Nielsen Lab

Systems and Synthetic Biology

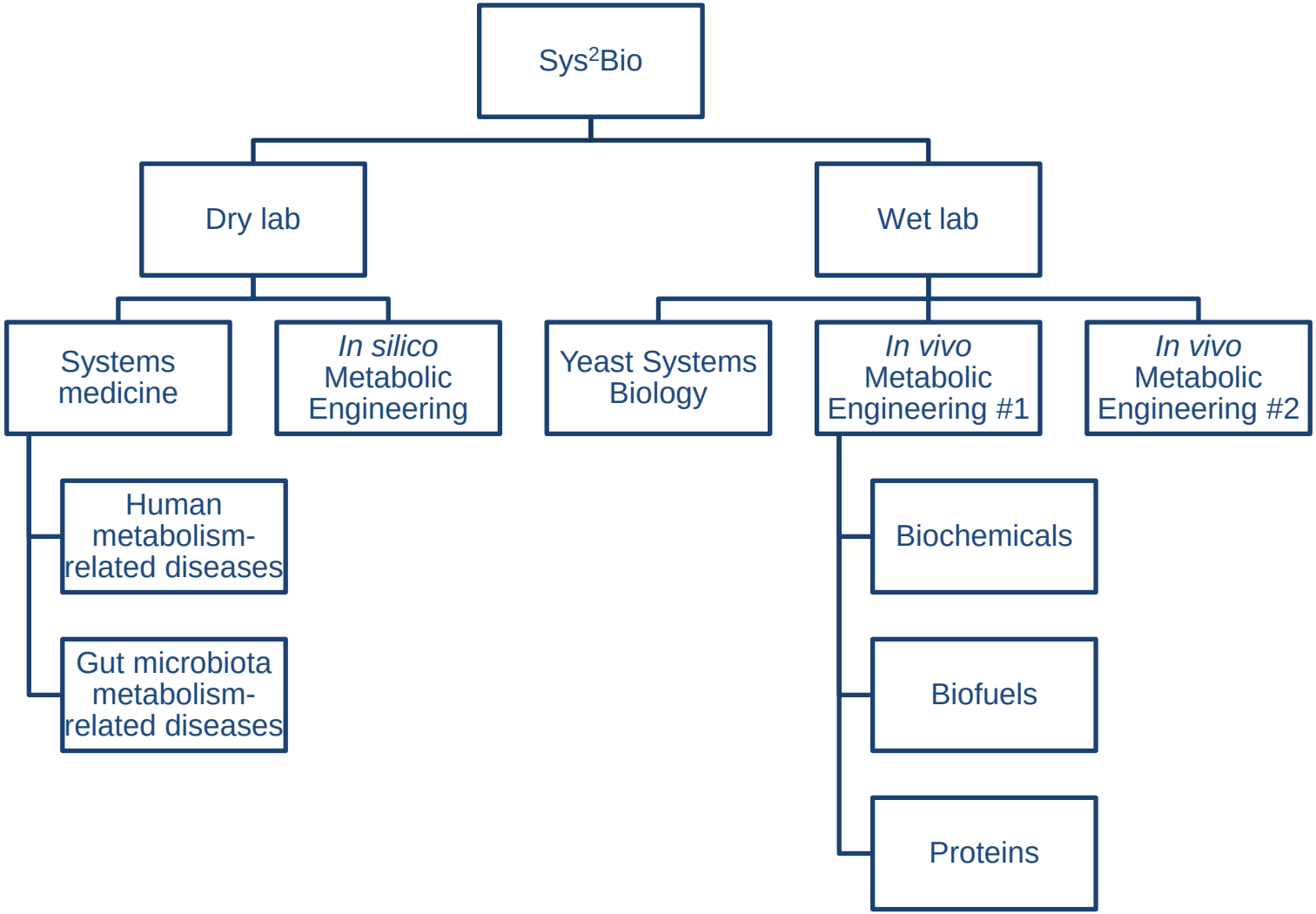
Chalmers University of Technology

Sweden

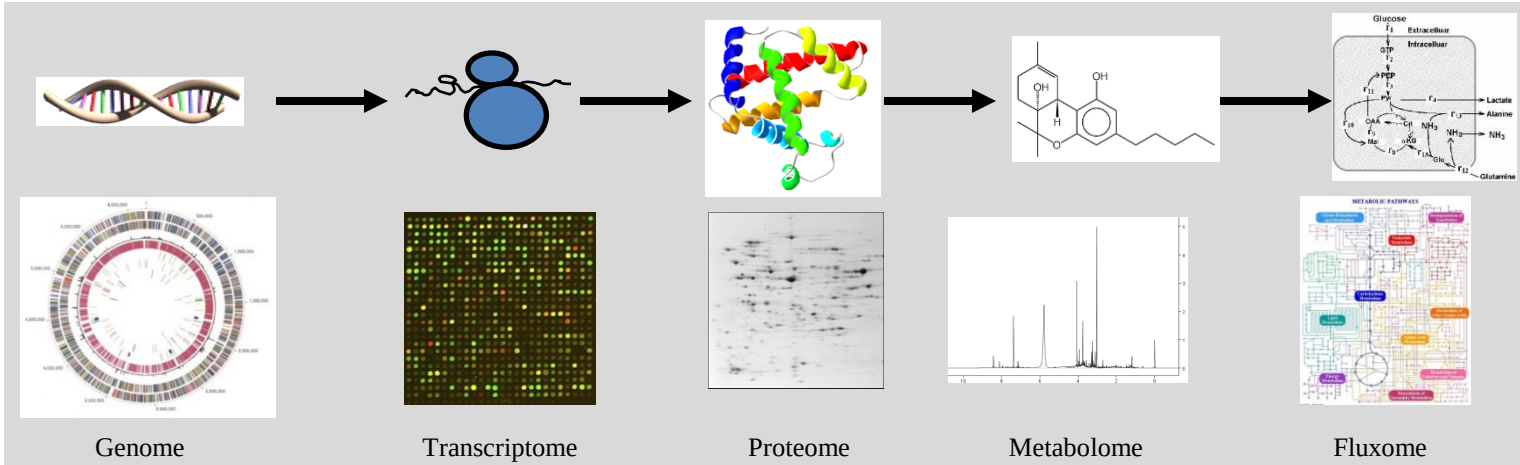
Systems and Synthetic Biology

19 Post Docs
25 PhD students
5 Visiting PhD students
8 MSc students
7 Research Engineers/Technicians
2 Admin

Nielsen Lab Organization

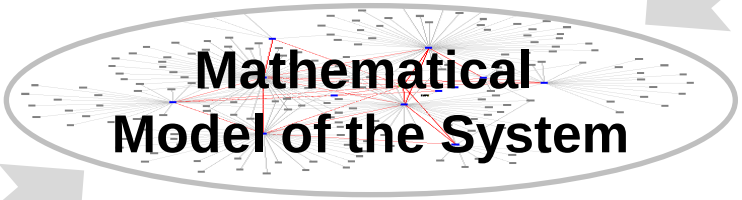


Top-Down & Bottom-Up SB



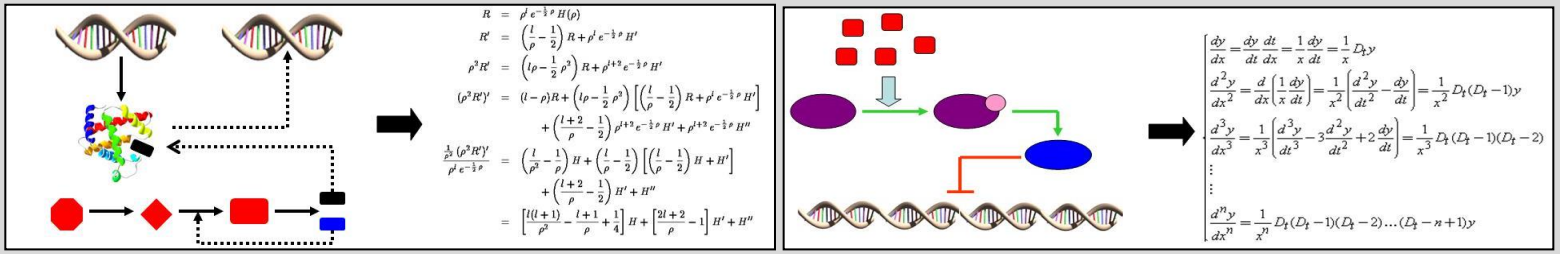
High throughput –*omics* data analyzed by bioinformatics

Discovery Based
Top-down Approach



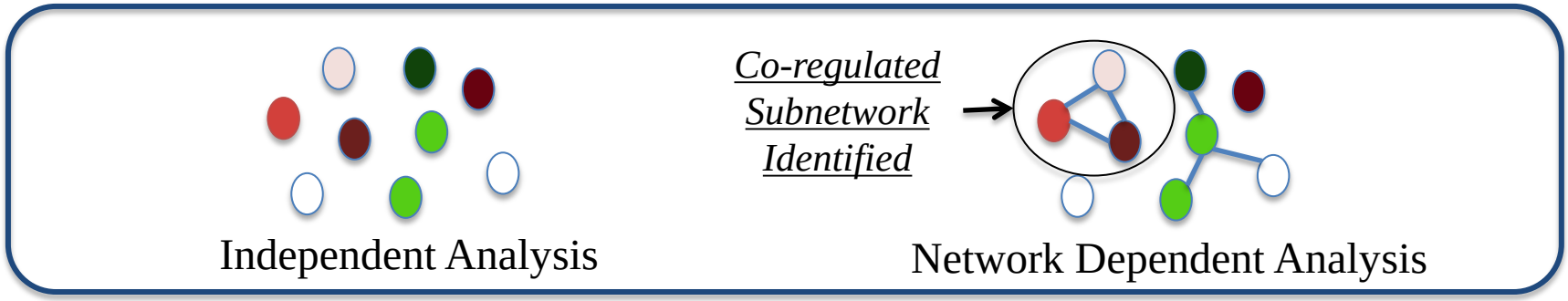
Hypothesis Based
Bottom-up Approach

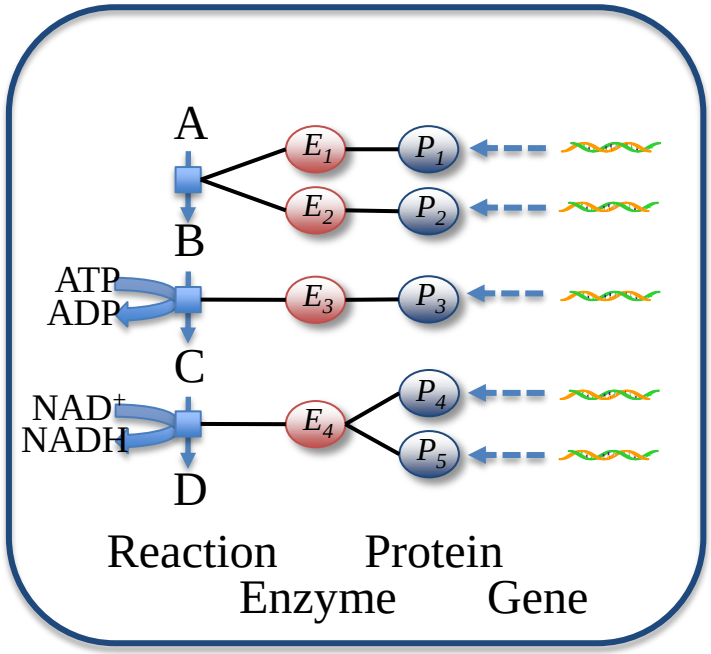
Cellular modules

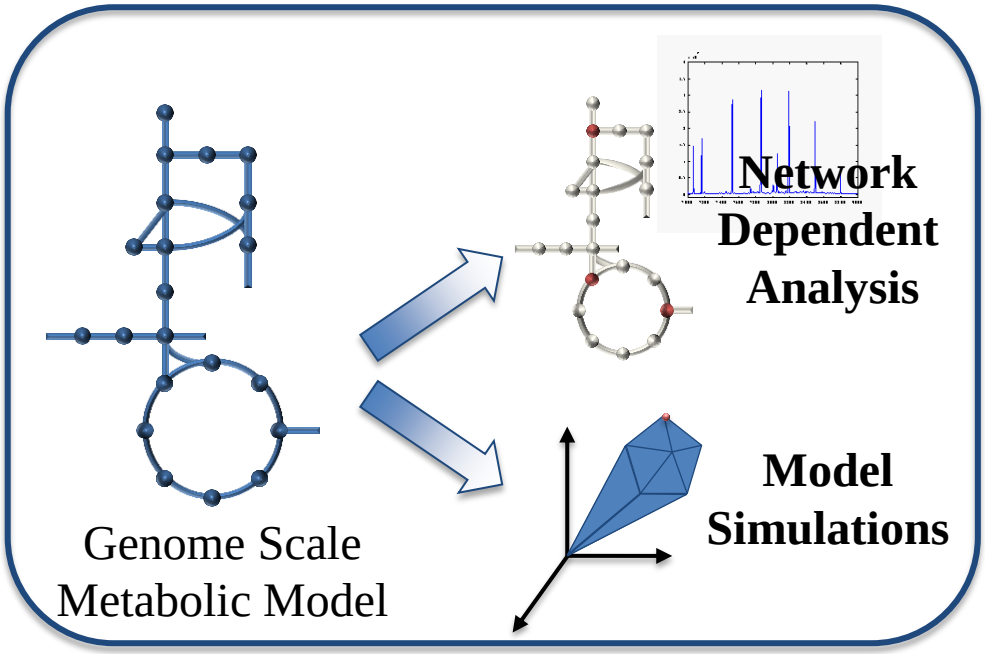


Genome-Scale Metabolic Models

Genome-scale metabolic models (GEMs) provide gene-protein-reaction connections and hereby allow for context dependent analysis



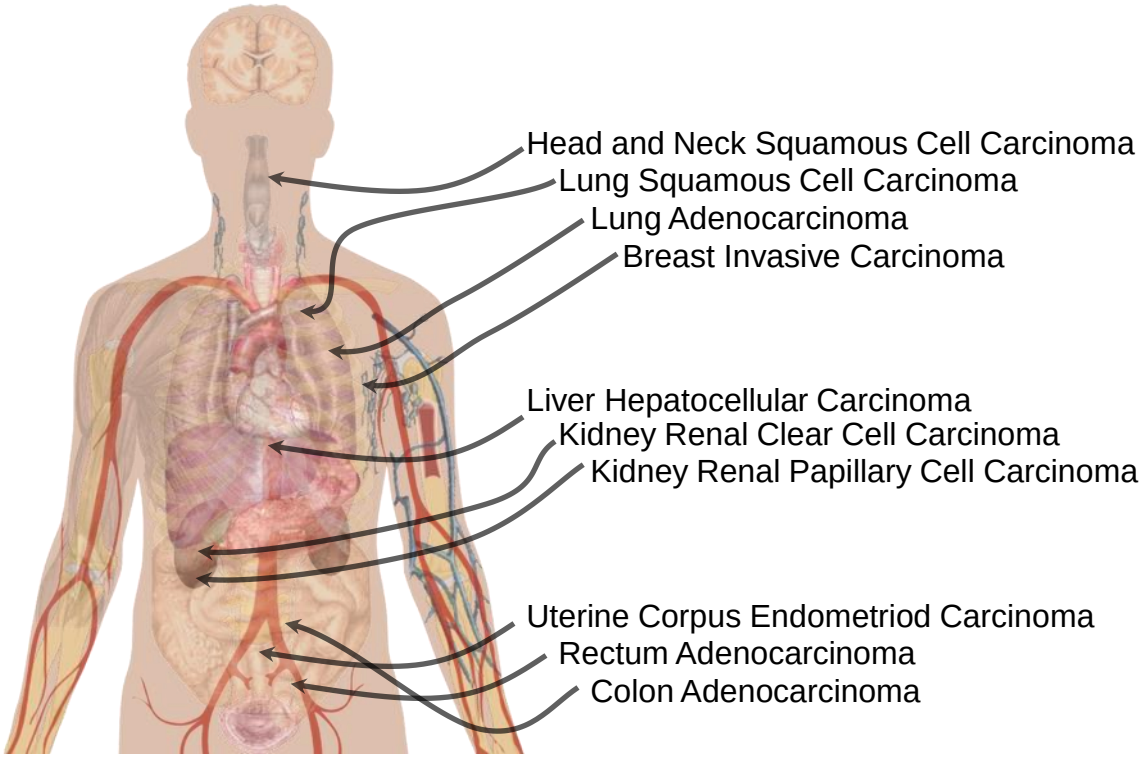


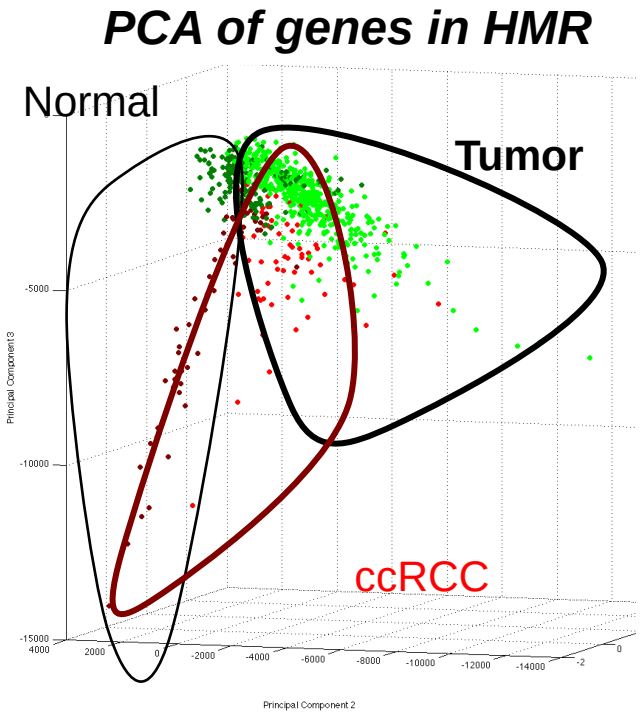


PNAS (2005) **102**:2685-2689; *Mol. Sys. Biol.* (2006) **2**:50
Curr. Op. Microbiol. (2010) **13**:255-262; *J. Int. Med.* **271**:142-154

Is Metabolism of Cancer Cells Different from Normal Cells

RNAseq "*metabolic*" transcriptome of 10 cancers and control



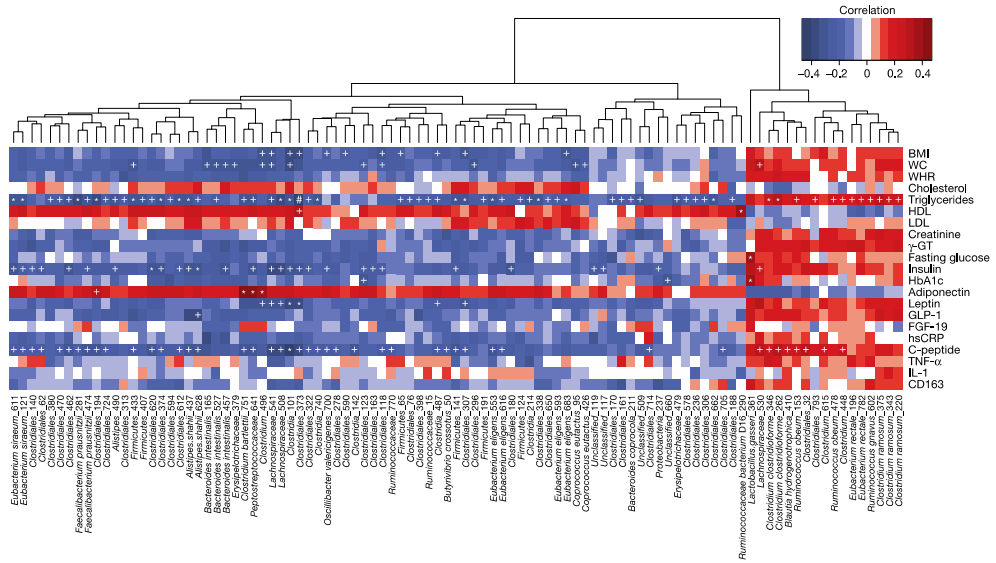


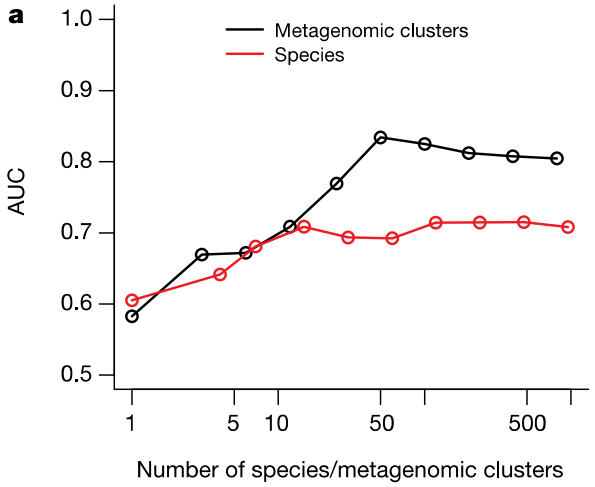
Clear cell Renal Cell Carcinoma (kidney cancer) deviates and do not show a clear distinction in terms of transcription of metabolic genes

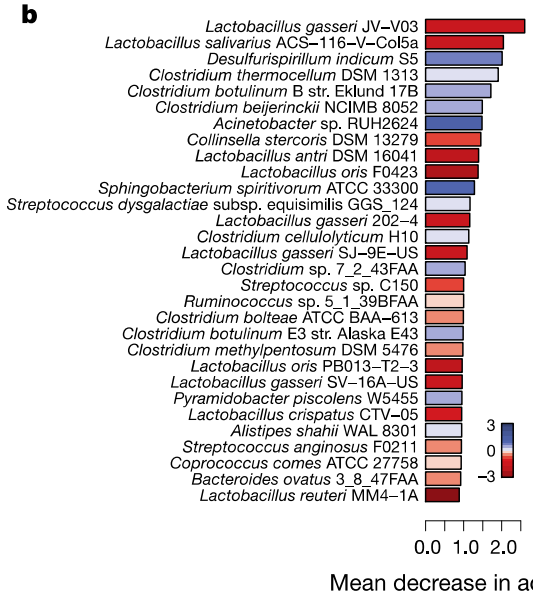
Analysis of Type 2 Diabetes Patients

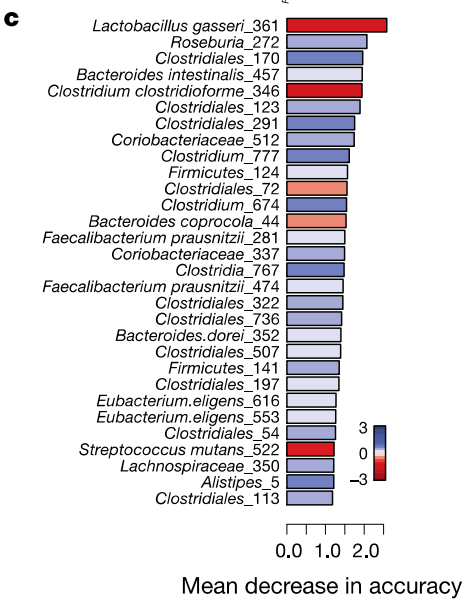
We could correlate gut bacteria with plasma chemistry

We could further predict T2D based on the gut metagenome









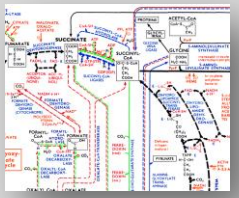
Nature. (2013) **498**:99-103

Metabolic Engineering

sys²bio www.sysbio.se

Modeling & Design

Genome-scale metabolic models
Kinetic models
Thermodynamics
Flux balancing

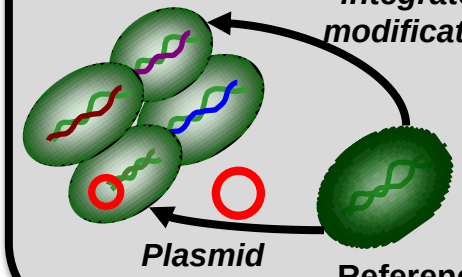


$$Tv = b$$

$$\frac{dC^{prod}}{dt} = k_f C_{react} - k_r C_{prod}$$

Strain Construction

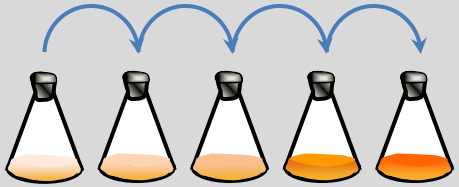
Chromosome integrated modification



Plasmid

Reference

Directed Evolution

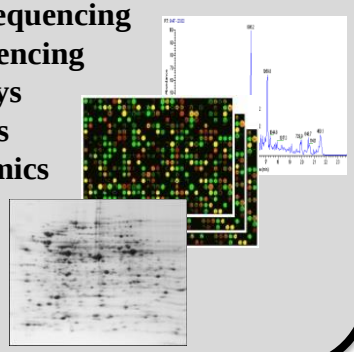


Growth rate selection
Adaption to media
Increasing tolerance

Inverse Metabolic Engineering

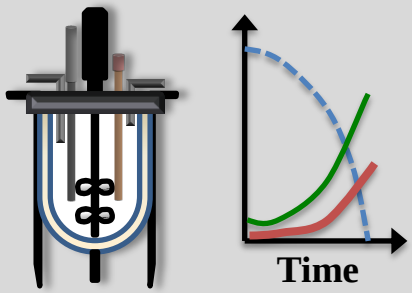
Omics Analysis

Genome sequencing
RNA sequencing
DNA arrays
Proteomics
Metabolomics
Fluxomics



Fermentation

Phenotypic characterization

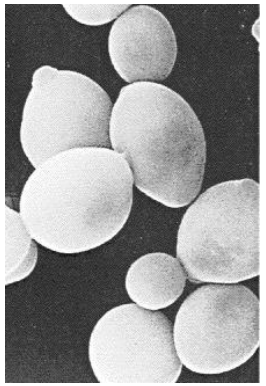


Directed evolution is today often integrated into metabolic engineering

Yeast as a Platform Cell Factory

Many advantages:

- Extremely well-characterized
- Many online databases with information on genome, as well as different omics data
- Genetically tractable
- GRAS (Generally Regarded as Safe)
- **Robust industrial organism**



A widely used cell factory

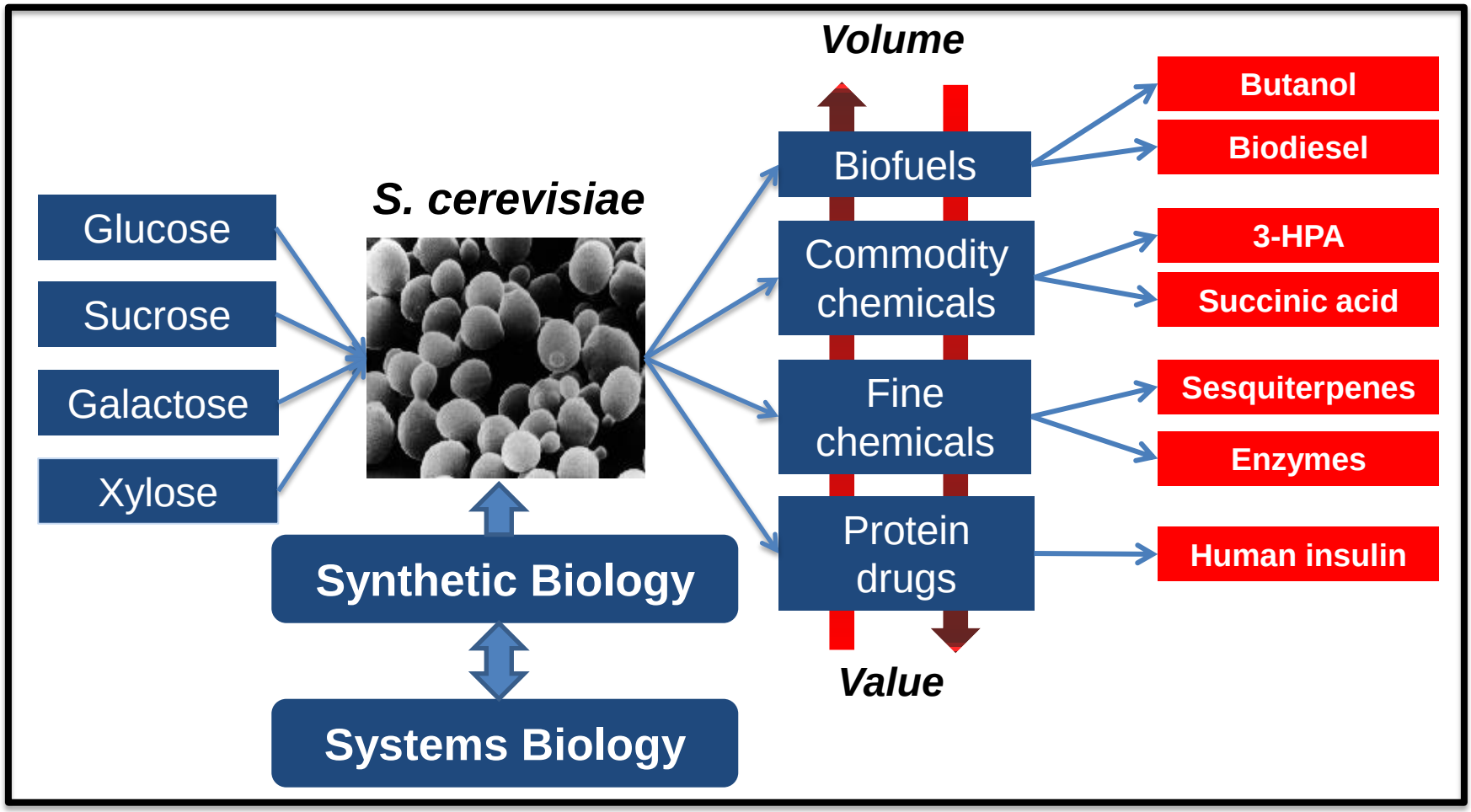
- Bioethanol
- Baker's yeast
- Wine & Beer
- Resveratrol
- Insulin precursors
- Vaccines (HPV, Hepatatis)

Ongoing developments of novel cell factories:

- **Fuels** (butanol, biodiesel)
- **Commodity chemicals** (malate, succinate, 3-OH propionic acid)
- **Fine chemicals** (isoprenoids**)**
- **Food ingredients** (PUFAs)
- **Protein drugs**

Metabolic Engineering of Yeast

Our objective is to establish an extensive technology base for wider use of yeast as platform cell factory and demonstrate its use for development of diverse products



Butanol production and tolerance in *Saccharomyces cerevisiae*

Payam Ghiaci

Francisca Lameiras

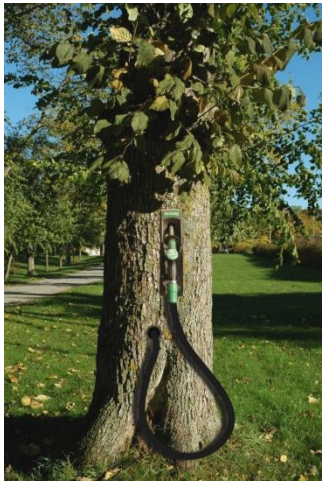
Joakim Norbeck

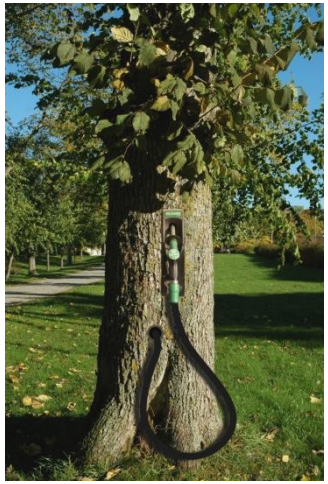
Intawat Nookaew

Christer Larsson

Chalmers University of Technology, Dept Chemical and Biological Engineering - Systems Biology, Gothenburg, Sweden

Bio-fuels; Butanol vs Ethanol





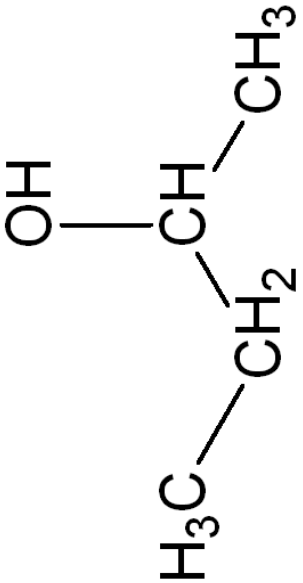
Why butanol?

•Butanol has a higher energy content compared to ethanol

• Lower water absorption and volatility compared to ethanol

• Existing distribution systems can be used

• Can be used in conventional engines without or with less modifications



Why not butanol?

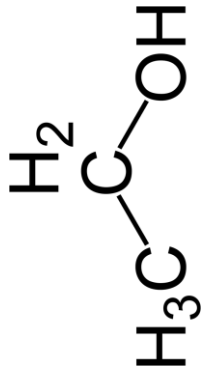
Butanol is very toxic to the producing organisms

Why yeast?

•Outstanding history of human usage and exploitation under large-scale industrial conditions

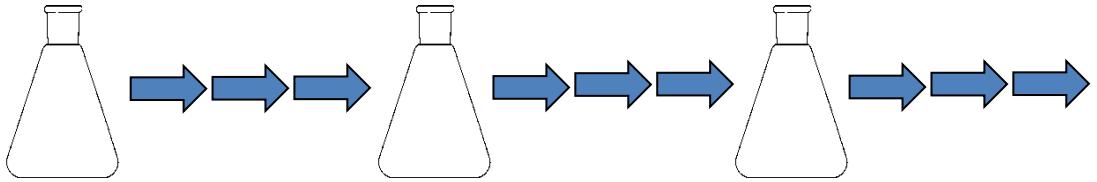
•Can cope with harsh and/or nutrient poor conditions such as, *e.g.* Lignocellulosic substrates

• Amenable to genetic manipulations



Part 1: Tolerant yeast

Evolving a 2-butanol tolerant yeast via evolutionary engineering



Batch culture 1.9% Butanol

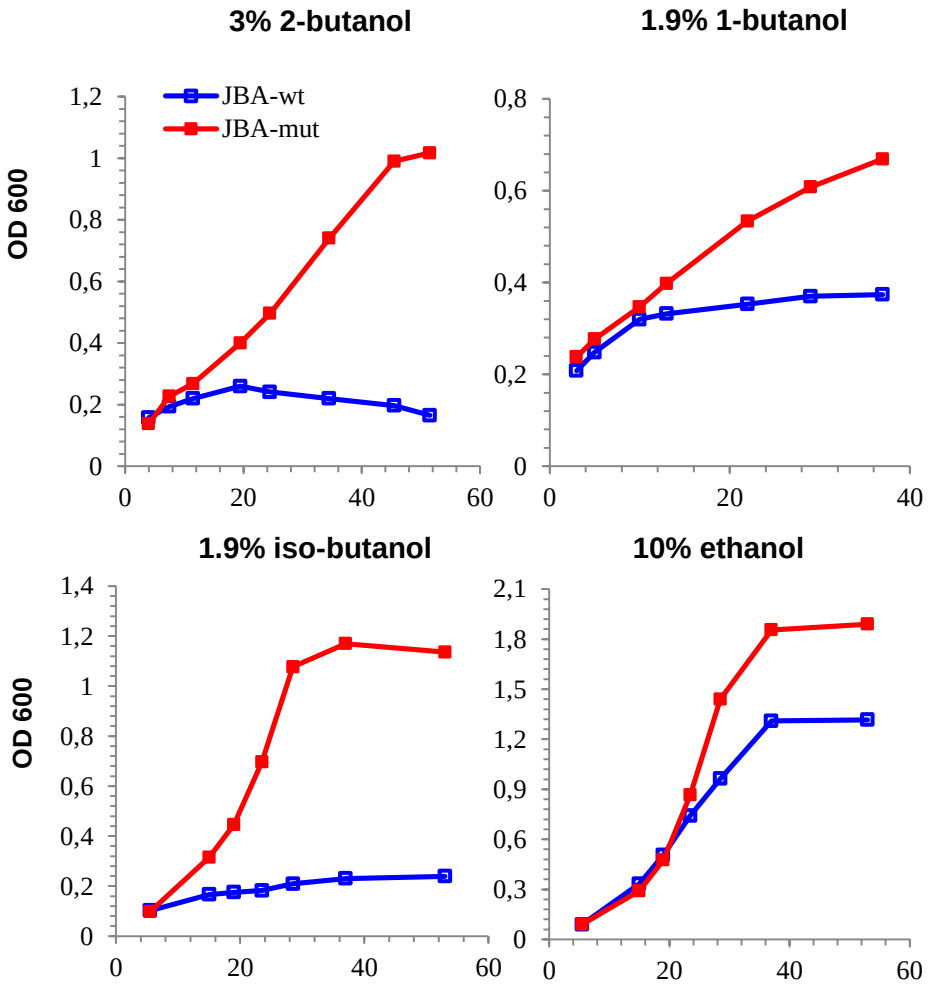
Batch culture 2.5% Butanol

Batch culture 3.0% Butanol

Tolerant strain

- 30 sequential batch cultures
- About 100 generations within 24 days
- Industrial baker's yeast strain

Comparison of growth in the presence of various alcohols between wild-type and the evolved 2-butanol tolerant strain



Characterization of evolved mutant; protein expression and lipid analysis.

Growth conditions

1. Chemostat culture, D = 0.1 h^{-1}, with 2.5% 2-butanol

2. Batch culture with 1.2% 2-butanol (μ identical between mutant and wild-type)

Protein expression profile by MS; comparison wild-type vs evolved mutant

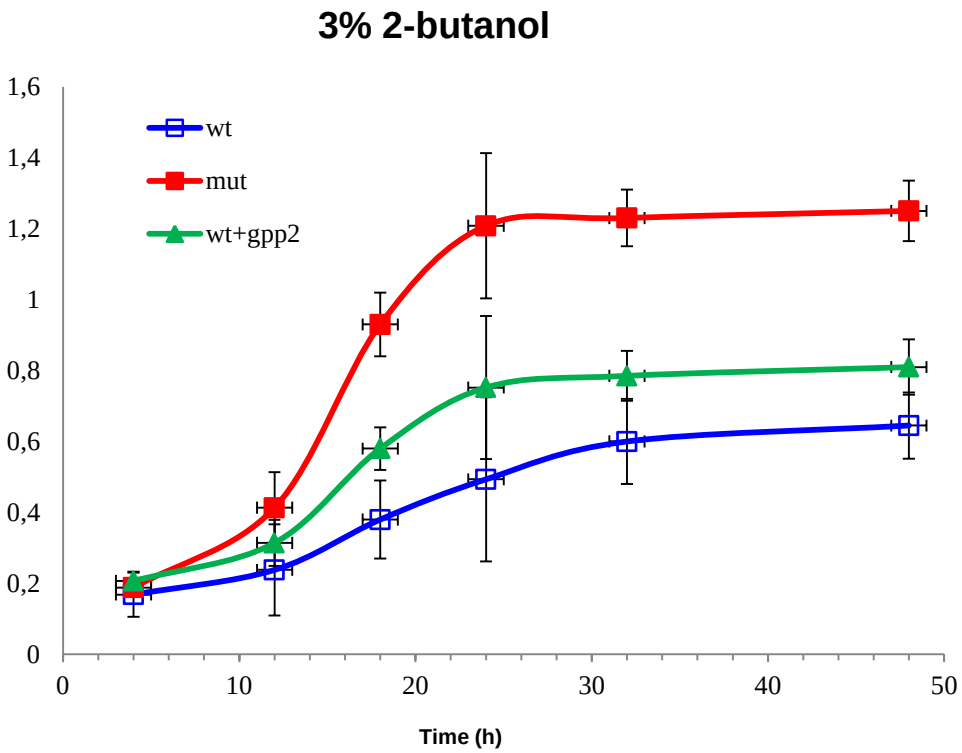
Gene	Corresponding protein	Fold change	Mitochondrial location
PIM1	Lon protease homolog	2.86	X
MAM33	Mitochondrial acidic protein MAM33	2.72	X
CYT1	Cytochrome c1, heme protein	2.09	X
GLO1	Lactoylglutathione lyase	2.01	
HSP42	Heat shock protein 42	1.97 *	
AIM2	Protein AIM2	1.94	X
FUN30	Uncharacterized ATP-dependent helicase FUN30	1.88 *	X
HOR2	(DL)-glycerol-3-phosphatase 2	1.81 *	
MCR1	NADH-cytochrome b5 reductase 2	1.80 *	X
GLK1	Glucokinase-1	1.74 *	
MRPL38	54S ribosomal protein L38	1.70 *	X
QCR6	Cytochrome b-c1 complex subunit 6	1.67	X
EDE1	EH domain-containing and endocytosis protein 1	1.65	
MSS116	ATP-dependent RNA helicase MSS116	1.65 *	X
YPL088W	Putative aryl-alcohol dehydrogenase YPL088W	1.64 *	
ATP4	ATP synthase subunit 4	1.62	X
ATP17	ATP synthase subunit f	1.62 *	X
PEP4	Saccharopepsin	1.62	
LSP1	Sphingolipid long chain base-responsive protein LSP1	1.62	
QCR2	Cytochrome b-c1 complex subunit 2	1.61	X
COX4	Cytochrome c oxidase subunit 4	1.59	X
ZWF1	Glucose-6-phosphate 1-dehydrogenase	1.59	
ECM33	Cell wall protein ECM33	1.58	
GVP36	Protein GVP36	1.57	
CCP1	Cytochrome c peroxidase	1.57	X
CAR2	Ornithine aminotransferase	1.57 *	
AAC2	ADP, ATP carrier protein 2	1.56	X
CYC1	Cytochrome c iso-1	1.56 *	X
ATP1	ATP synthase subunit alpha	1.55 *	X
ATP2	ATP synthase subunit beta	1.54 *	X
CPR3	Peptidyl-prolyl cis-trans isomerase C	1.54	X
KGD1	2-oxoglutarate dehydrogenase	1.54	X
QCR7	Cytochrome b-c1 complex subunit 7	1.53	X
MRP8	Uncharacterized protein MRP8	1.51 *	

- ~1100 proteins quantified (also membrane proteins)
- Expression of ribosomal proteins was not changed
- 34 proteins were up-regulated 1.5 fold or more (≥95% significance)
- 21 of these were mitochondrial and 12 components of respiratory chain
- Glo1, Hsp42 and Gpp2 were the most up-regulated non-mitochondrial proteins

- 12 proteins down-regulated 1.5 times or more (≥95% significance)

P. Ghiaci, J. Norbeck and C. Larsson. Biotechnology for biofuels, 2013, **6:**101

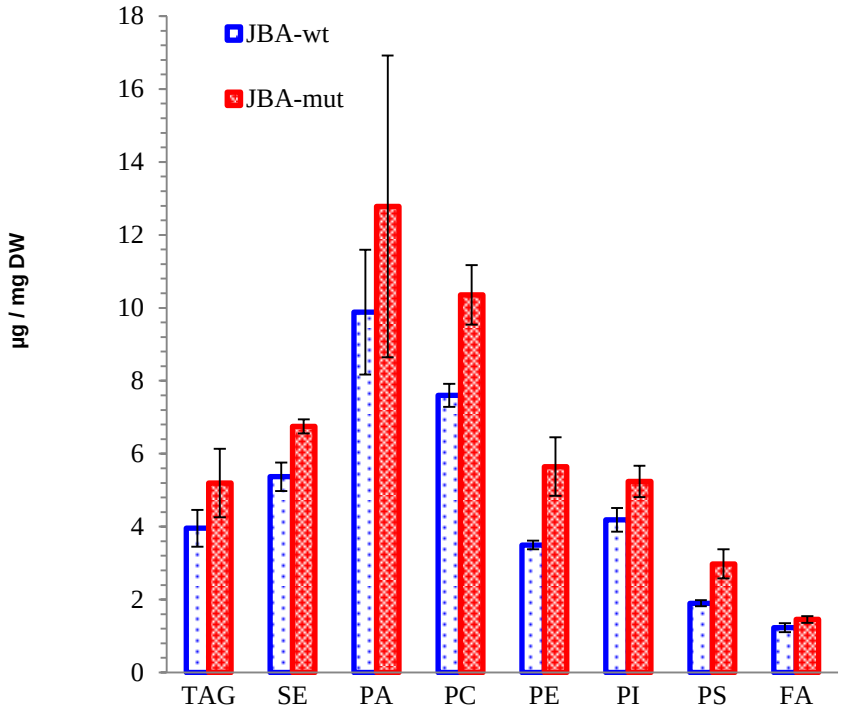
Verification of protein expression data by individual overexpression of Glo1, Hsp4, Gpp2 and Hap4



Gpp2 overexpression improved 2-butanol tolerance

Glo1, Hsp42 and Hap4 overexpression did not improve 2-butanol tolerance

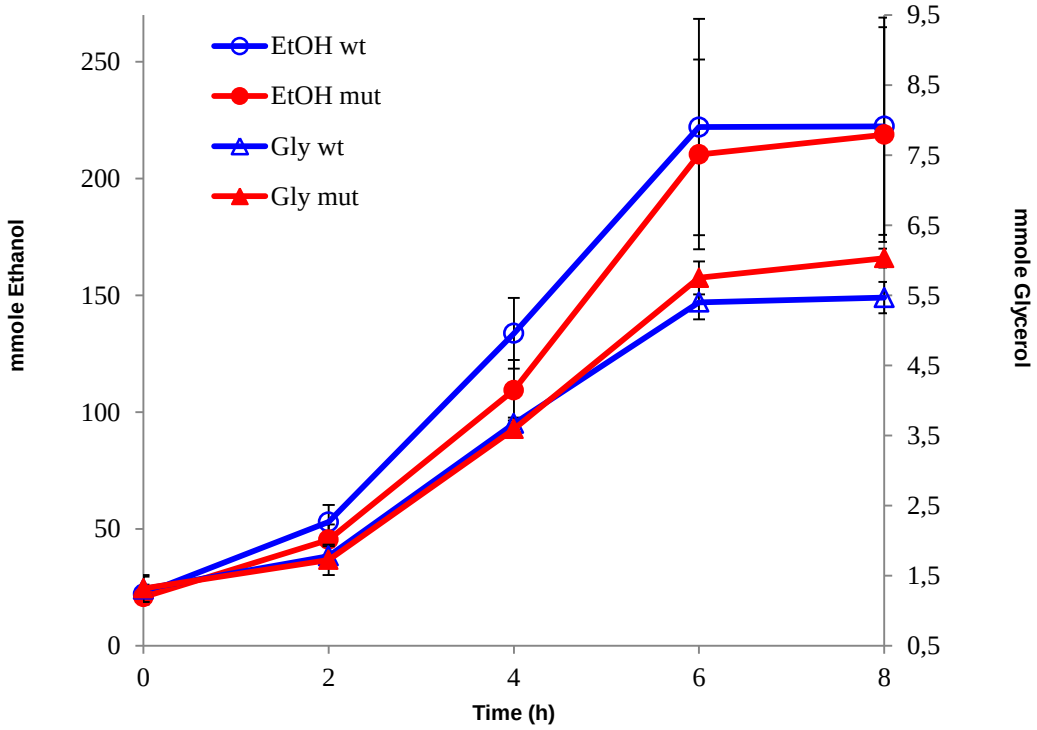
Lipid analysis; comparison wild-type vs evolved mutant



Tendency to a somewhat higher content of lipids in the tolerant mutant (not significant)

TAG = triacylglycerol
SE = steryl ester
PA, PC, PE, PI, PS = different phospholipids
FA = free fatty acid
ES = ergosterol

Growth characteristics; comparison wild-type vs evolved mutant during growth with 1.2% 2-butanol



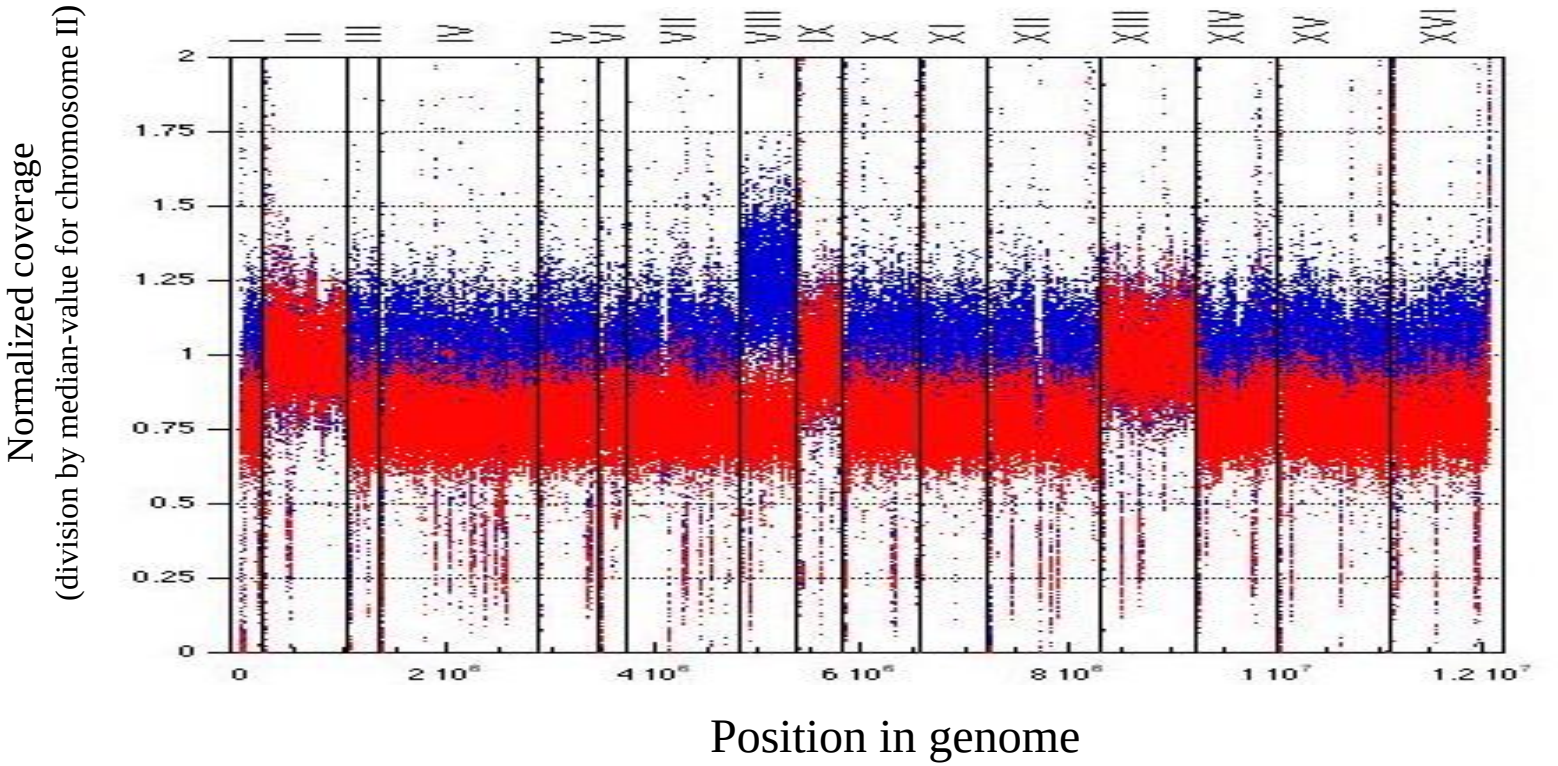
Growth characteristics were very similar

2-butanol tolerant mutant had a slightly lower ethanol and higher glycerol production consistent with proteomics data

Whole genome sequencing and comparison between wild-type vs evolved mutant

Butanol tolerance correlates with a loss of ploidy for most chromosomes.

(we have sequenced with approximately 1000 reads/bp)



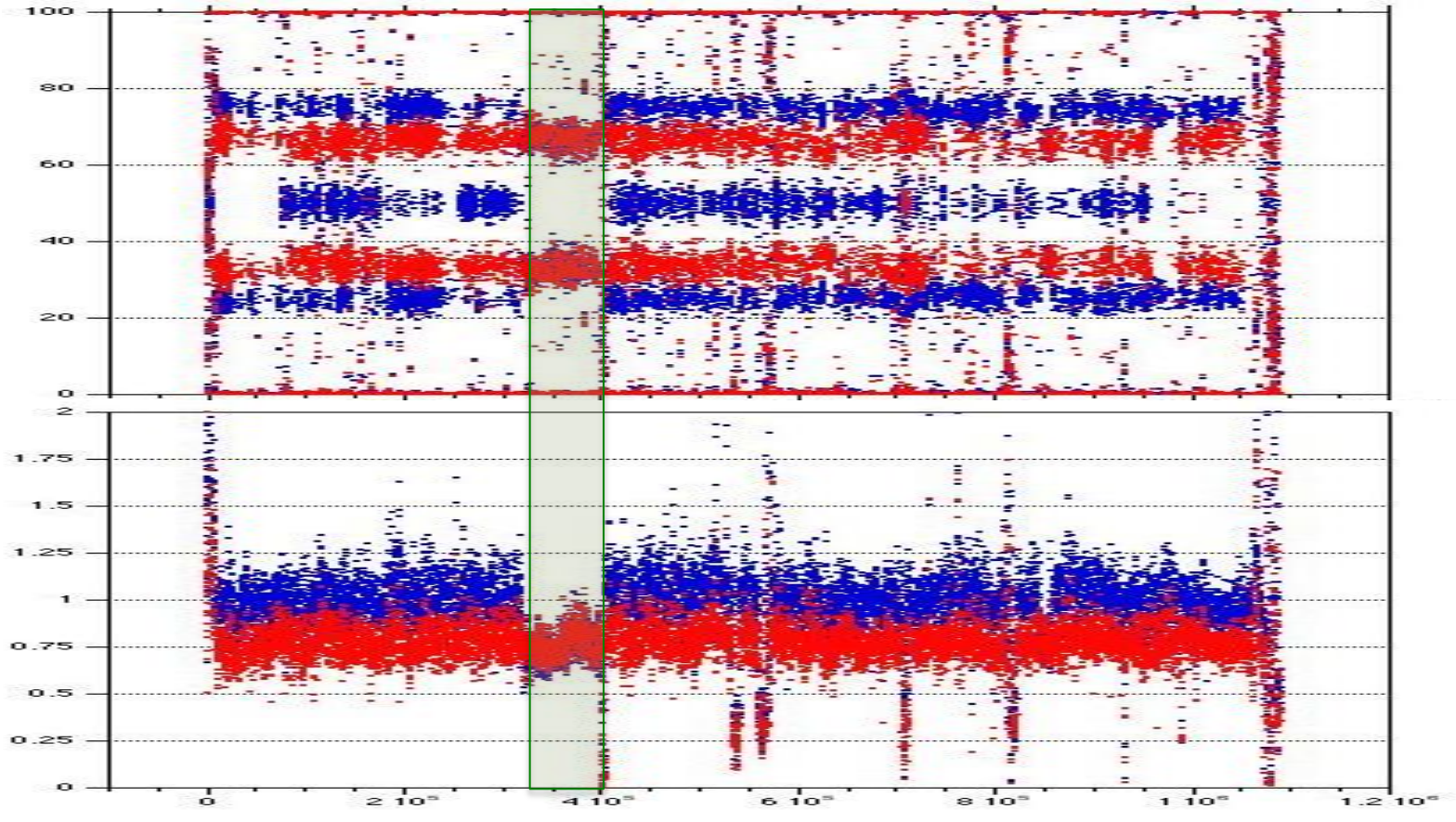
Plot of normalized sequence coverage (100 bp average) against position in genome.
Blue is JBA-wt and red is JBA-mut.

Chromosomes II, IX and XIII are tetraploid in both strains,
chromosome VIII is pentaploid in JBA-wt.
All other chromosomes are triploid in JBA-mut and tetraploid in JBA-wt.

Chromosome VII as a detailed example:

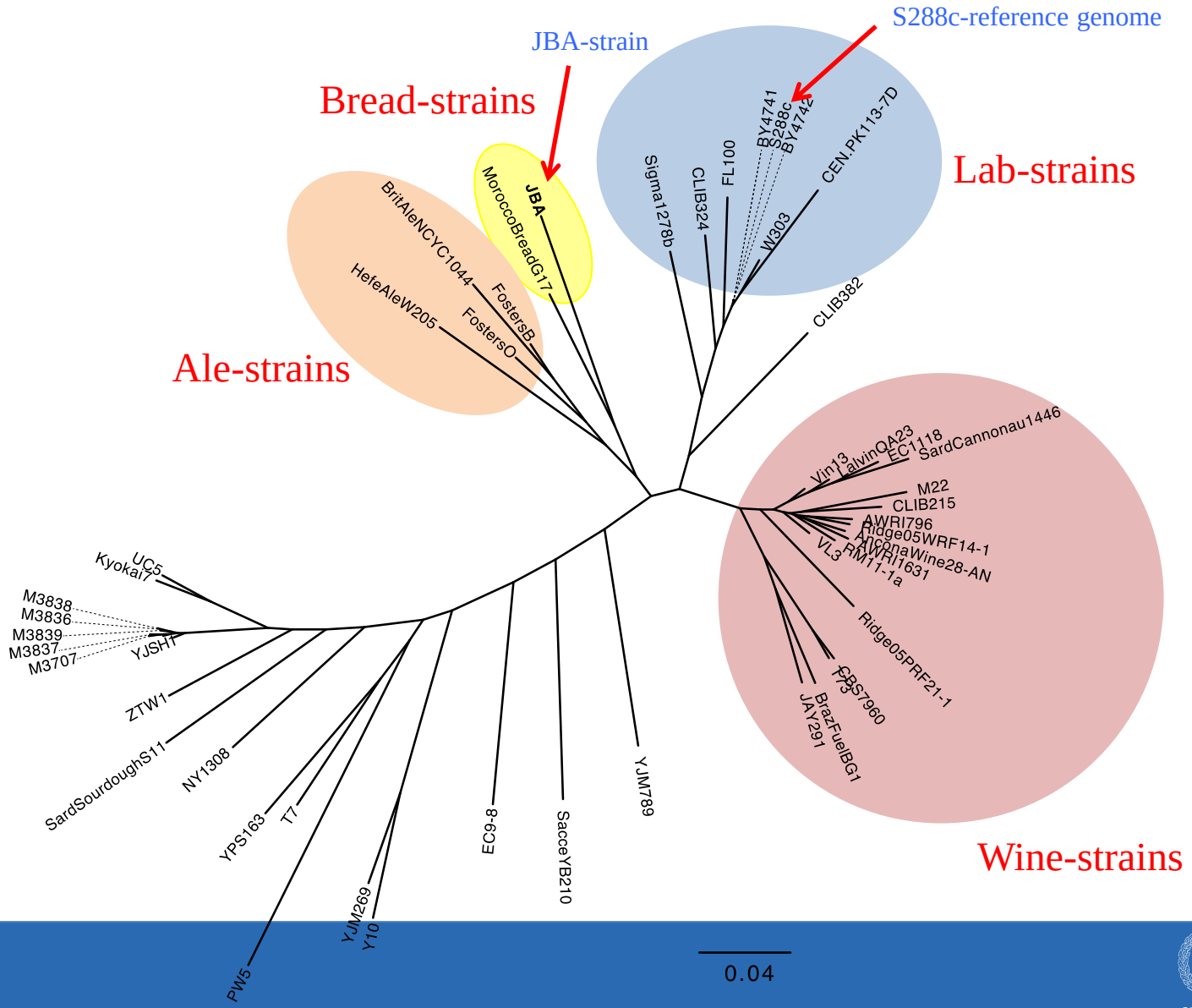
Upper plot shows frequency of each nucleotide at SNV-site. The wild type (blue) mainly has values being multiples of 25%
indicative of a tetraploid. For the mutant (red) the values are multiples of 33%, in agreement with triploid.

Lower plot shows normalized coverage values (blue-wt, red-mut), green square indicates a region of local triploidy in wt. Several regions of lower coverage, corresponding to regions lacking from one or more chromosome copies can be seen. (these are mostly transposable elements)



JBA is closely related to bread-baking strains

JBA-wt differs from JBA-mut by ~ 16000 heterozygous SNV's (1.3 SNV/1000 bp)
JBA-wt differs from the S288c reference genome by ~ 100 000 heterozygous SNV's (8.6 SNV/1000 bp)



Conclusion from genome sequencing:

There are major changes in the genomic composition of JBA-wt and JBA-mutant which was surprising since there were few changes in the proteome

No differences in genes corresponding to proteins with an altered expression level between wild-type and mutant could be detected

Part 2: 2-butanol production in yeast

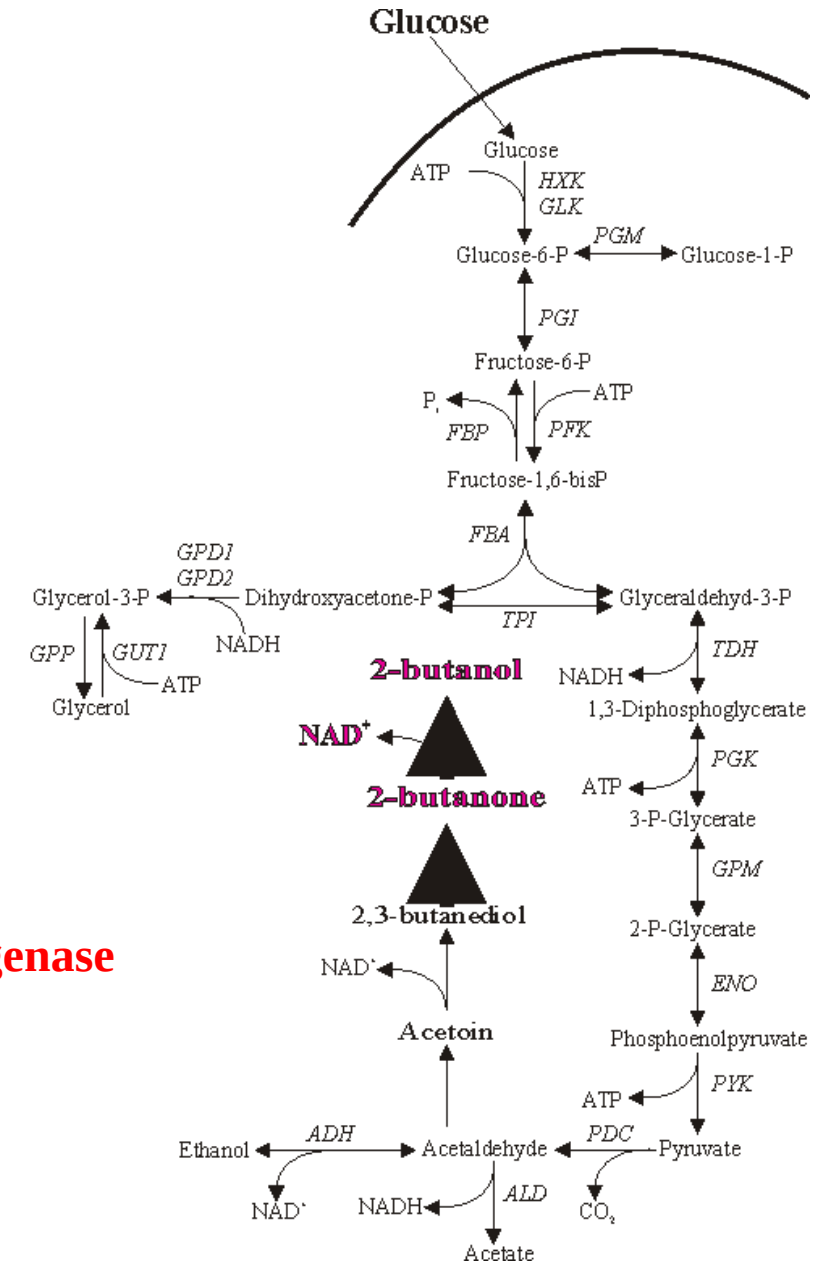
2,3-Butanediol

↓ **Diol-dehydratase**

2-Butanone

NADH → NAD+ **Butanone-dehydrogenase**

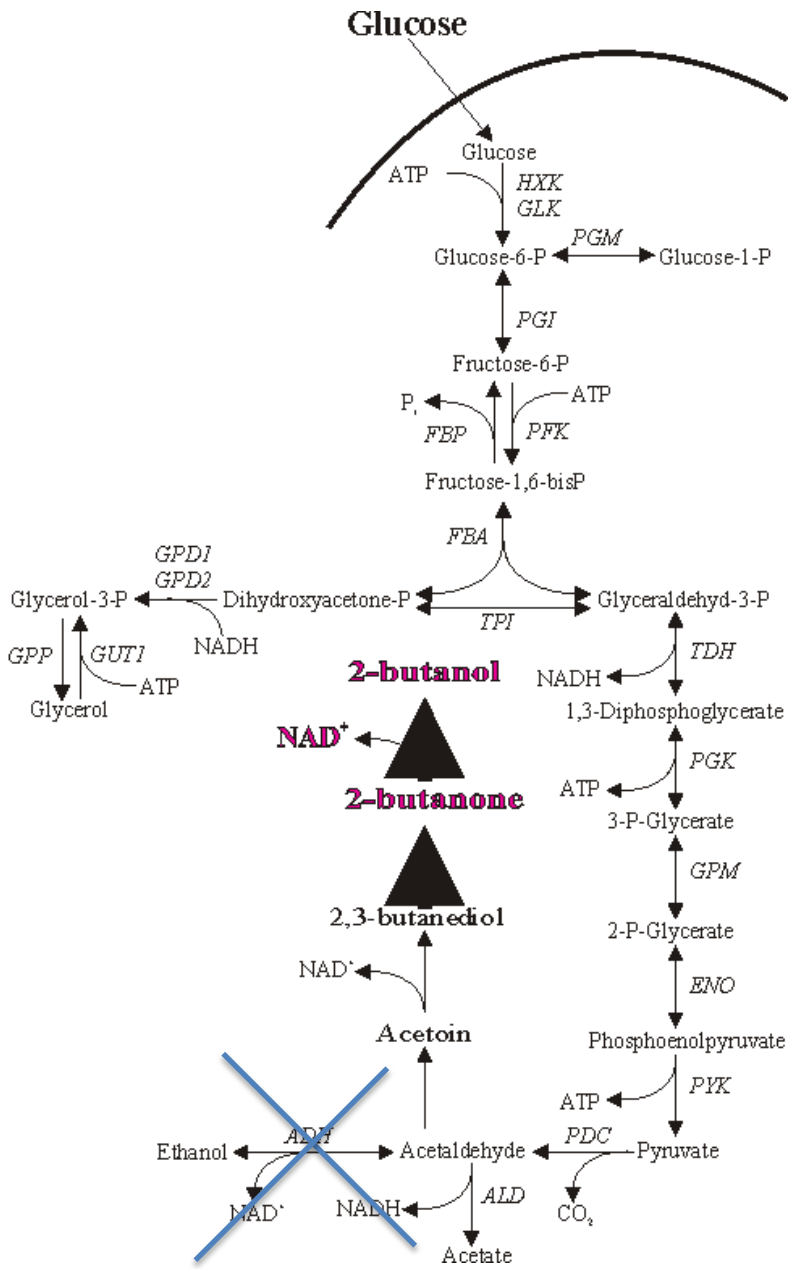
2-Butanol



Part 2: 2-butanol production in yeast

Butanol producing pathway that is very similar to the ethanol production pathway, e.g:

- Redox neutral
- Energetics is similar
- 2 ATP/Glucose
- Formation of one 4-carbon compound instead of two 2-carbon compounds



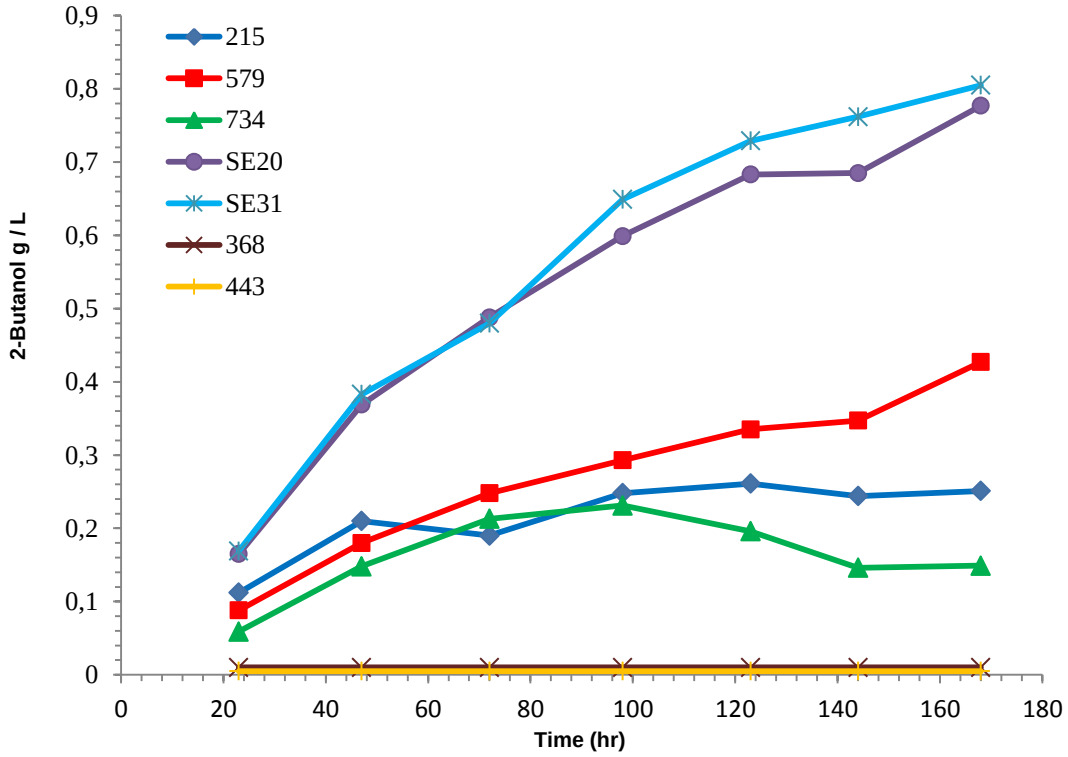
How to find the required enzymes?

The required enzyme activities are reported to be present in Lactobacilli and specifically *L. Brevis*

No conversion of 2,3-butanediol into 2-butanol in MRS medium but when using a defined medium (SM2).....

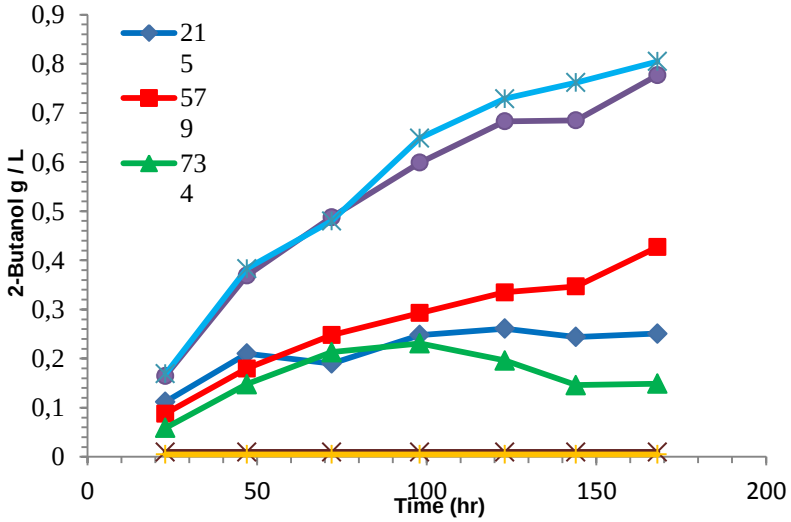
Strain		Source	*meso*-2,3-butanediol consumption	2-butanone consumption
L. plantarum	16 strains	ethanol pilot plant, Örnsköldsvik	No	Not tested
L. pantheris	8 strains	ethanol pilot plant, Örnsköldsvik	No	Not tested
L. rossiae	2 strains	ethanol pilot plant, Örnsköldsvik	No	Not tested
L. paracasei	1 strain	ethanol pilot plant, Örnsköldsvik	No	Not tested
L. fermenum	1 strain	ethanol pilot plant, Örnsköldsvik	No	Not tested
L. paracollinoids	1 strain	ethanol pilot plant, Örnsköldsvik	No	Not tested
L. malefermentas	1 strain	CCUG 32206	No	Not tested
L. buchneri	LB 12	ethanol pilot plant, Örnsköldsvik	**Yes**	**Yes**
	LB 16	ethanol pilot plant, Örnsköldsvik	**Yes**	**Yes**
L. brevis	SE 20	ethanol pilot plant, Örnsköldsvik	**Yes**	**Yes**
	SE 31	ethanol pilot plant, Örnsköldsvik	**Yes**	**Yes**
	LB 215	CCUG 21531	**Yes**	**Yes**
	LB 219	CCUG 21959	**Yes**	**Yes**
	LB 350	CCUG 35039	**Yes**	**Yes**
	LB 368	CCUG 36840	**Yes**	**Yes**
	LB 399	CCUG 39980	**Yes**	**Yes**
	LB 443	CCUG 44317	**Yes**	**Yes**
	LB 579	CCUG 57950	**Yes**	**Yes**
	LB 734	CNRZ 734	**Yes**	**Yes**

Production of 2-butanol from 2,3-butanediol by different isolates of L. brevis



Ghiaci, Lameiras, Norbeck and Larsson (2014) FEMS Microbiol. Lett. (under revision)

Production of 2-butanol from 2,3-butanediol by different isolates of L. brevis



The diol-dehydratase is induced by the presence of propanediol as well as butanediol and shows activity also with glycerol

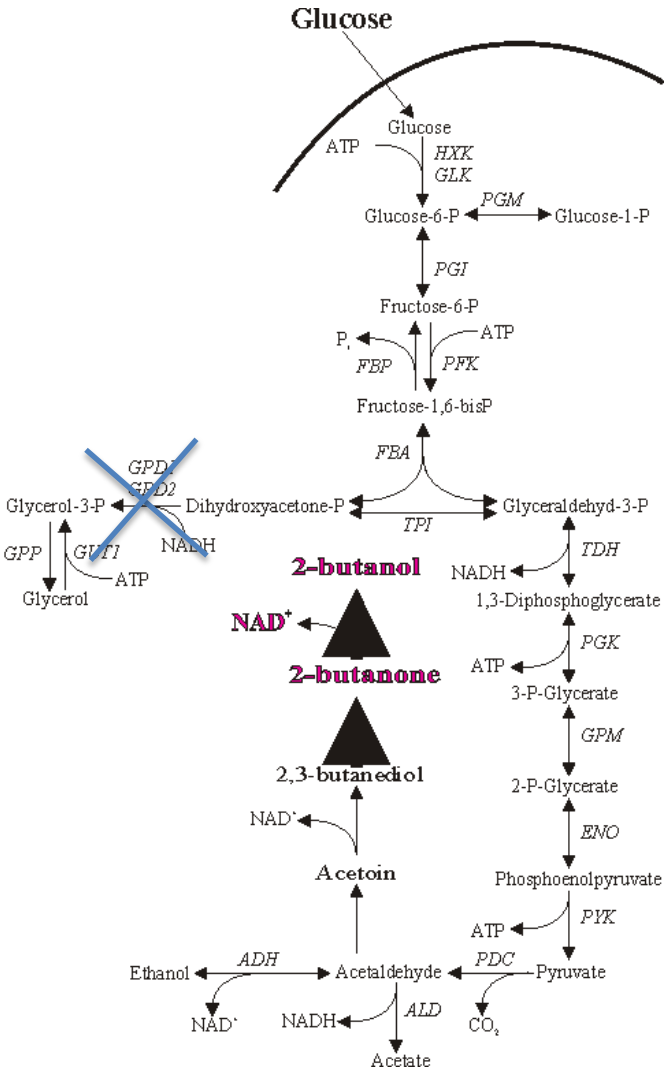
	Specific activity (µmol min^{-1} g^{-1})			
Grown in	1,2-propanediol	2,3-butanediol	glycerol	1,3-propanediol
SM2	< 0.1	< 0.1	< 0.1	< 0.1
SM2 + 1,2-propanediol	0.19 ± 0.001	0.08 ± 0.001	0.10 ± 0.001	0.11 ± 0.001
SM2 + 2,3-butanediol	0.13 ± 0.001	0.10 ± 0.002	0.22 ± 0.001	0.16 ± 0.001

Introduction of diol-dehydratase and a secondary alcohol dehydrogenase into *S. cerevisiae*

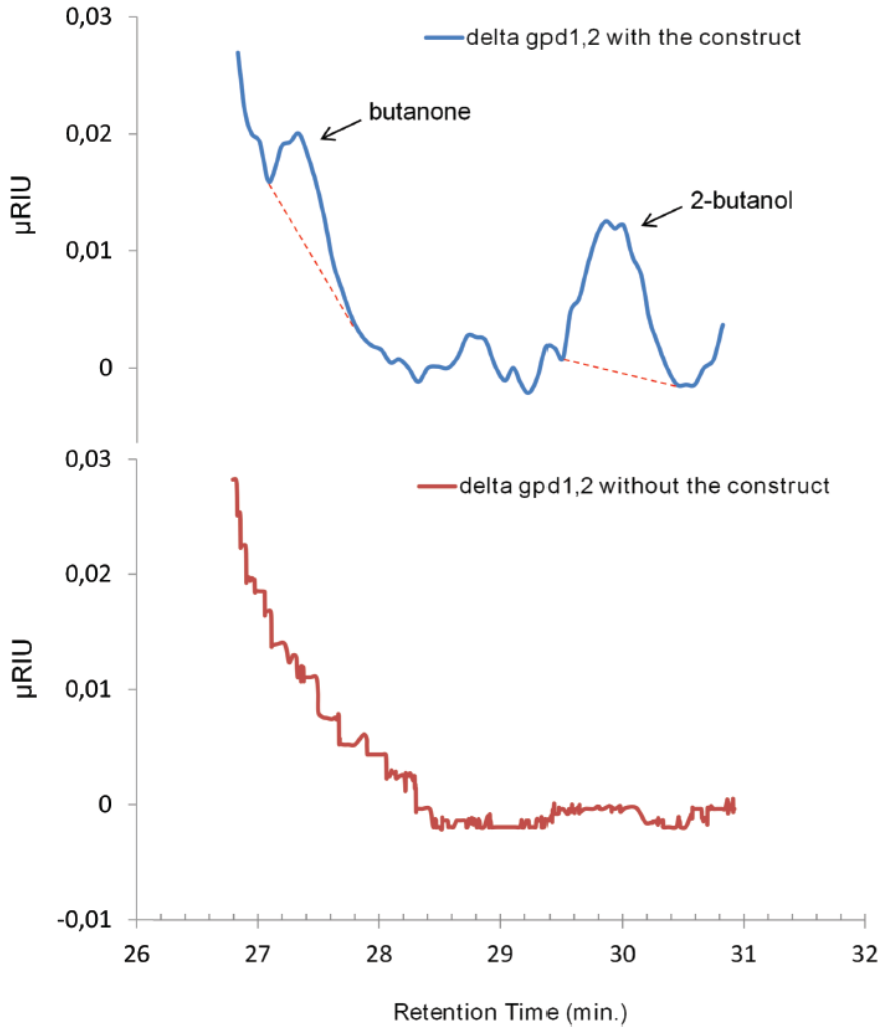
2-butanol production from 2,3-butanediol in a Δ*gpd1,2* double mutant

Wild type strain did not produce any 2-butanol

Diol-dehydratase is a vitamin B_{12} dependent enzyme



2-butanol production from 2,3-butanediol in a Δ*gpd1,2* double mutant



Ghiaci, Norbeck and Larsson (2014) Plos One 9:7

Conclusions

• Evolutionary engineering increased the 2-butanol tolerance with more than 50%

• The evolved strain showed an enhanced tolerance to other isomers of butanol as well as other alcohols

• The evolved strain showed an enhanced expression of many mitochondrial and respiratory proteins

• Increased expression of glycerol-3-phosphate phosphatase (Gpp2) increased 2-butanol tolerance in yeast

• The capacity to produce 2-butanol from 2,3-butanediol seems widespread among strains of *L. Brevis*

• B_{12} dependent 2-butanol production is established in *S. cerevisiae*

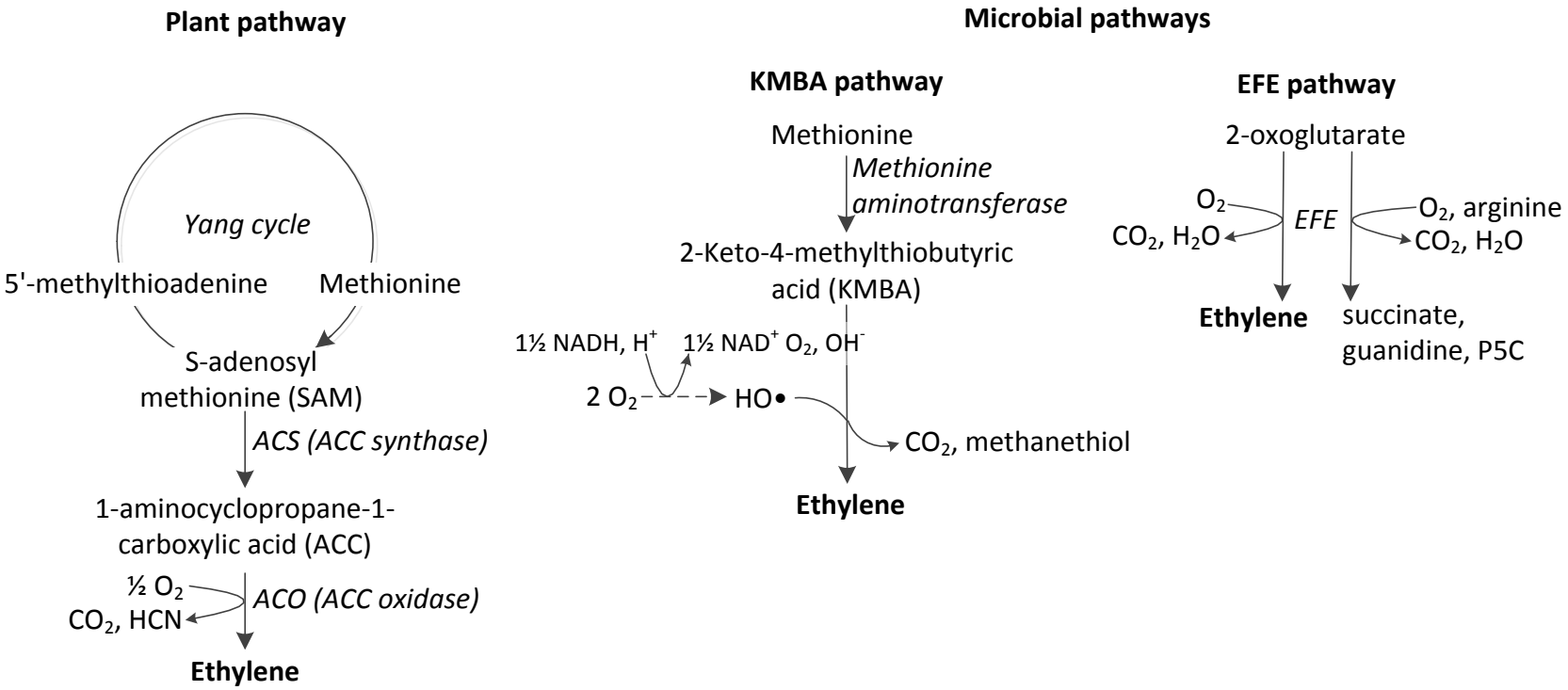
Transforming *Saccharomyces cerevisiae* into an ethylene producing organism

- **Nina Johansson**
- **Paul Quehl**
- **Karl Persson**
- **Joakim Norbeck**
- **Christer Larsson**
- *Chalmers University of Technology, Dept Chemical and Biological Engineering - Systems Biology, Gothenburg, Sweden*

Why ethylene in yeast?

- Bulk chemical (124 million tons/year)
- Fossil based production
- Yeast has an outstanding history of human usage and exploitation under large-scale industrial conditions
- Can cope with harsh and/or nutrient poor conditions such as, *e.g.* Lignocellulosic substrates
- Amenable to genetic manipulations

Three different ethylene producing pathways are identified



EFE = Ethylene Forming Enzyme and the EFE pathway

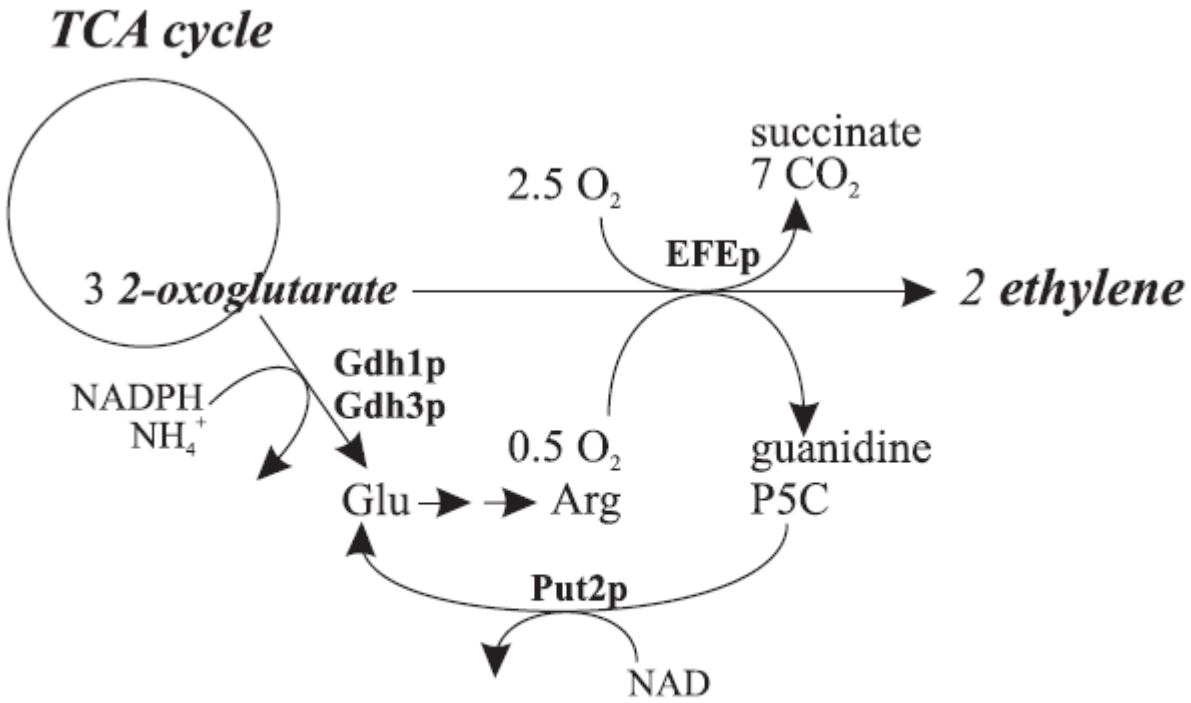
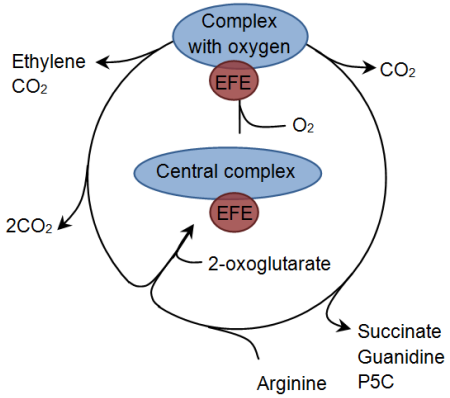


Fig. 1. Ethylene biosynthetic metabolism by insertion of the ethylene forming enzyme (EFE) from the plant pathogenic bacterium, *Pseudomonas syringae* (Fukuda et al., 1992) in the yeast *S. cerevisiae*.

Dual circuit mechanism of EFE



1: 2-oxoglutarate + O_2 → ethylene + 3 CO_2 + H_2O

2: 2-oxoglutarate + O_2 + arginine → succinate + guanidine + 1-Pyrroline-5-carboxylic acid + CO_2 + H_2O

(Proposed by Fukuda et al. (1992) Biochem. Biophys. Res. Com. 188:483-489)

Metabolic modelling identified oxygen and respiration of NADH as key factors for efficient ethylene production

Ethylene production at different levels of dissolved oxygen. Chemostat cultivation at D = 0.1h^{-1}

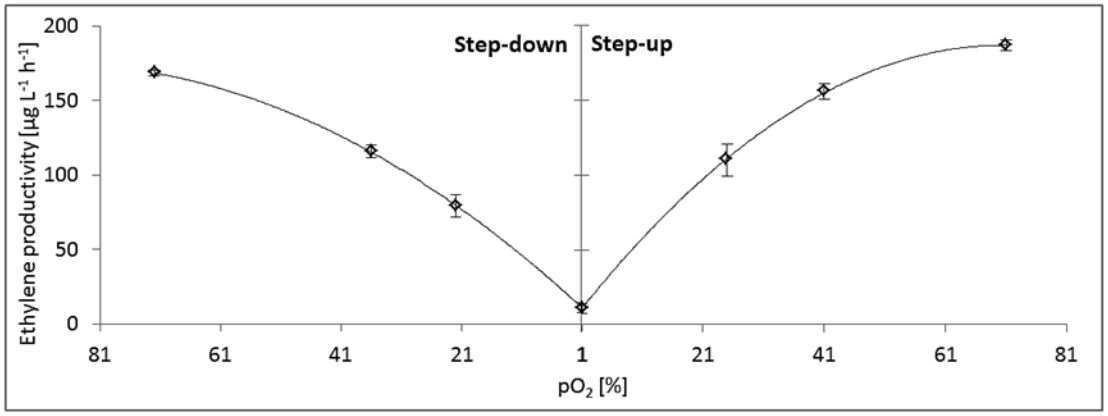


Table 2
The effect of respiration rate on ethylene formation

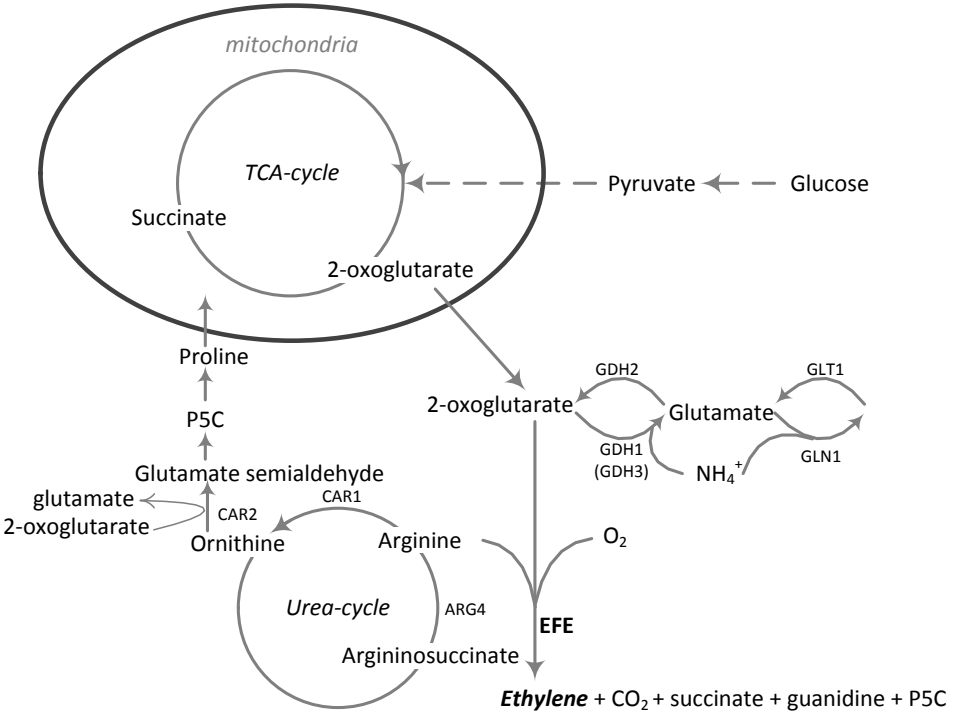
Condition	Ethylene	
	Specific productivity [µg g_{DW}^{-1} h^{-1}]	Productivity [µg $L_{Culture}^{-1}$ h^{-1}]
Reference condition	30.4 ± 2.8	178 ± 25
+ 7.5 mM Benzoate	50.3 ± 1.3	37.7 ± 0.9
+ 1 mM Azide	0	0

Oxygen important due to:

1. **EFE poor affinity for O2**
2. **Required for NADH oxidation**

Larsson et al (2011) IET Systems biology 5:245
Johansson et al (2013) Microbial cell factories 12:89

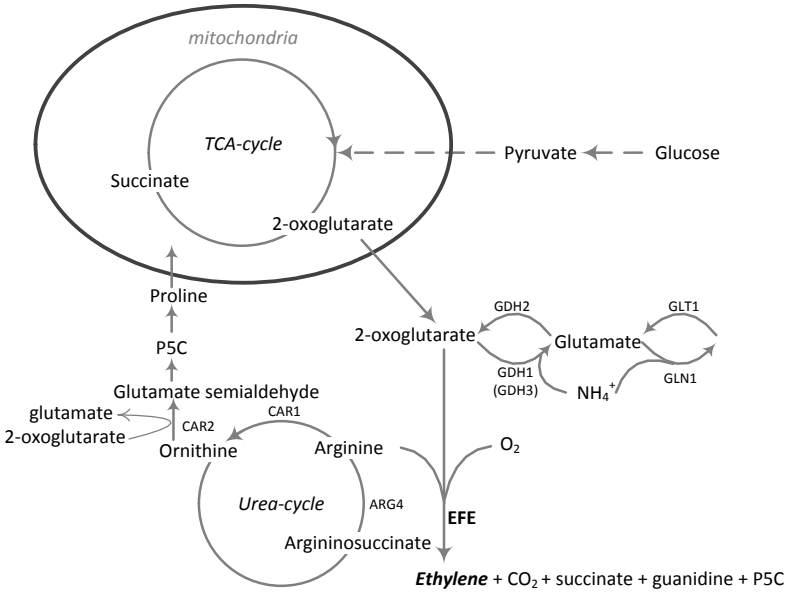
Close connection between ethylene formation and carbon as well as nitrogen metabolism



- Glutamate instead of NH4 drastically improves productivity and yield of ethylene
- Arginine drastically reduces productivity and yield
- Absence of arginine blocks ethylene formation

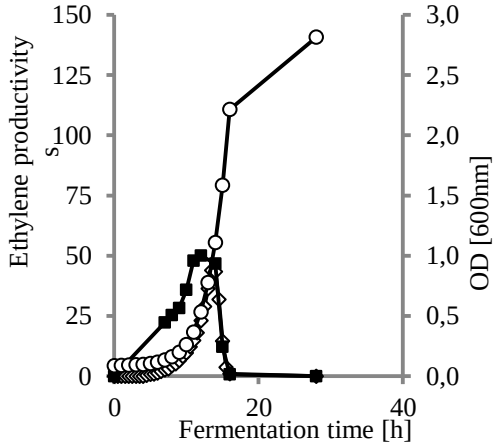
2-oxoglutarate + O_2 → ethylene + 3 CO_2 + H_2O (Arginine co-factor)
2-oxoglutarate + O_2 + arginine → succinate + guanidine + 1-Pyrroline-5-carboxylic acid (Arginine substrate)

Metabolic engineering strategies to enhance ethylene productivity and yield by decreasing intracellular levels of arginine



Overexpression of CAR1 ⟹ No effect

Deletion of ARG4

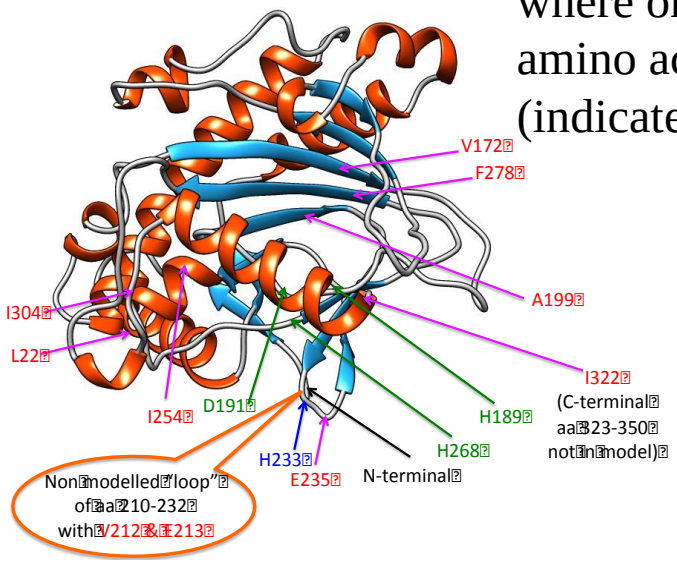


Arginine as a nitrogen source induced formation of pyruvate? Extracellular levels above 1 g/l

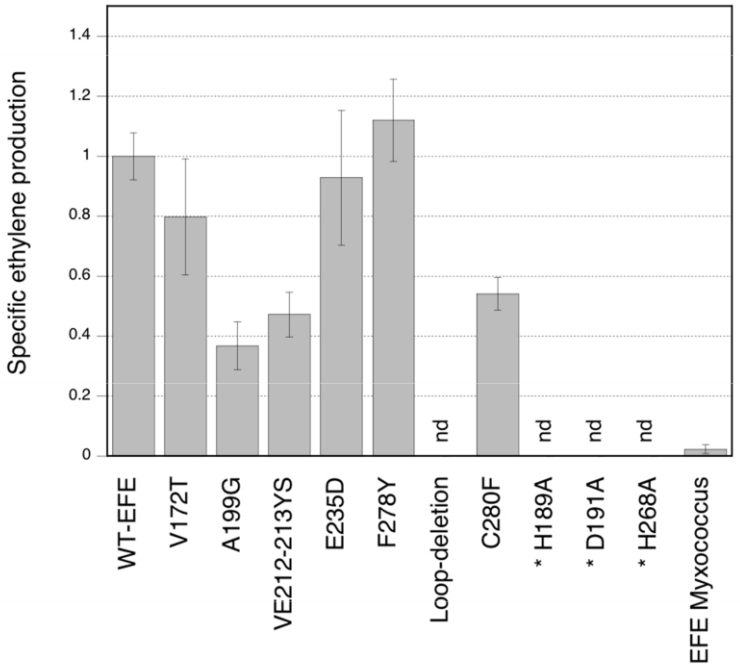
Johansson et al (2014) Fems Yeast research (under revision)

Structure function studies of EFE

Attempts to determine structure using NMR and crystallography failed!
But
Predicted structure from sequence homology with a dioxygenase from *Arabidopsis thaliana*



Comparison of amino acid sequence of three EFE's where one did not produce ethylene identified amino acids correlating with ethylene production (indicated in the figure)



Johansson et al BMC Biochemistry (submitted)

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Thank you and questions?