Artficial promoters and minimal cells for whole cell biocatalysis

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Im Rahmen des
K *plus* Programms gefördert durch:

Pichia pastoris

- Methylotrophic yeast
- >1000 proteins produced
- high cell density
- Very high yields (>20 g/L)
- efficient secretion (>13 g/L)

www.invitrogen.com

S. cerevisiae *P. pastoris*

J Lin Cereghino, and J Cregg, FEMS Microbiol Rev 24 (2000).

Independent new expression plattform developed by TU Graz, VTU Technology & Research Centre Applied Biocatalysis in Graz

1) **License free plattform**
2) **Advanced system**

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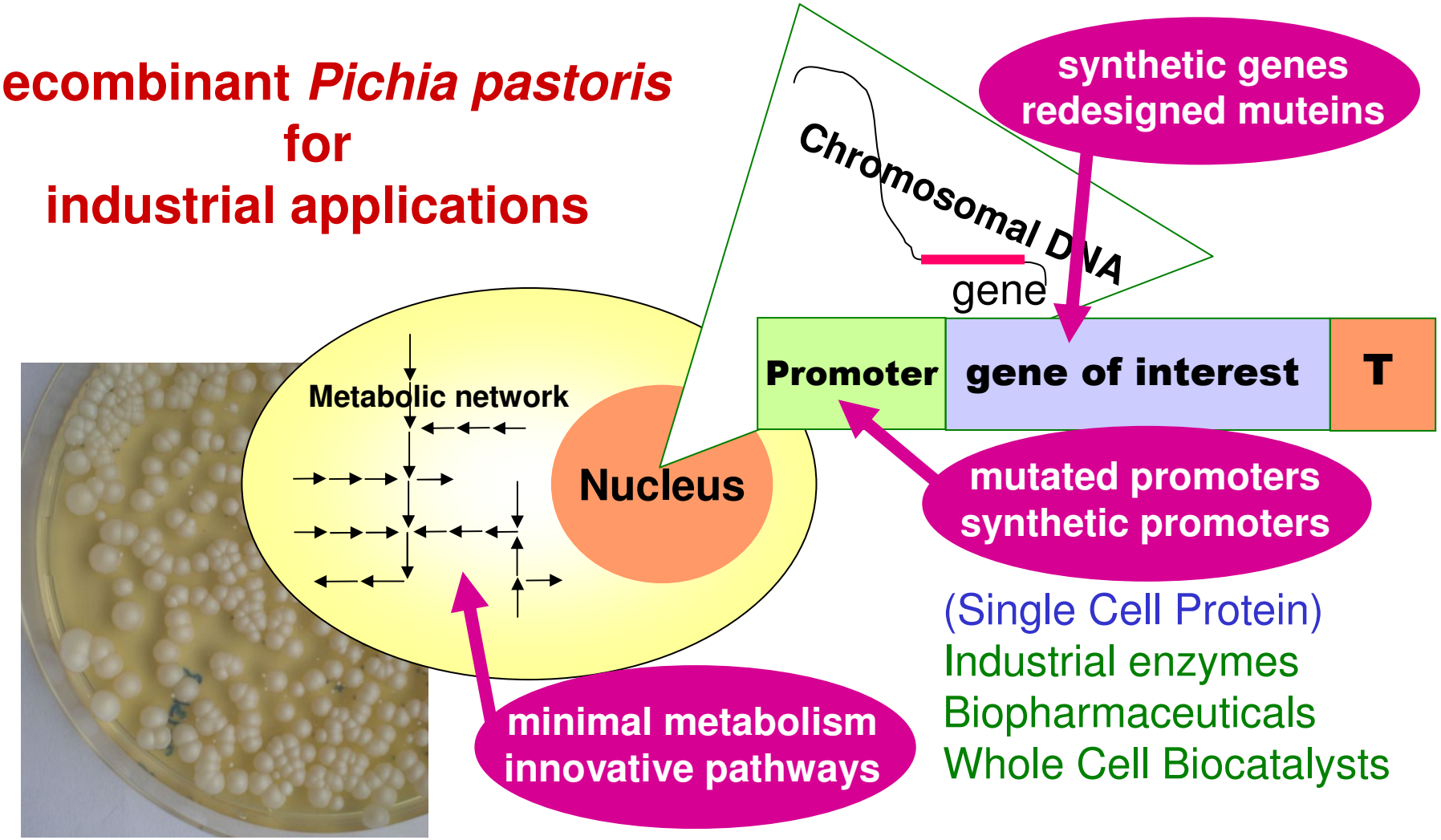
Institute for Molecular BioTechnology

Synthetic Biology
Pichia pastoris

Applied Biocatalysis
Research Centre

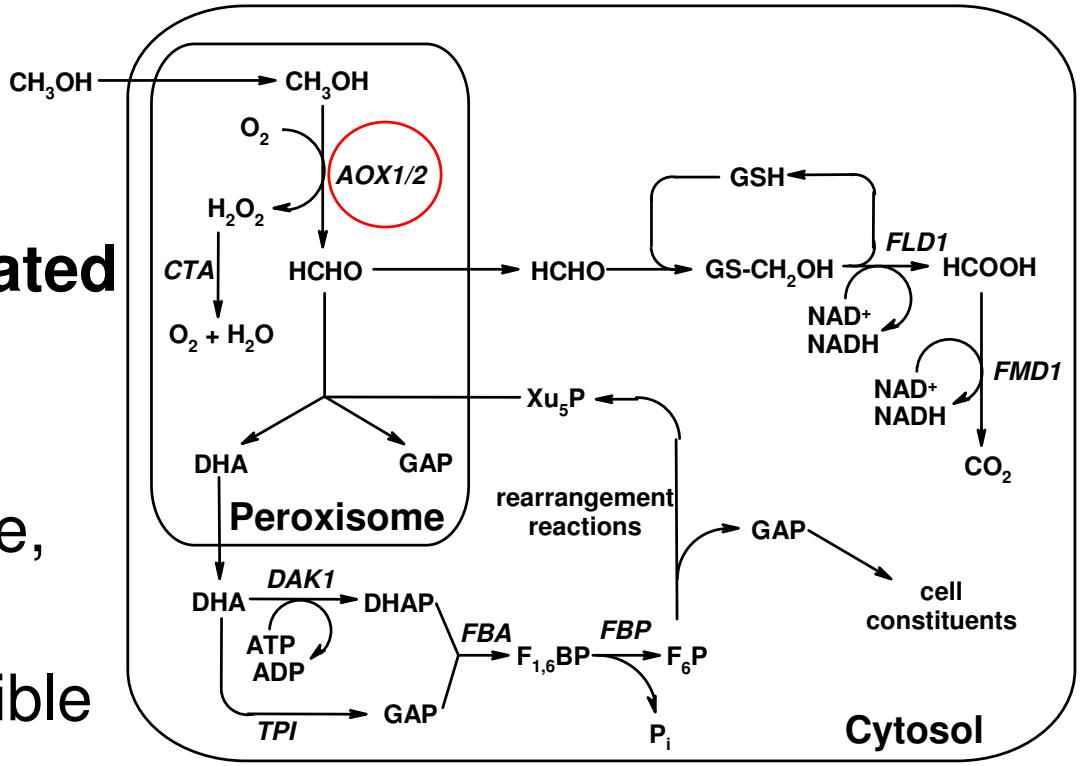
Recombinant *Pichia pastoris* for industrial applications



From the wooden boat to a racing boat

Methanol utilisation

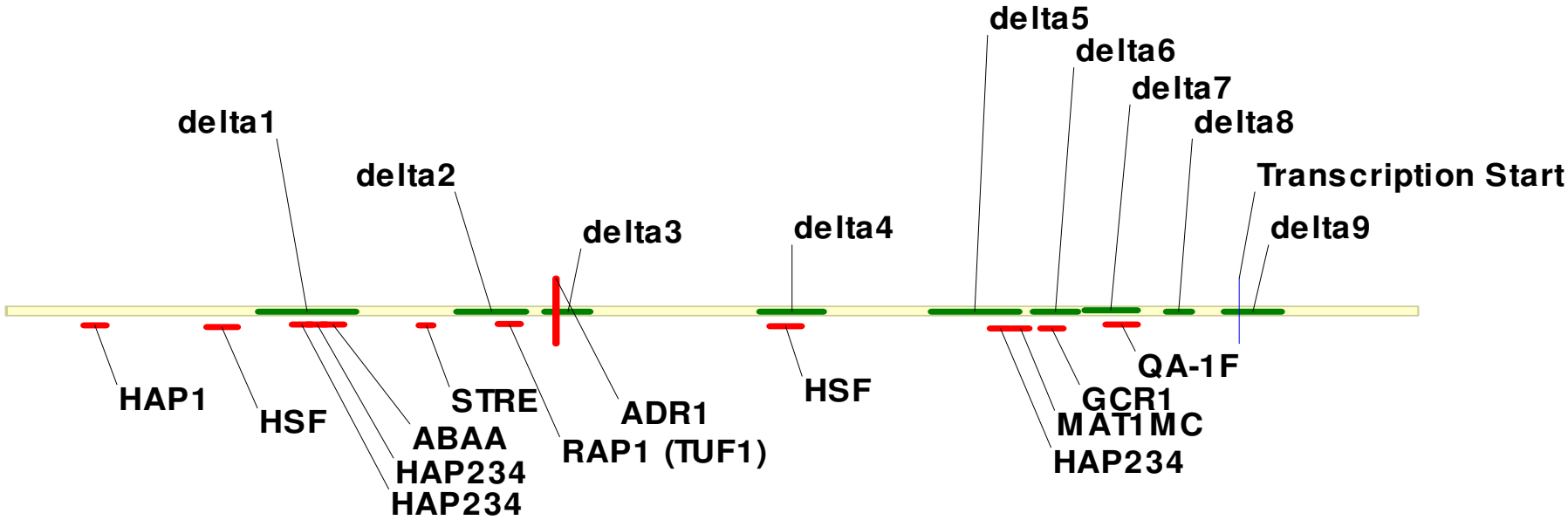
- **Methanol utilisation** pathway is **tightly regulated**
  - *AOX1* : majority of alcohol oxidase
  - Repressed by glucose, glycerol, ethanol, …
  - P_{AOX1}: Strongly inducible by methanol



Tschopp, J.F., et al. Nucleic Acids Res 15 (1987).

One for all promoter system?

P_{AOX1} sequence analysis

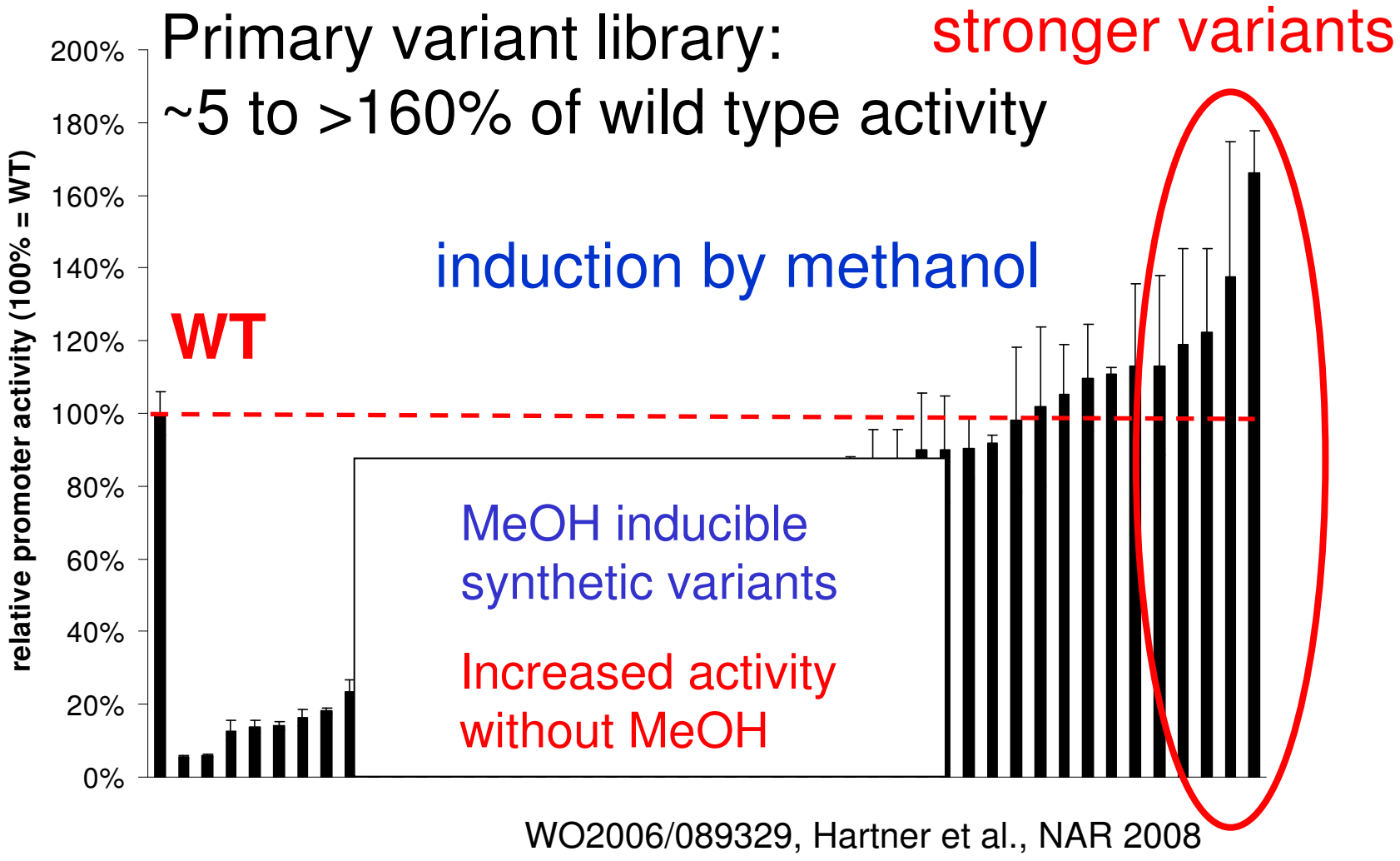


Transcription factor binding sites:
HSF.....heat shock factor
STRE.....stress response element
HAP.....O_2 and glucose regulation
GCR....regulator of glycolysis
ADR....inductor of ScADH2 and peroxisomal genes

➢ **Primary promoter variant library:**

short deletions (5-60 bp), covering putative transcription factor binding sites

Promoter library



WO2006/089329, Hartner et al., NAR 2008

Institute for Molecular BioTechnology

Knowledge based synthetic promoters

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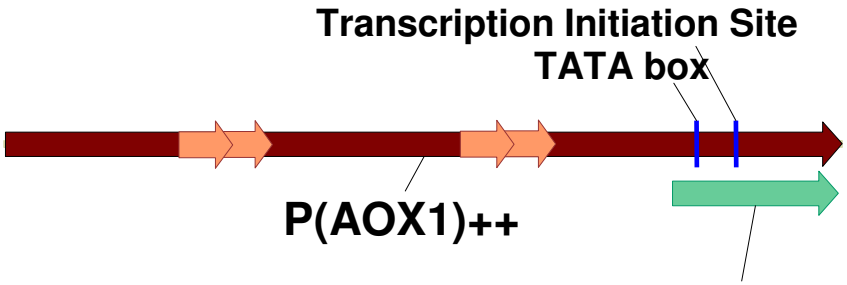
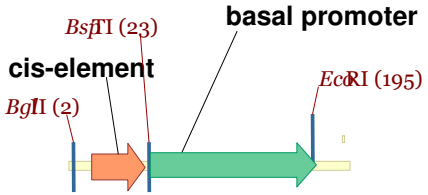
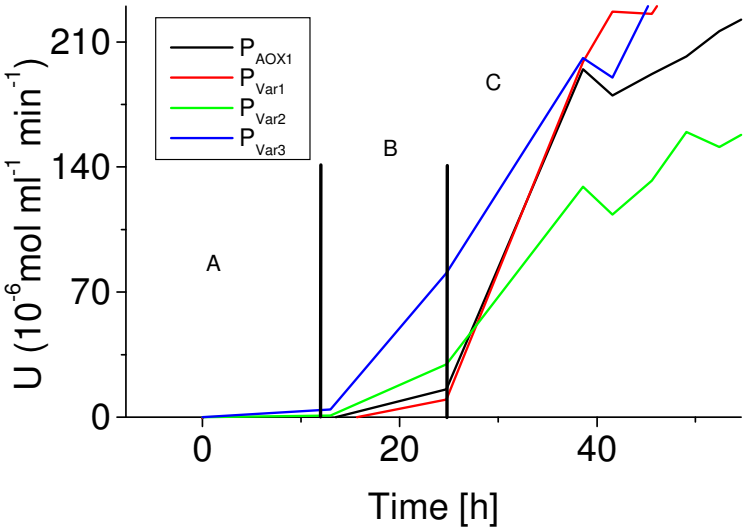
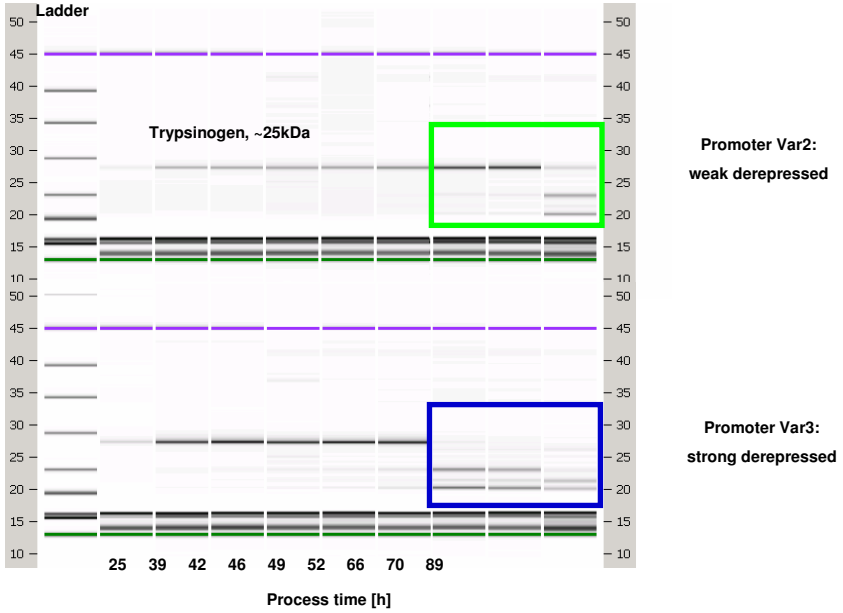




Basal promoter + cis element



MeOH inducible synthetic variants
Increased activity without MeOH



Expression cascades

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Classical strategy

Biomass production

Glucose, glycerol

production

methanol

helper protein expression

constitutive

Cascade strategy

Biomass production

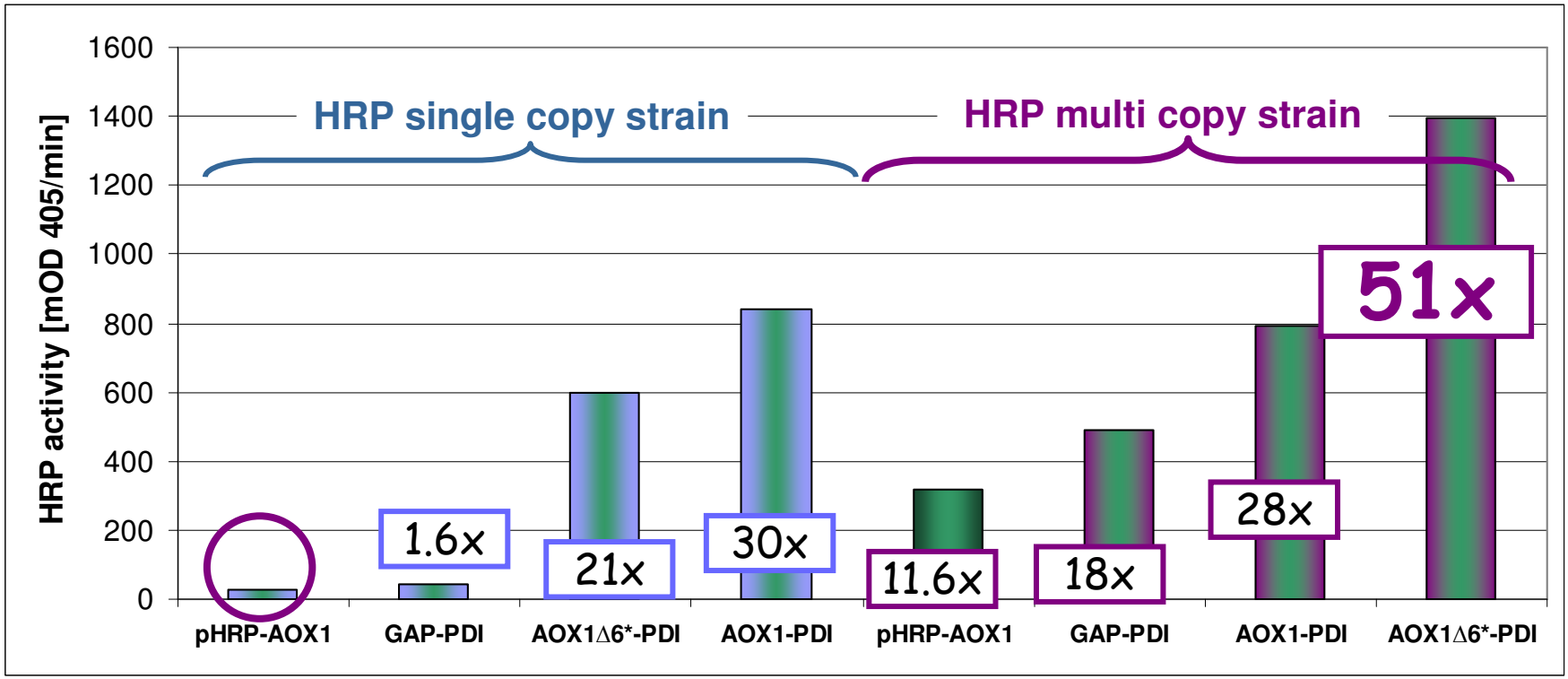
Glucose, glycerol

helper protein expression

production

methanol

HRP Overexpression



GAP: constitutive
Δ6* : derepression, medium induction
AOX1: strong induction by methanol

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VTU technology

synthetic promoters

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- More efficient expression
- Derepression / No need for methanol for induction
- Improved space/time yields
- Improved protein quality
- Tuneable expression/enhanced folding
- Fine tuned expression cascades

Many different opportunities with one single well known system

VTU *Pichia* Protein Expression

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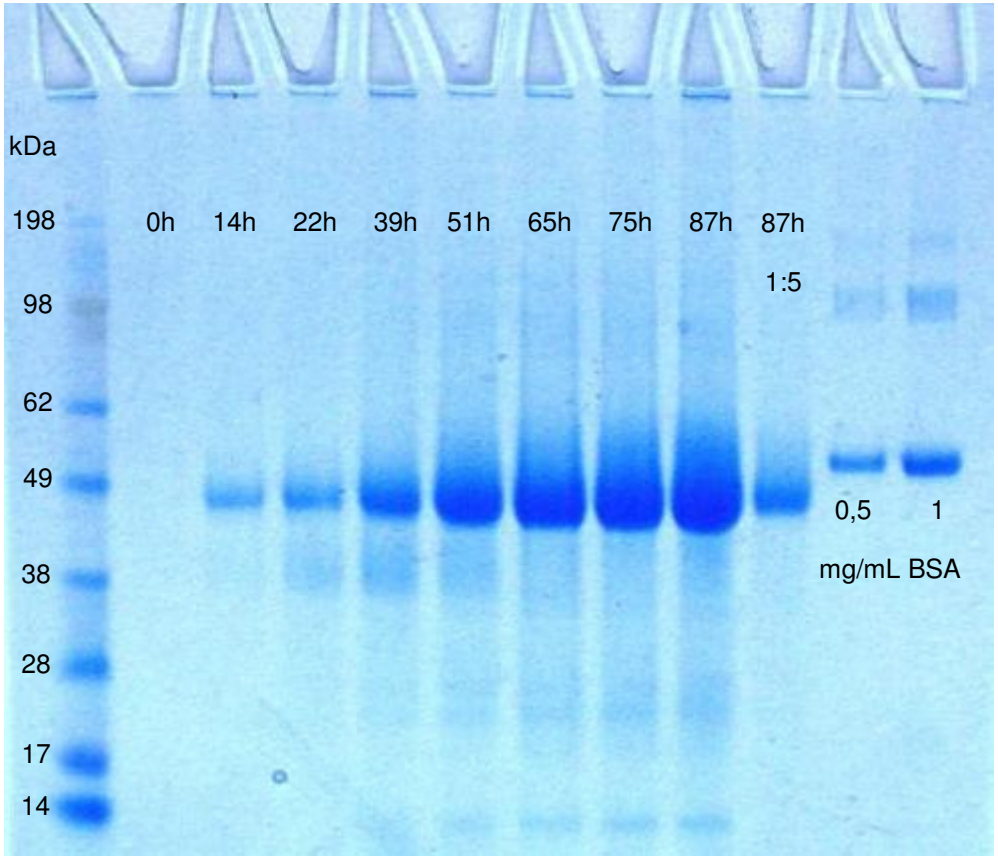
Yield depends on perfect promoter/target combination

- **Different requirements for high yield secretion CalA vs. CalB**
  - One organism
  - Two secretory lipases
  - 30% identity – 50% similarity

Target	Copy number	Promoter characteristics	Auxiliary proteins	Yield secreted
CalB	low	medium & derepressed	high	> 5 g/L
CalA	high	strong & induced	high	> 5 g/L

VTU *Pichia* Protein Expression

Fermentation – secretion



Synthetic gene design competition

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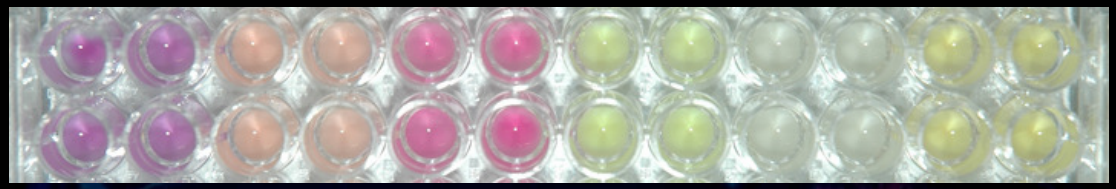

Artwork by Kerstin Kitz 2007

Whole Cell Biocatalysis

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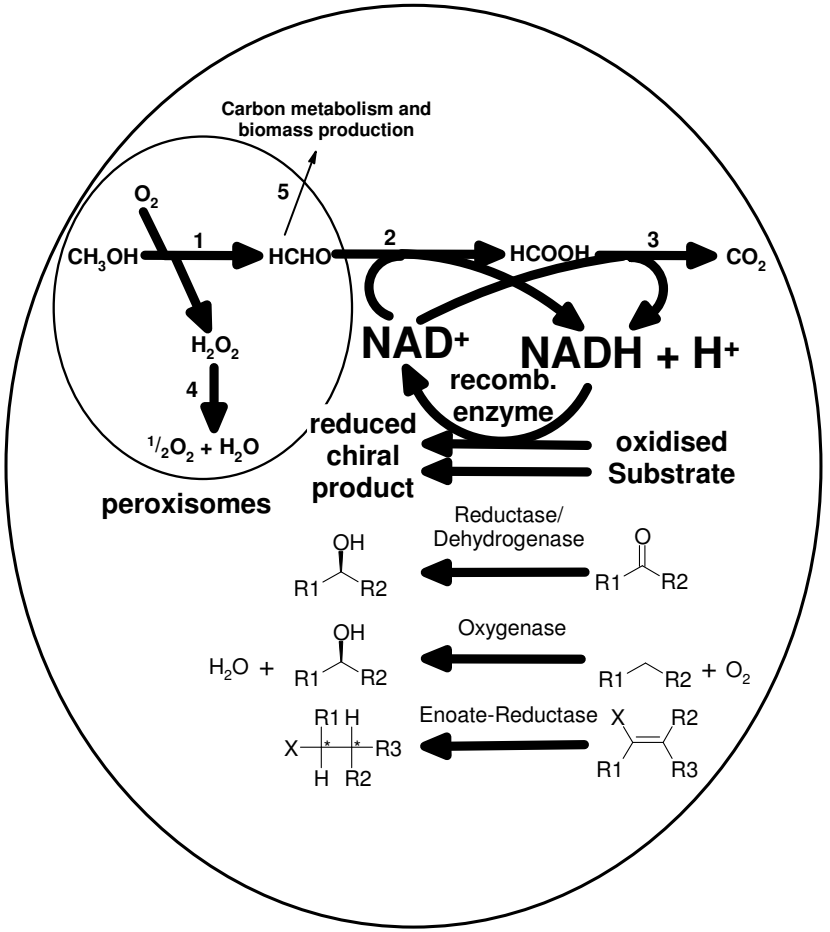
Classic: Growing Cells
By-product: BIOMASS

Minimal Cell for NAD(P)H dependent catalysis

Classic: Resting Cells
No catalyst regeneration



Engineered Methylotrophic Yeast

NADH regeneration

The lucky four.....

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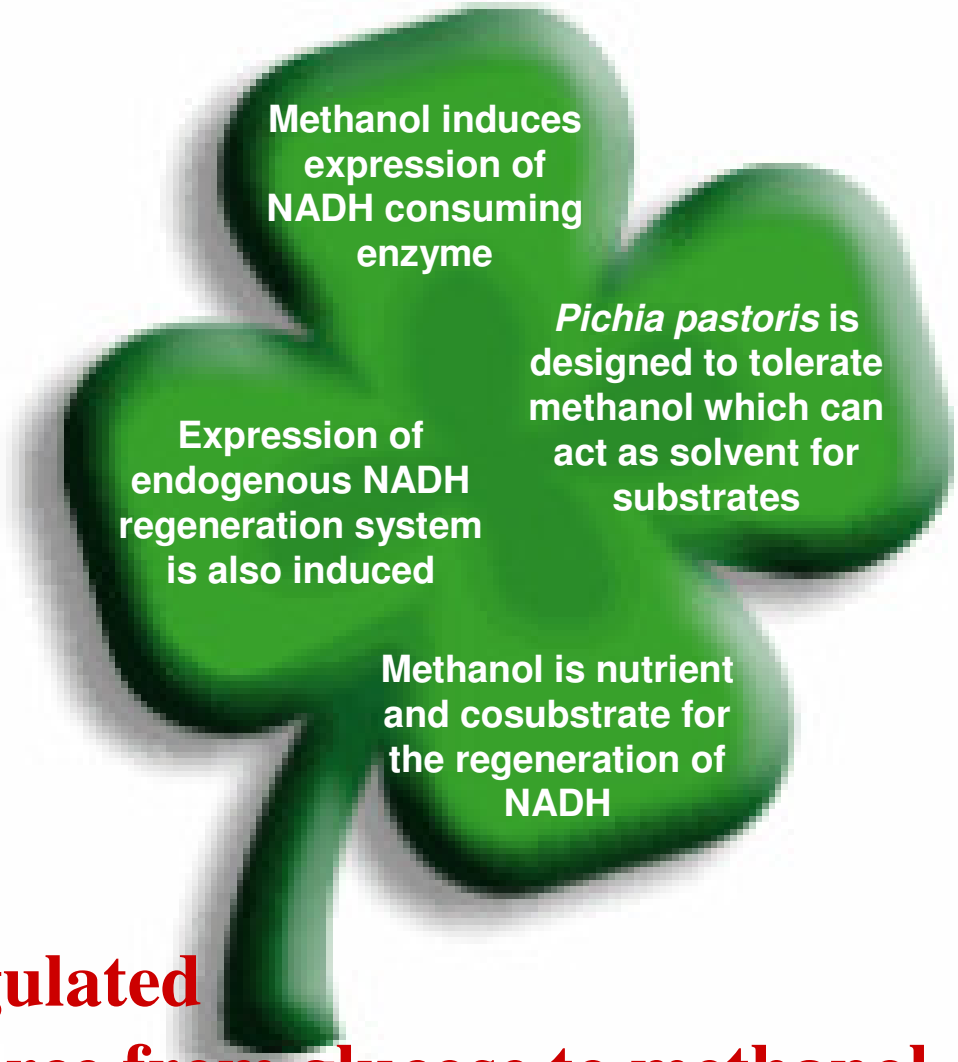
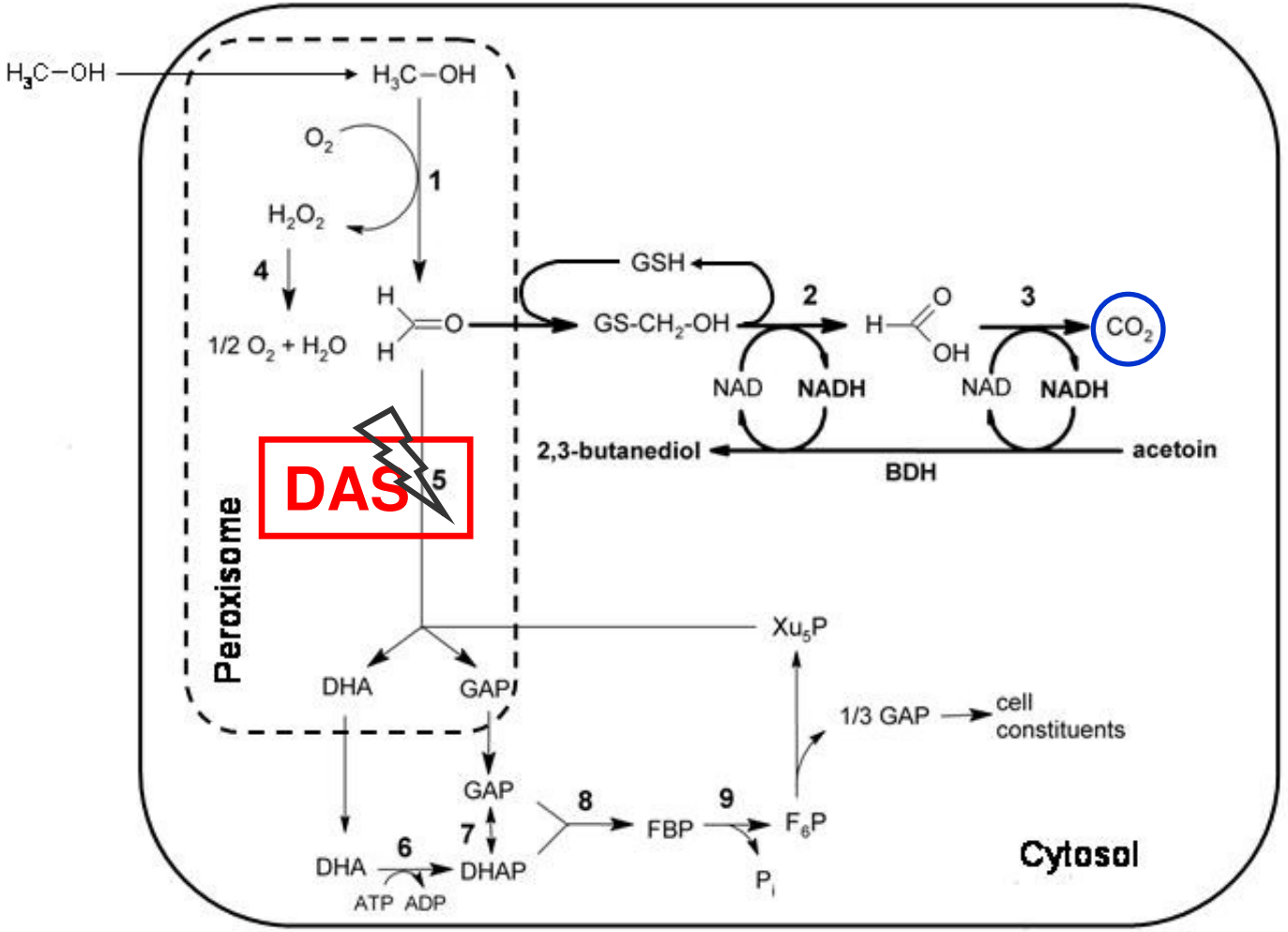
Why *Pichia pastoris*?

at least **4 positive features** from methanol utilization pathway



~ 1400 genes downregulated when switching C-source from glucose to methanol

Cofactor regeneration



P. pastoris on methanol $q_{S,max}$ 10-17 mmol g^{-1} h^{-1}

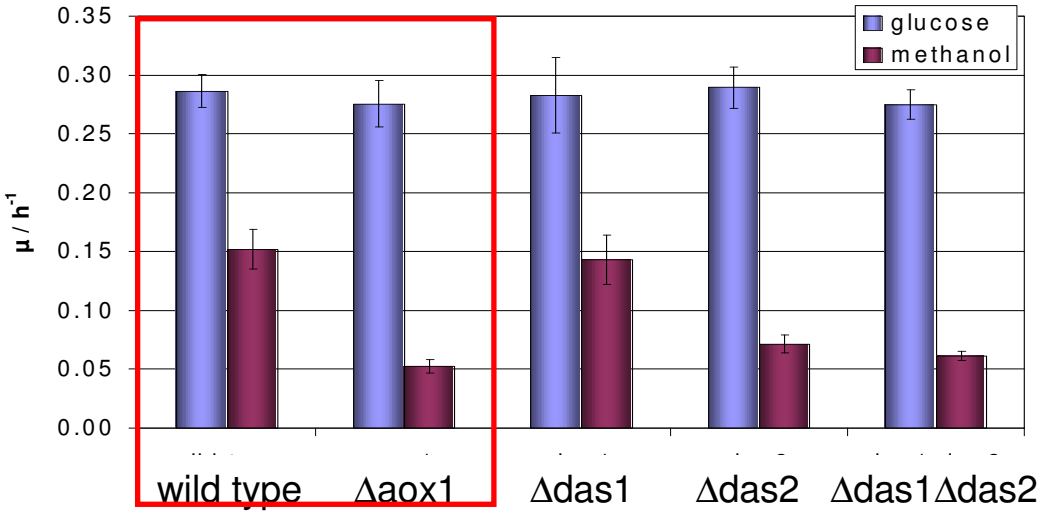
S. cerevisiae on glucose $q_{S,max}$ 18 mmol g^{-1} h^{-1}

2 NADH per MeOH

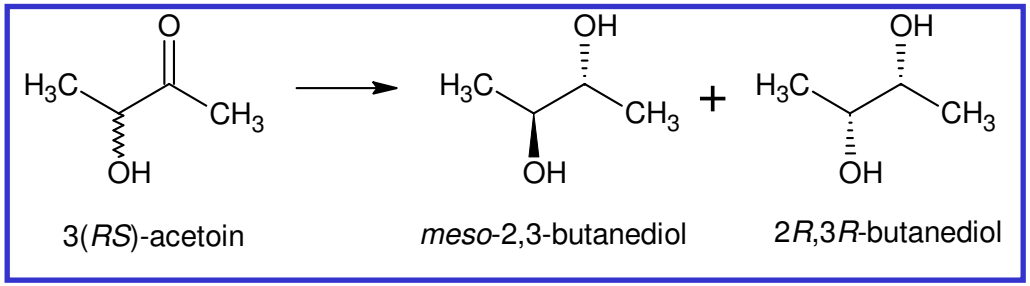
CO_2 as byproduct → irreversible reaction

1. Alcohol oxidase
2. Formaldehyde DH
3. Formate DH
4. Catalase
5. Dihydroxyacetone synthase (DAS)
6. Dihydroxyacetone kinase
7. Triosephosphate isomerase
8. F-1,6-BP Aldolase
9. F-1,6-B-Phosphatase

ΔDAS –
Conversion in shake flasks

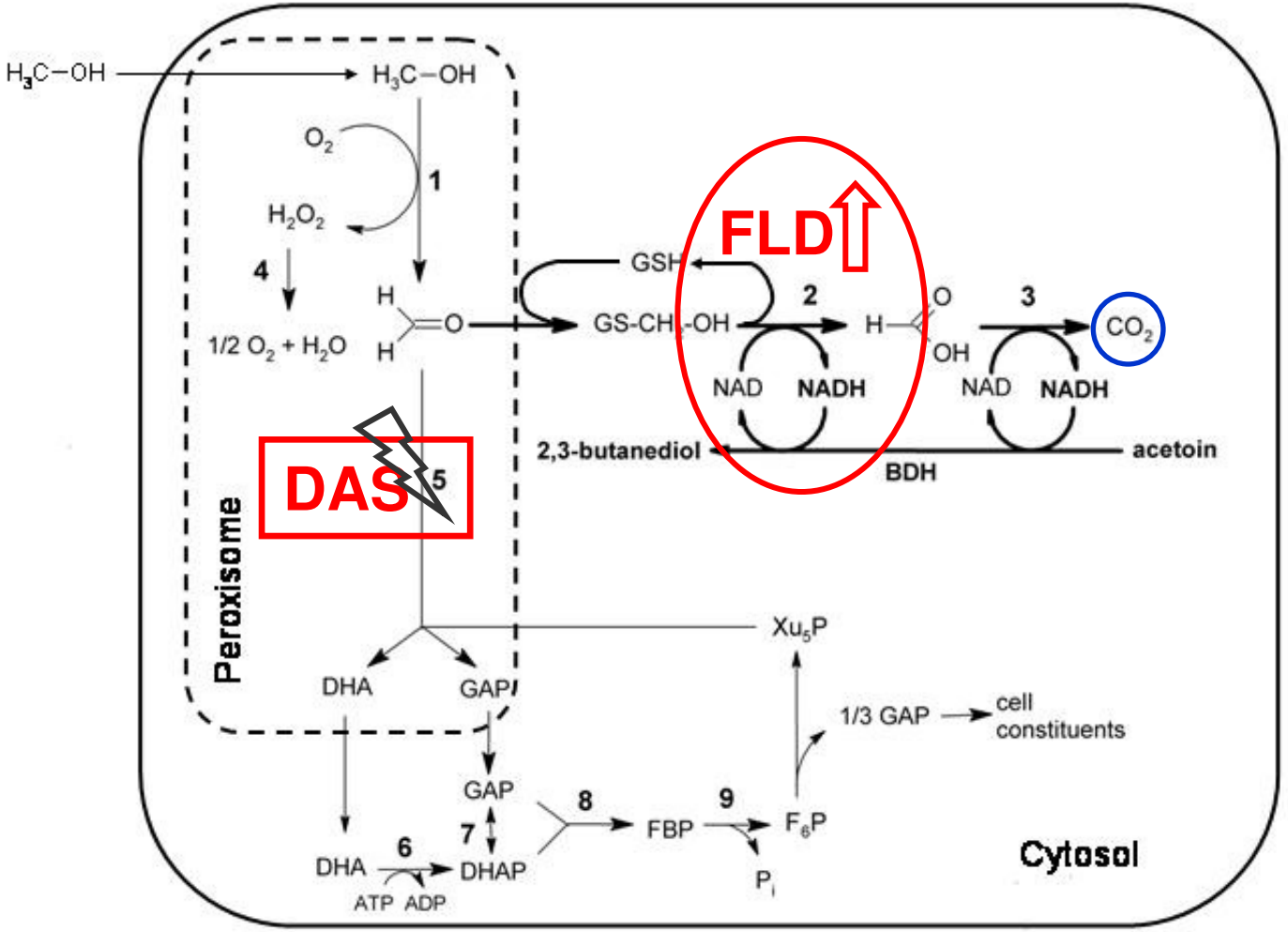


- Increased cell density (60 g/L)
- 25 g/L substrate concentration
- 6% methanol



Strain	STY	Specific conversion rate
742D4 (*BDH1*)	91 mmol L^{-1} h^{-1}	1.30 mmol (g CDW)$^{-1}$ h^{-1}
764D10 (*das1 das2 BDH1*)	95 mmol L^{-1} h^{-1}	1.42 mmol (g CDW)$^{-1}$ h^{-1}

Cofactor regeneration



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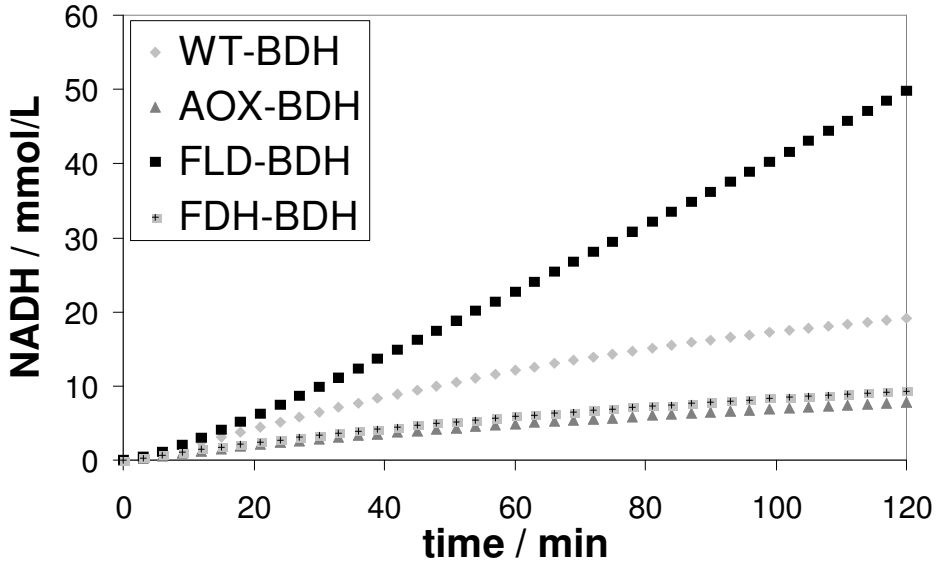
FLD ↑

Characterization of recombinant *P. pastoris* strains

P. pastoris with overexpressed
- model oxidoreductase *BDH1*
- and *AOX1*, *FLD* or *FDH*
- under control of P_{AOX1}

- Determination of kinetic properties of AOX, FLD and FDH
- Simulation of NADH formation rates

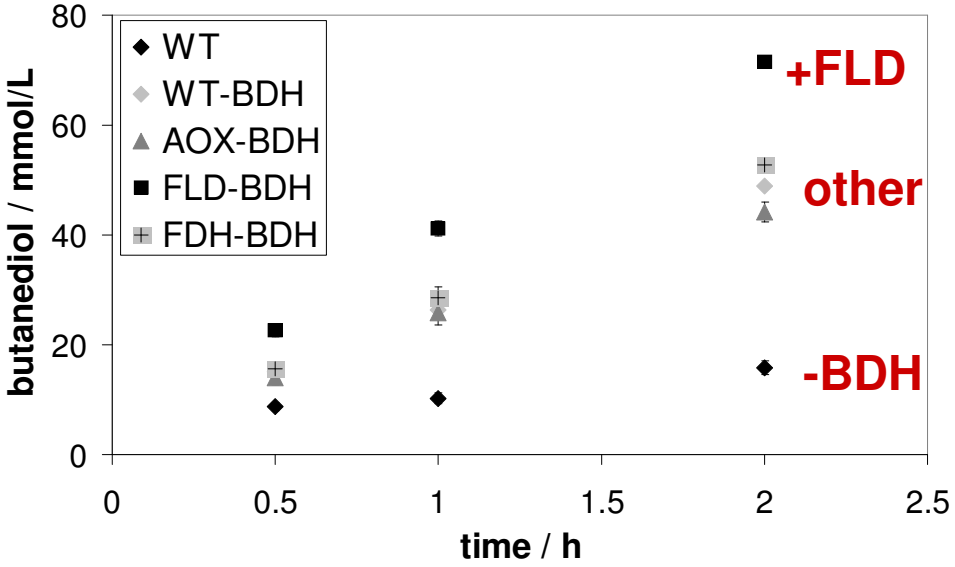


- Whole-cell biotransformations converting acetoin to 2,3-butanediol

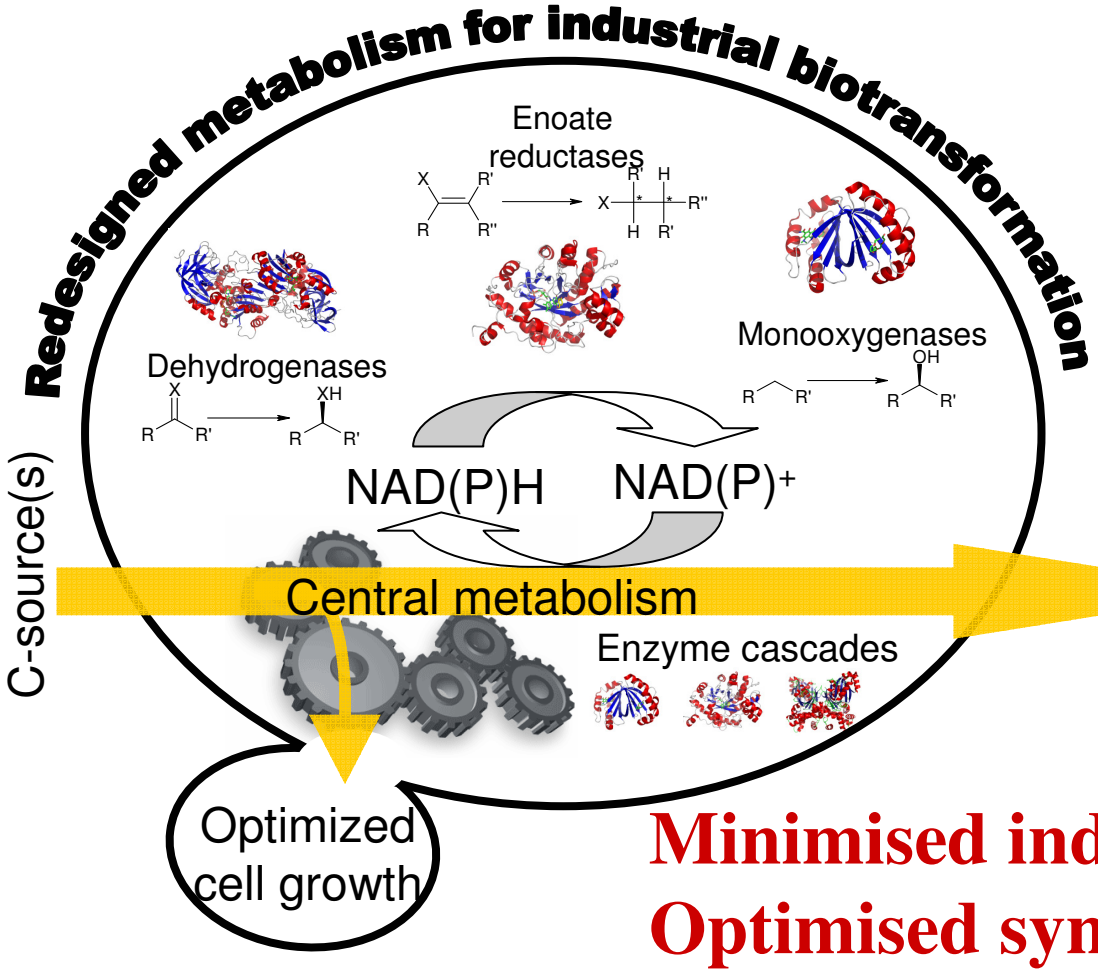
➢ ***P. pastoris+BDH+FLD***

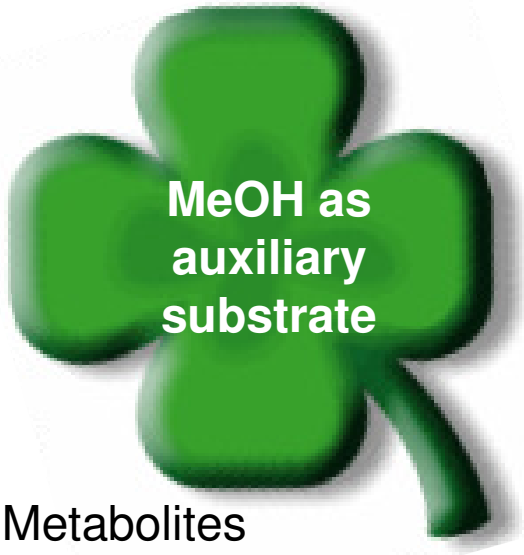
90% conversion after 2 h

compared to 55-65% for other strains



Summary and Outlook





Metabolites
Bulk chemicals

Minimised industrialised metabolism
Optimised synthetic genes
Better synthetic promoters

Special Thanks

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AB Centre

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ACIB

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Claudia Ruth
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Roland Weis (VTU)
Thomas Purkarthofer (VTU)

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