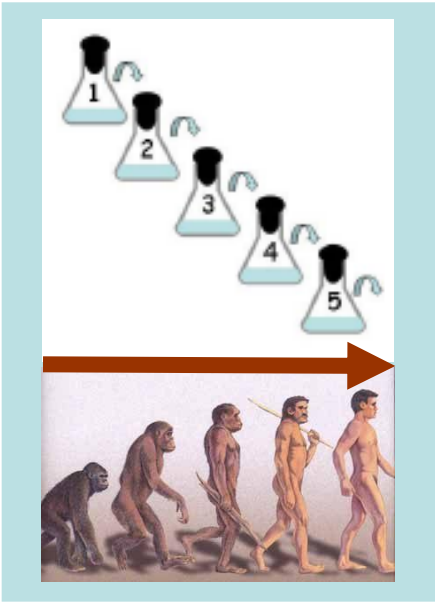
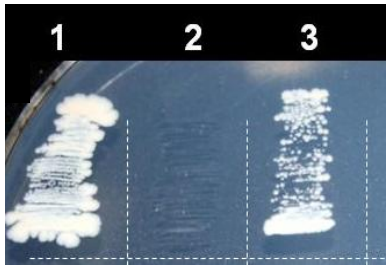
Adaptive evolution of *pyc1,2Δ pdc2Δ* strain expressing *E. coli sfcA* (malic enzyme) gene

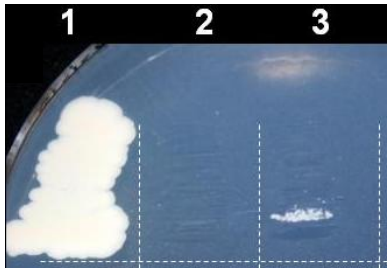


- Precultivation in CO_2-sparged chemostats with aspartate, switch to medium without aspartate
- Before complete washout stop glucose feed, continue as batch culture
- CO_2-dependent growth observed after ca. one week
- After several sequential batch cultures, single-cell line isolated

CO_2



air



Evolved strain grows anaerobically at 0.06 ± 0.01 h^{-1} (wild type: 0.30 h^{-1})

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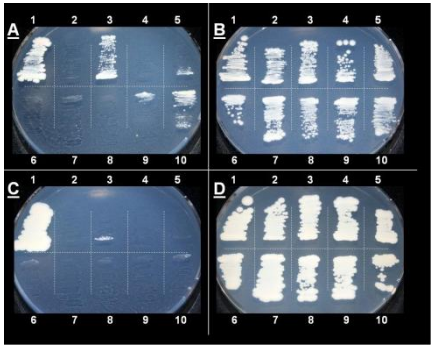
Plasmid recovery and retransformation:
mutation on *sfcA* plasmid essential for growth of evolved strain



→ Single G1006A (Asp336Gly) point mutation in *sfcA*….

….with dramatic impact on cofactor specificity

Allele	Malic enzyme kinetic parameters			
	NAD^+		$NADP^+$	
	V_{max}	K_m (mM)	V_{max}	K_m (mM)
sfcA (reference)	1.8	0.14	0.6	9
sfcA G1006A	1.0	4	0.7	0.2

Rintze Zelle *et al*. 2010
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Conclusions

Product export is crucial in engineering *S. cerevisiae* for C_4-dicarboxylic acid production

Carbon dioxide concentration is an essential parameter for product yield and titers

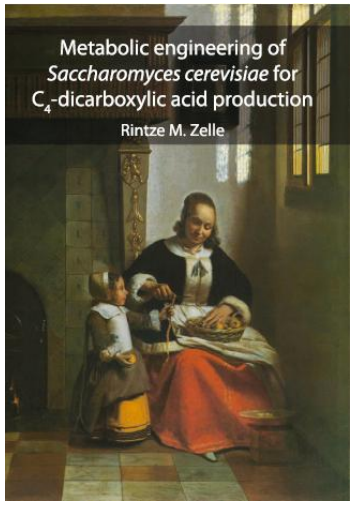
In vivo carboxylating activity of PEPCK & malic enzyme requires **optimization of thermodynamic driving force**:

- high CO_2 concentration
- elevated concentration of PEP (PEPCK) or pyruvate (ME)
- use of NADPH as redox cofactor (ME)

Metabolic engineering strategies should not only incorporate pathway **stochiometry** but also **kinetics and thermodynamics**

PEP-carboxykinase and malic enzyme can now be implemented in *S. cerevisiae* metabolic engineering strategies

Acknowledgements



Rintze Zelle (→ Allylix)

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Stan Bower (→ Integrated Genomics)

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WAGENINGEN UR — Cor Dijkema, Pieter de Waard

Wouter van Winden (then TUDelft, now DSM)

Kluyver CENTRE | Kluyver Centre for Genomics Of Industrial Fermentation

Netherlands Genomics Initiative

TU Delft

Industrial Microbiology Delft
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Pascale Daran-Lapujade
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Bart Oud
Jack Pronk
Daniel Ramos
Harmen van Rossum
Gabriele Romagnoli
Daniel Solis Escalante
Tânia Veiga
Han de Winde
Wouter Wisselink
Rintze Zelle

BIOEN – BE-Basic Joint Call

Themes :

Synthetic Biology for biofuels and bio-based chemicals (industrial microbiology, metabolic engineering, advanced bioprocessing, etc)

Sustainability (land use changes, socio-economic impacts, measuring tools, etc)

Event	Date
Call announcement	August 15, 2011
Closing date for submissions	October 13, 2011
Notification of successful proposals	from April 16, 2012

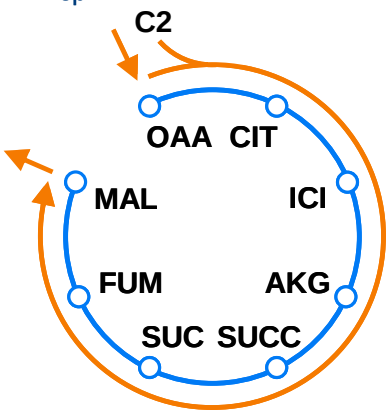
http://www.fapesp.br/en/6510

chamada-fapesp-be-basic@fapesp.br
supportoffice@be-basic.org.

Rerouting metabolism for malate production

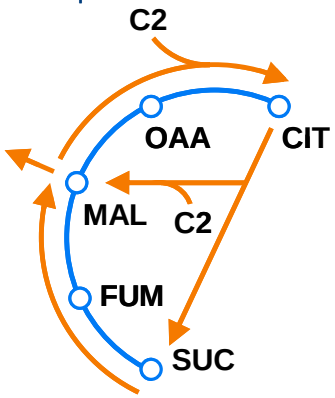
I TCA cycle

Y_{sp}^{max} 1 mol mol^{-1}



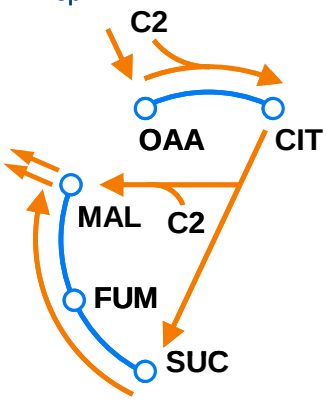
II Glyoxylate route (cyclic)

Y_{sp}^{max} 1 mol mol^{-1}



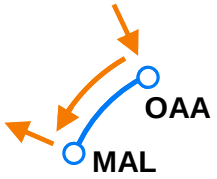
III Glyoxylate route (non-cyclic)

Y_{sp}^{max} 1⅓ mol mol^{-1}



IV Oxaloacetate reduction

Y_{sp}^{max} 2 mol mol^{-1}



Yield

- 0.42 mol malate per mol glucose