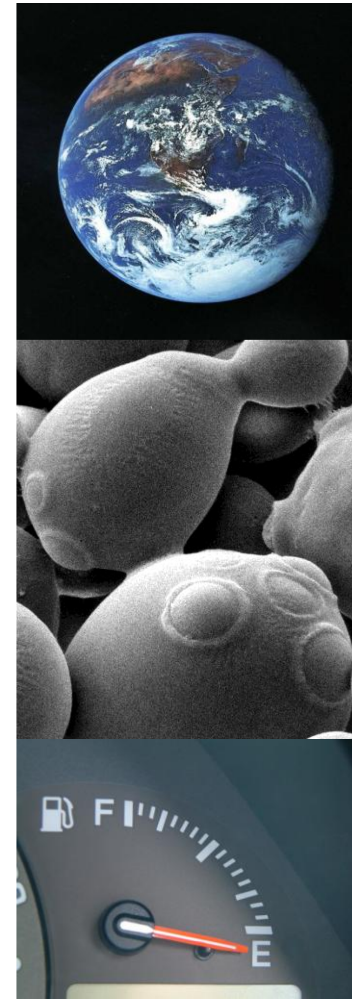
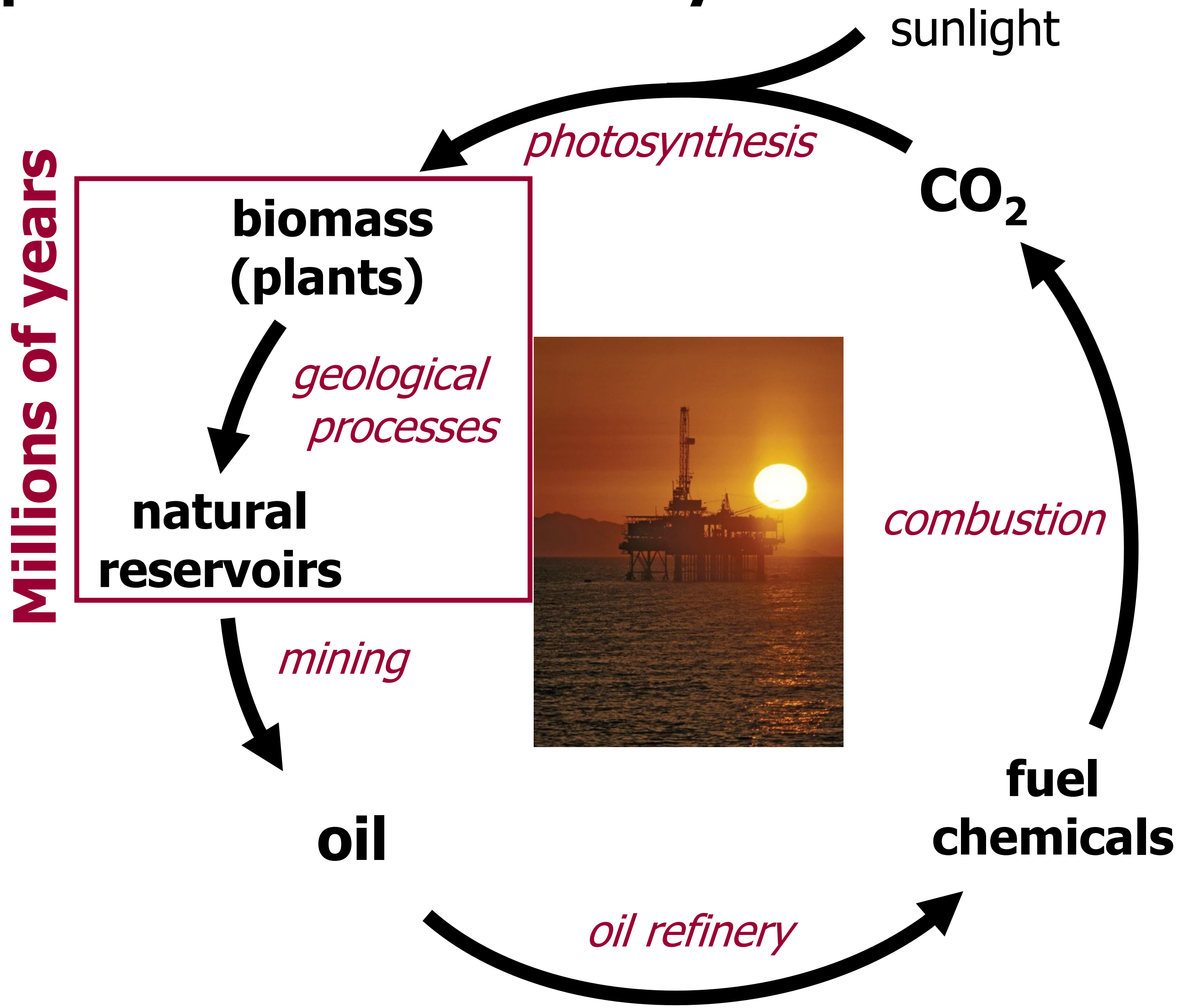
Engineering of *Saccharomyces cerevisiae* for efficient alcoholic fermentation of plant biomass hydrolysates



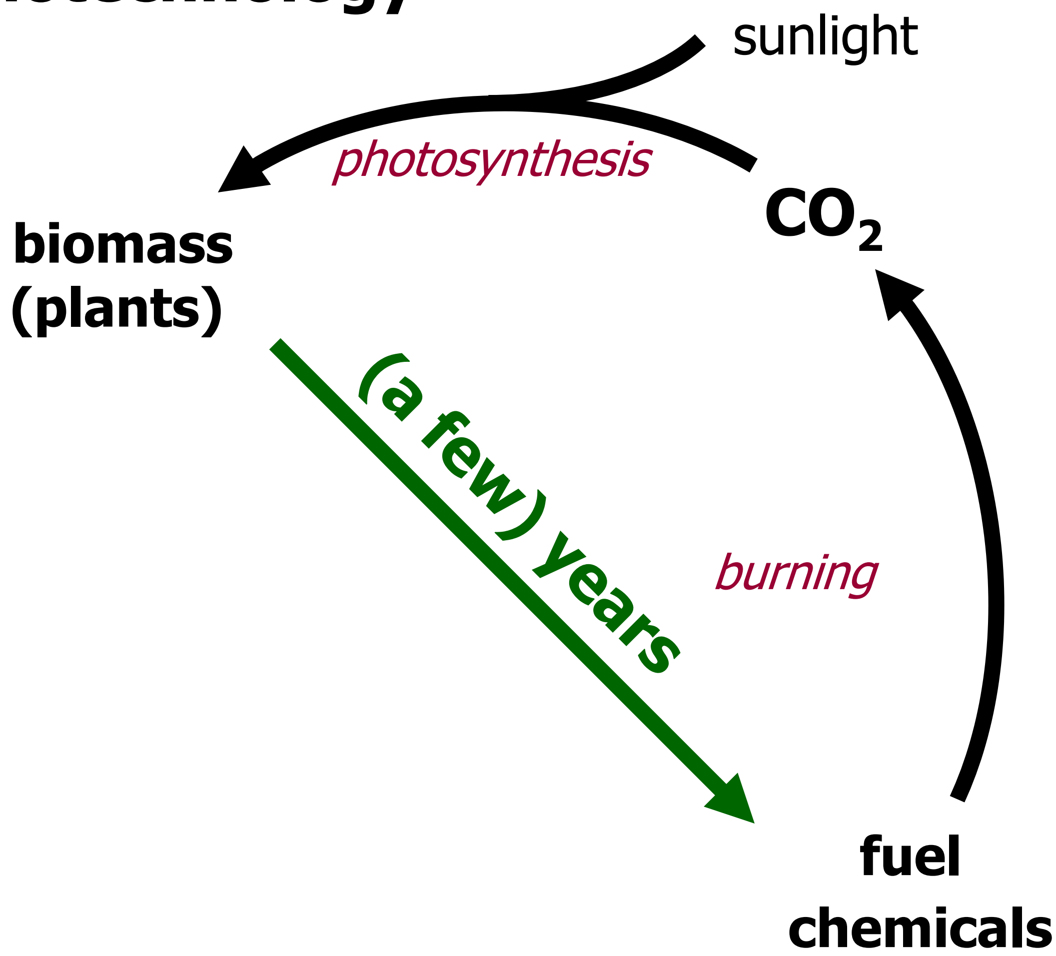
Ton van Maris
Delft University of Technology
Department of Biotechnology
Section Industrial Microbiology
Delft, the Netherlands

São Paulo, October 2010

The petrochemical carbon cycle



Industrial Biotechnology



Potential feedstocks for industrial biotechnology

current feedstocks
competing food/feed

agricultural residues
cheap/abundant

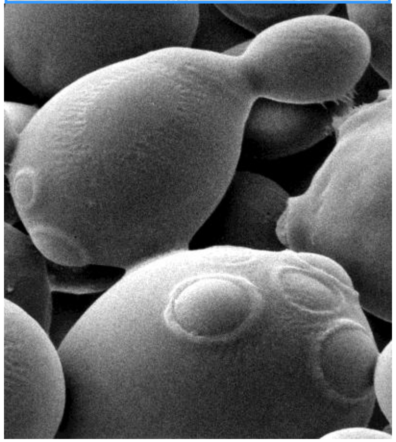
dedicated crops
use of less fertile land

Fuel Ethanol from Crop Residues: Strategy

Crop Residues
↓
Pretreatment → Cellulose Hydrolysis → **Sugar Mixture** → Fermentation

Cellulose Hydrolysis
↓
Lignin
↓
Combustion
↓
Heat, electricity

Fermentation
↑
Ethanol

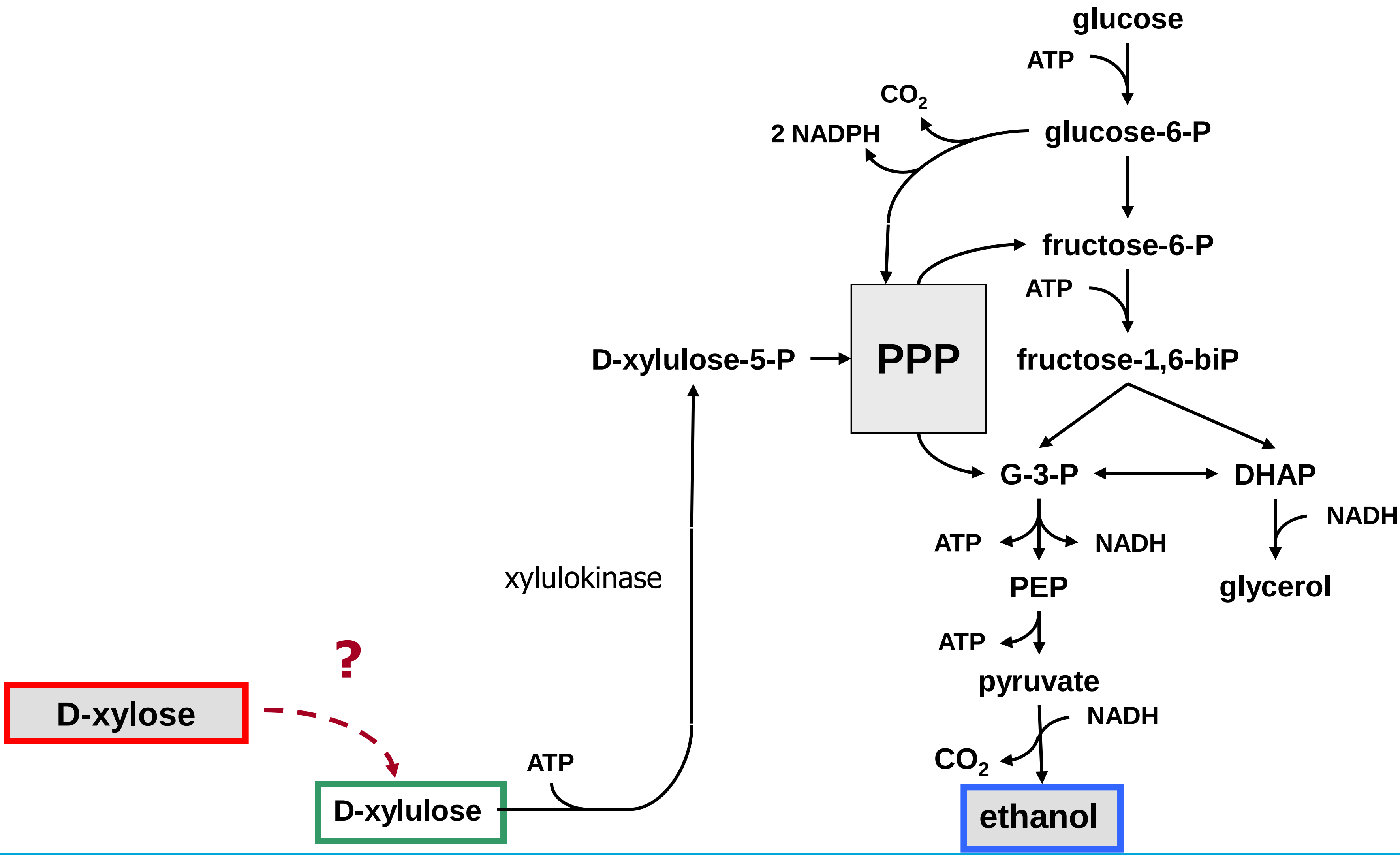


Saccharomyces cerevisiae

Sugars in Crop Residues: the Pentose Challenge

	Corn stover	Wheat straw	Bagasse
Sugars (%)			
glucose	34.6	32.6	39.0
mannose	0.4	0.3	0.4
galactose	1.0	0.8	0.5
xylose	19.3	19.2	22.1
arabinose	2.5	2.4	2.1
uronic acids	3.2	2.2	2.2
Other (%)			
lignin	17.7	16.9	23.1

Biochemistry of xylose metabolism in *S. cerevisiae*



Metabolic Engineering of *S. cerevisiae* for xylose fermentation: the beginning

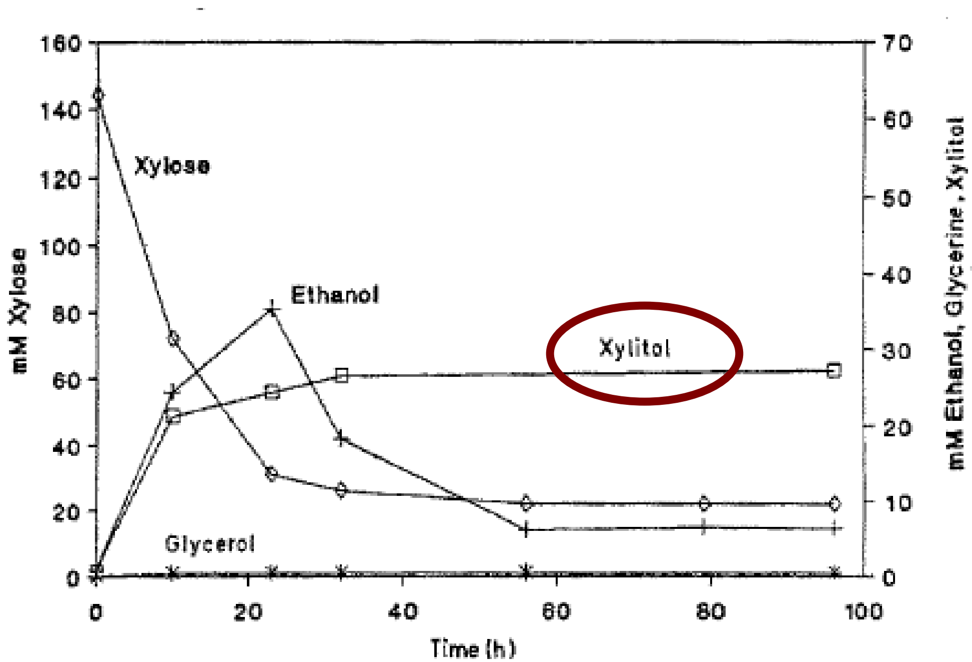
Appl Microbiol Biotechnol (1993) 38:776–783

Xylose fermentation by *Saccharomyces cerevisiae*

Peter Kötter*, Michael Ciriacy

Institut für Mikrobiologie, Heinrich-Heine-Universität, Universitätsstrasse 1, W-4000 Düsseldorf 1, Federal Republic of Germany

- Cloning of *Pichia stipitis* structural genes for XR and XDH in *S. cerevisiae*
- Slow (aerobic) growth on xylose
- Ethanol production accompanied by extensive production of **xylitol**



Xylose Isomerase from *Piromyces* in *S. cerevisiae*

Research at Nijmegen University, The Netherlands:

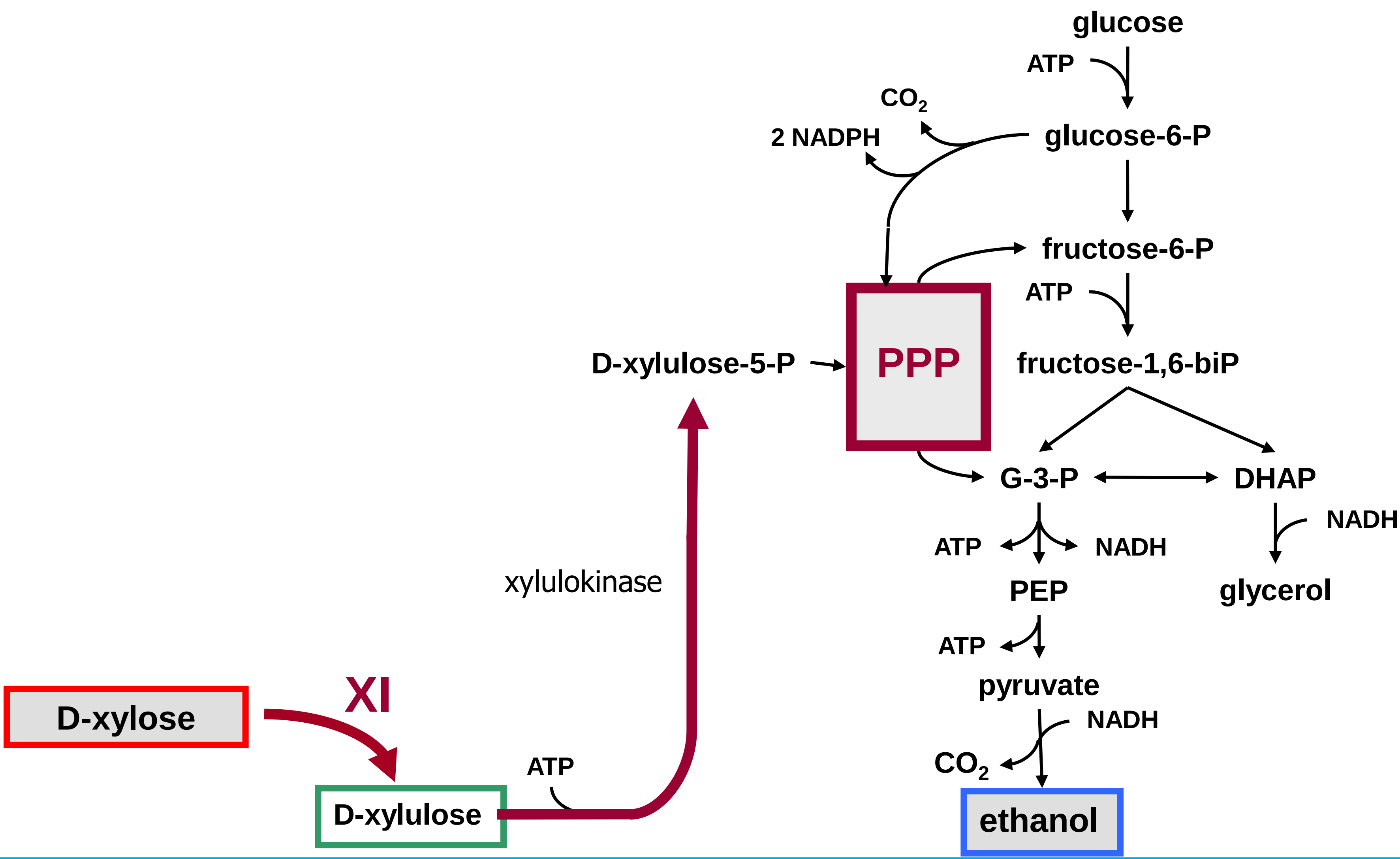
- Anaerobic *Piromyces* fungus from elephant dung
- Isolation of first fungal xylose isomerase gene (XylA)

Research at Delft University of Technology, The Netherlands:

- Overexpression of XylA in *S. cerevisiae*
- Overexpression of PPP (*XKS1, RKI1, RPE1, TKL1 &TAL1*)
- Deletion of aspecific aldose reductase (*GRE3*)

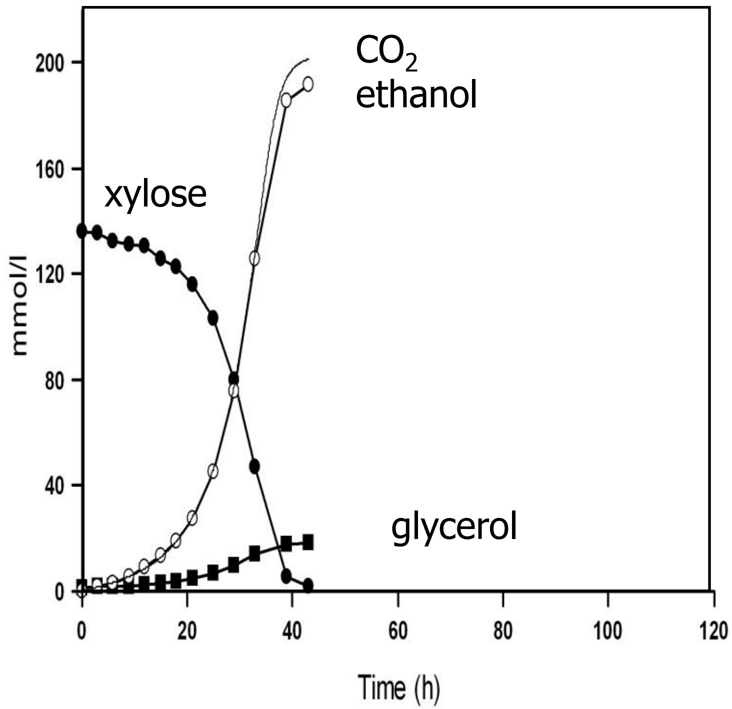
Harhangi *et al*. 2003
Arch. Microbiol. **180**:134

Biochemistry of xylose metabolism in *S. cerevisiae*



Xylose Fermentation by Engineered *S. cerevisiae*

XylA + *XKS1*↑ *TAL1*↑ *TKL1*↑ *RPE1*↑ *RKI1*↑ *gre3Δ*



(xylitol)

µ xylose anaerobic (h^{-1})	0.10
Ethanol yield ($g.g^{-1}$)	0.42
Xylitol yield ($g.g^{-1}$)	< 0.01

Anaerobic batch cultivation of the XI-expressing, metabolically engineered *S. cerevisiae* strain RWB 217 on xylose

Kuyper *et al*. 2005
FEMS Yeast Research **5**:399-409

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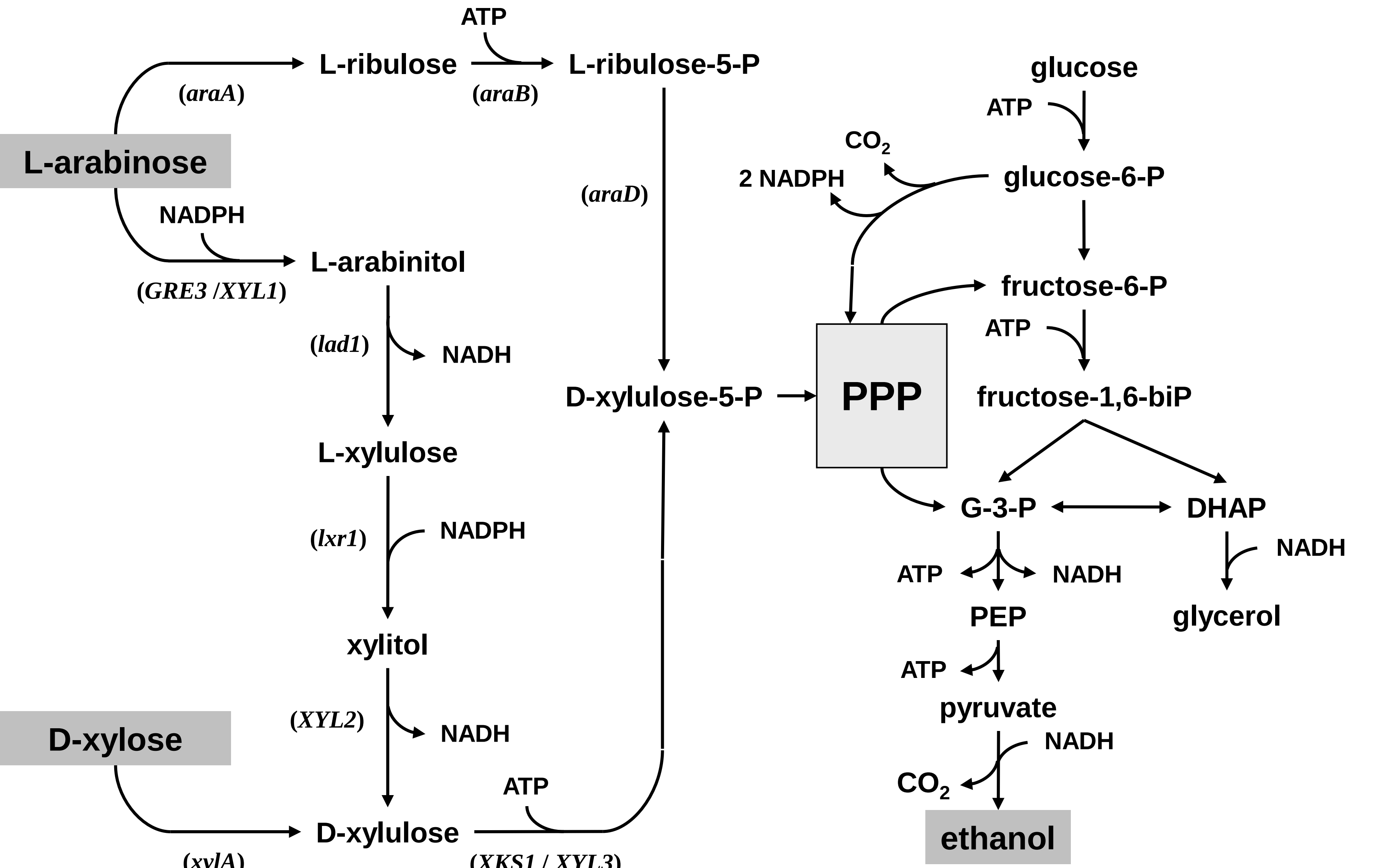
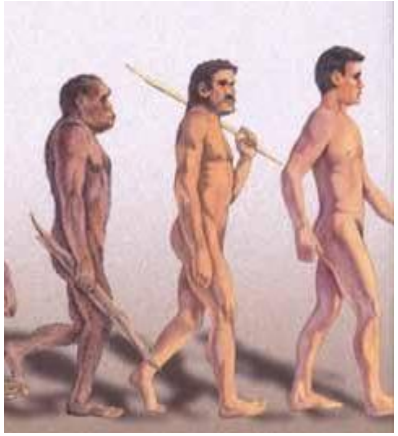
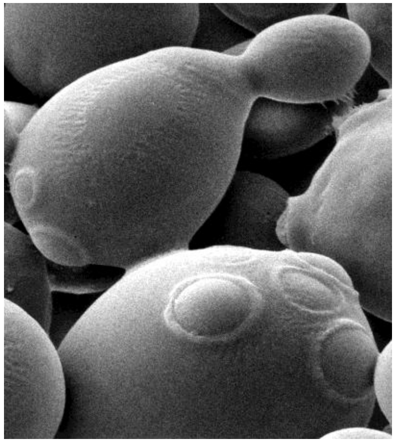
Pathways for L-arabinose fermentation



Further Improvements: 'Evolution in the lab'



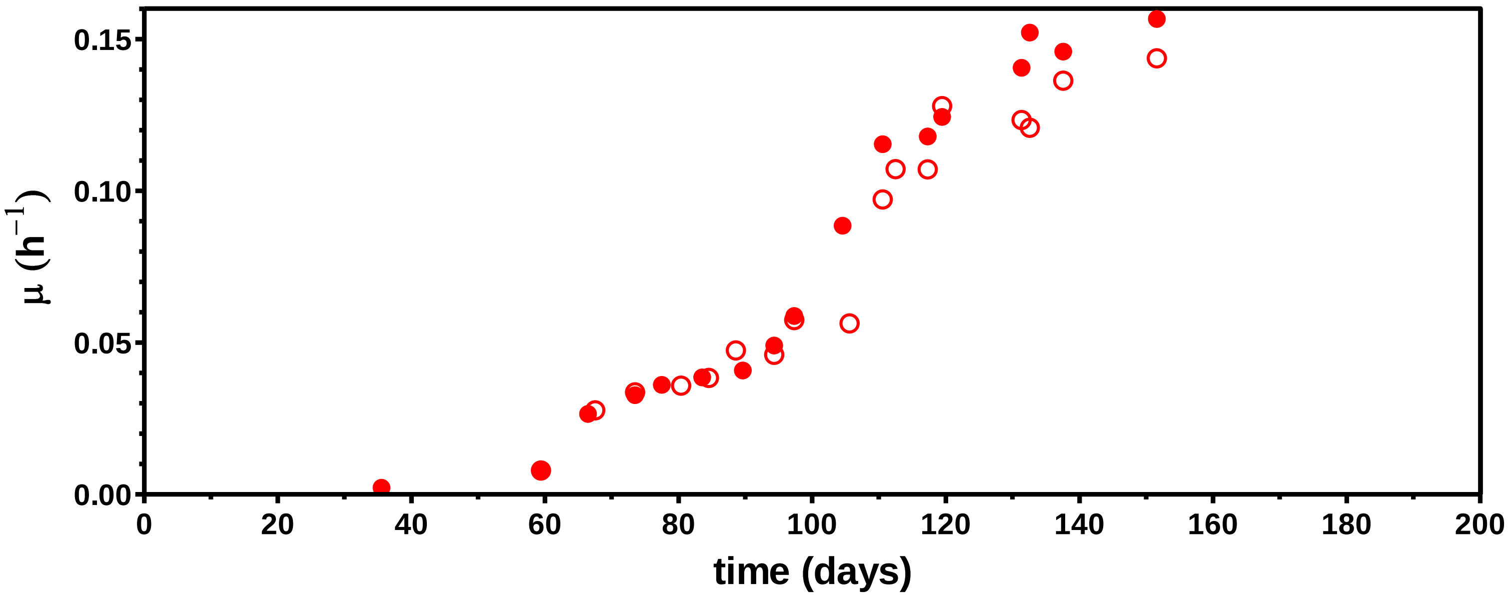
Generationtime
~ 20 years



Generationtime
~ 2 hour

evolutionary engineering

Evolutionary engineering for growth on L-arabinose

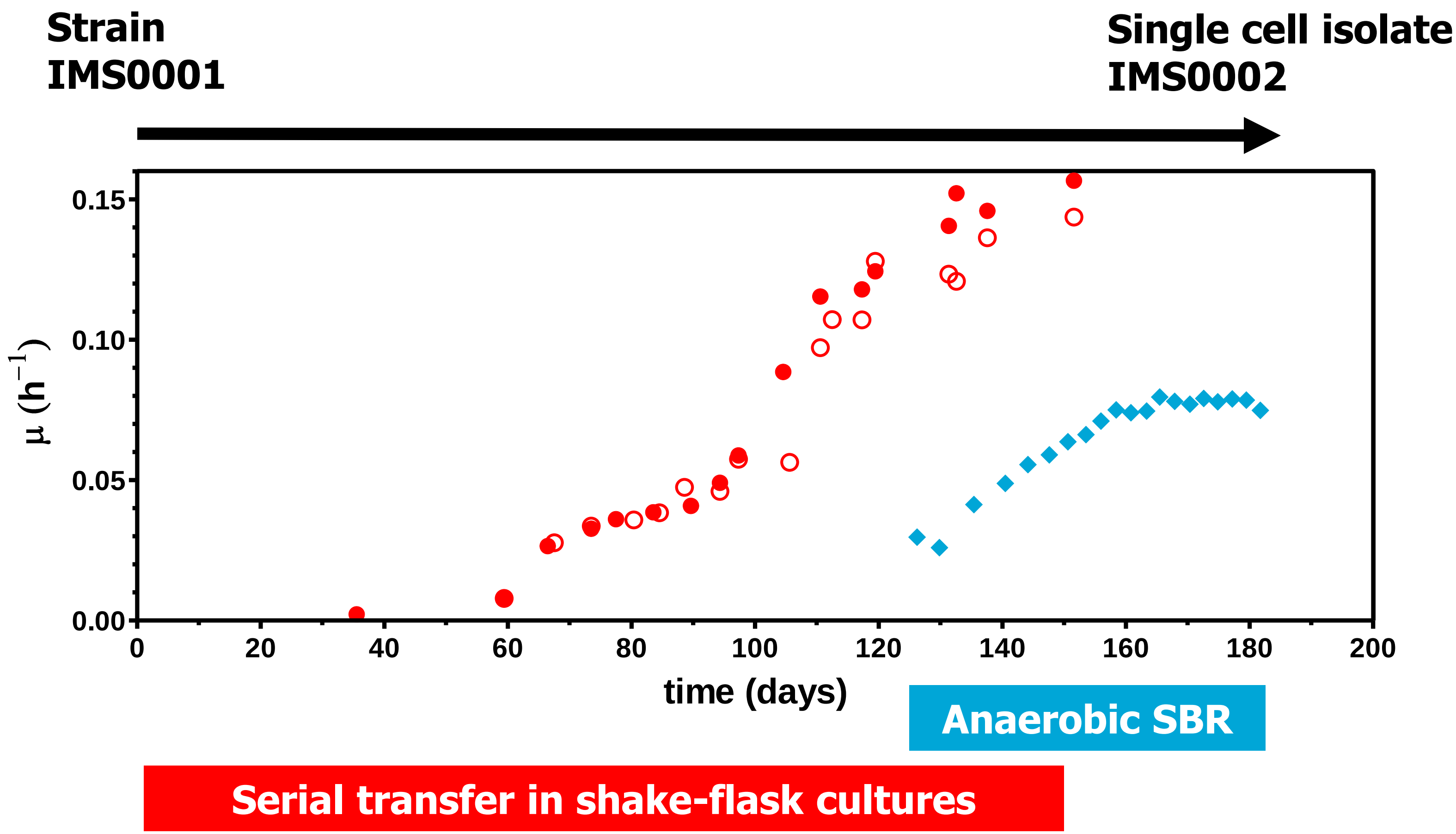


Serial transfer in shake-flask cultures

serial transfers in synthetic medium with 2% (w/v) arabinose

Wouter Wisselink *et al*. 2007

Evolutionary engineering for growth on L-arabinose



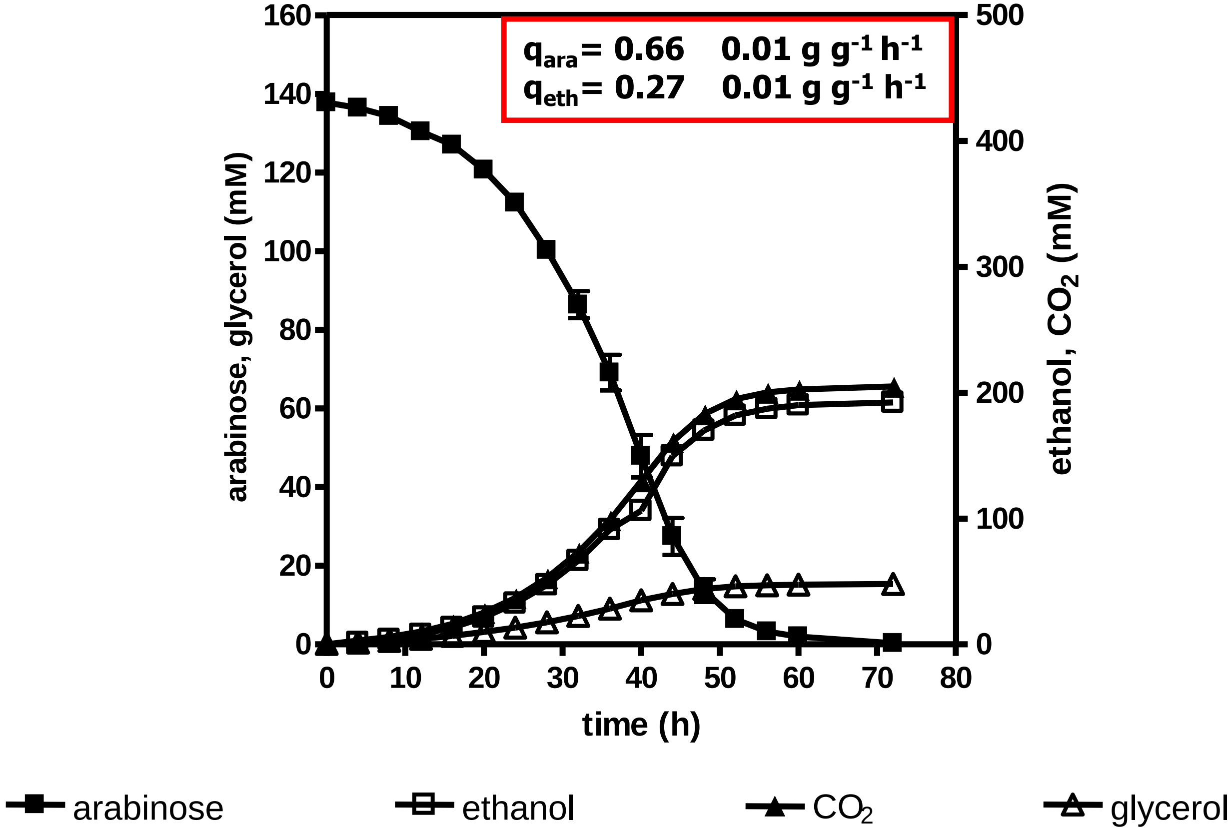
serial transfers in synthetic medium with 2% (w/v) arabinose

B-Basic
Bio-based Sustainable Industrial Chemistry

Wouter Wisselink *et al*. 2007

TU Delft

Successful metabolic and evolutionary engineering for arabinose fermentation



Wouter Wisselink *et al.* 2007
Appl. Environ. Microbiol.

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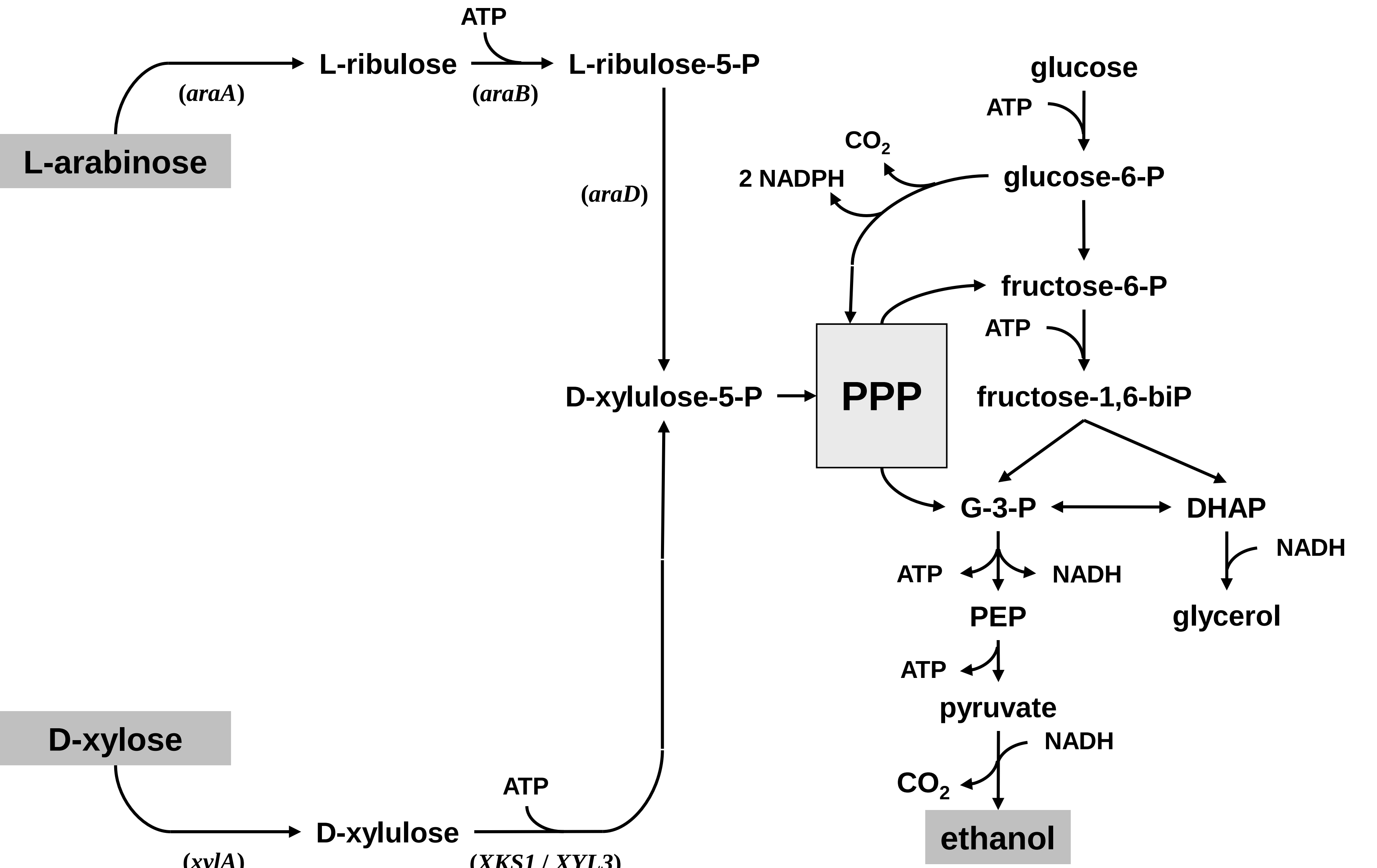
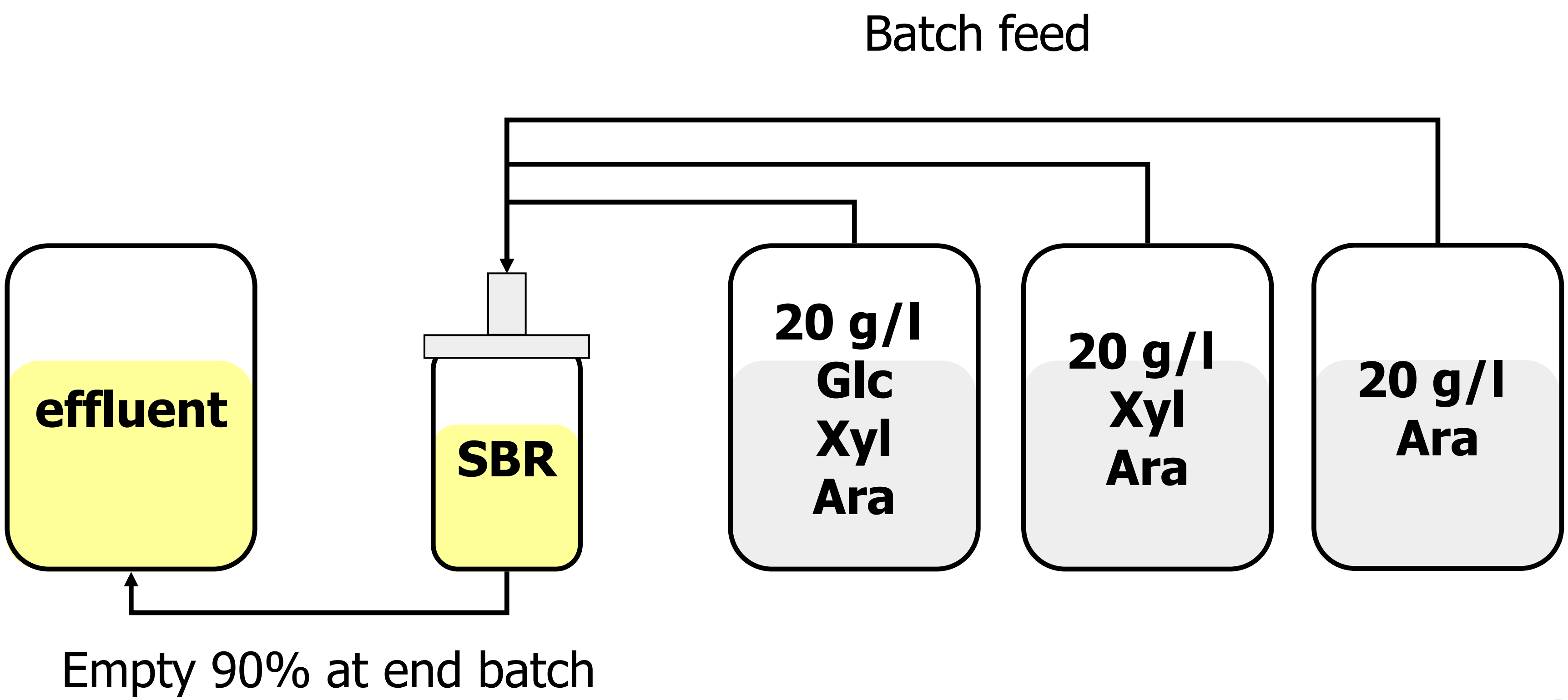
uronic acids	3.2	2.2	2.2
Other (%)			
lignin	17.7	16.9	23.1

Sugar mixtures: glucose, xylose and arabinose



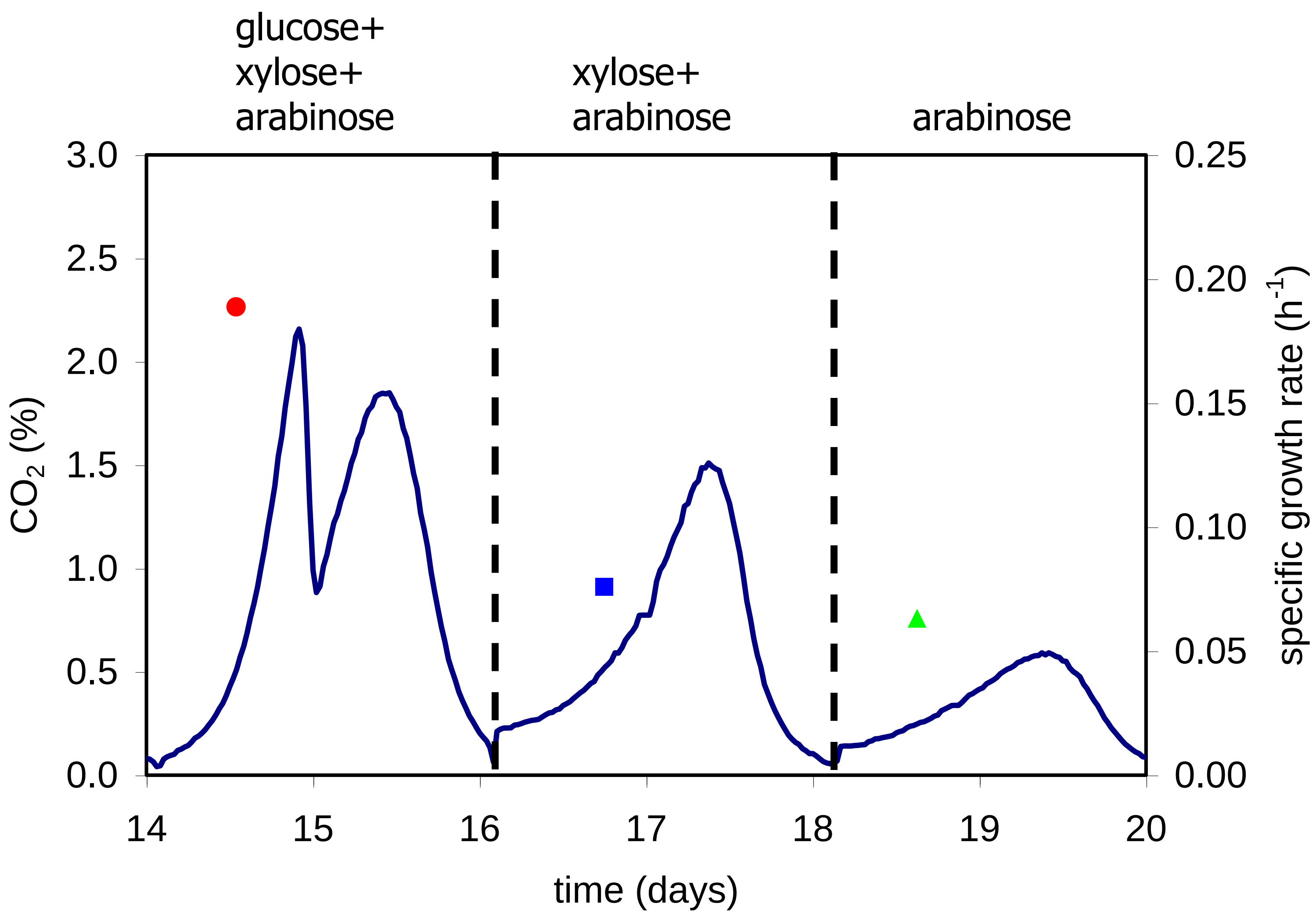
Evolution with Sequential Batch Reactor

designed to increase the generations on arabinose



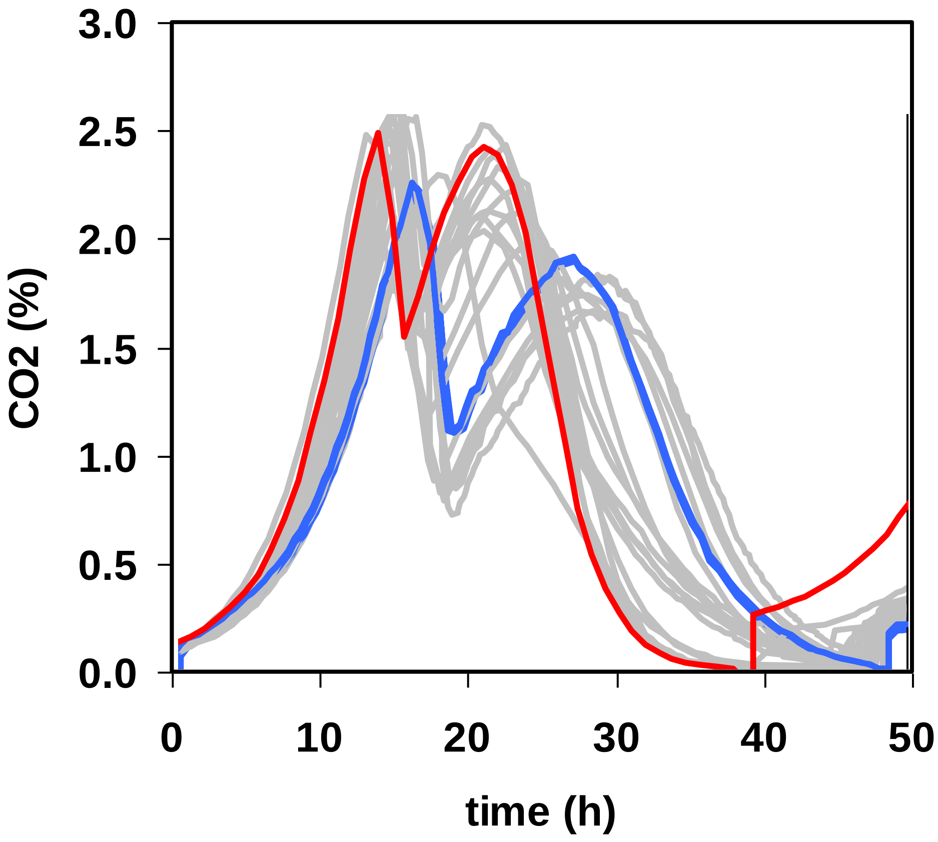
Wouter Wisselink *et al.* 2009
Appl. Environ. Microbiol.

CO_2 profile



Wouter Wisselink *et al*. 2009
Appl. Environ. Microbiol.

CO_2 profiles glucose-xylose-arabinose (20 cycles)



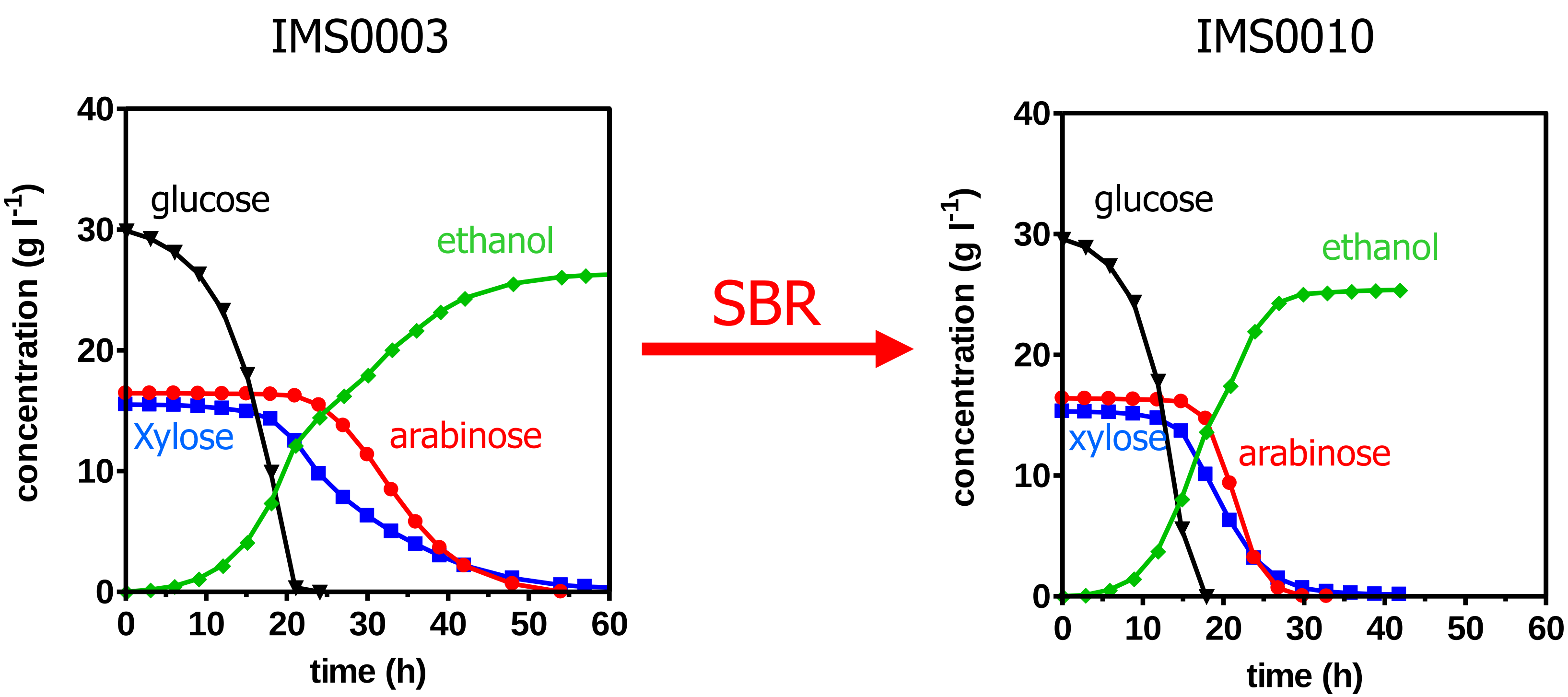
Wouter Wisselink *et al*. 2009
Appl. Environ. Microbiol.

B-Basic
Bio-based Sustainable Industrial Chemistry

TU Delft

Evolutionary engineering

consecutive batch cultivation in different sugar mixtures



- Increased specific consumption rates of xylose and arabinose → decreased total fermentation time

Does it also work in the real world?

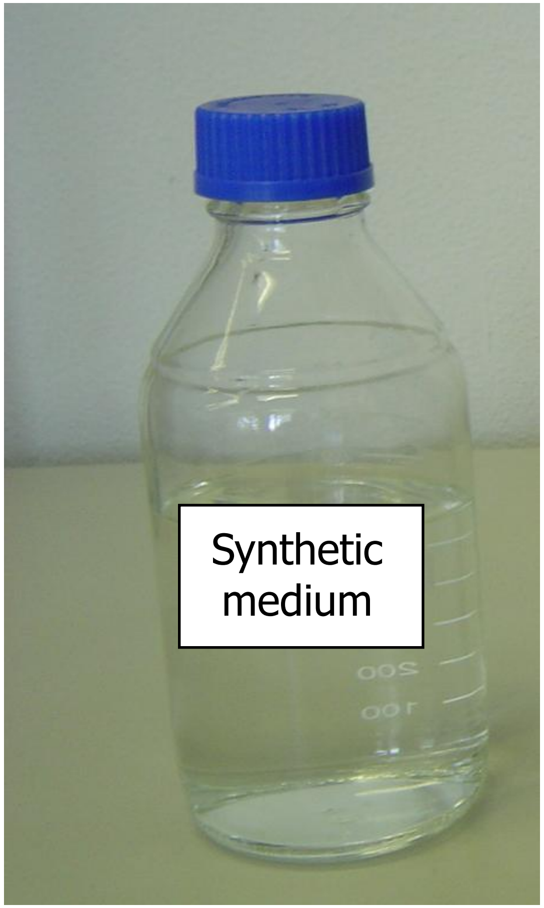
DSM background information

DSM, Corporate Communications
P.O. Box 6500, 6401 JH Heerlen, The Netherlands
Telephone (31) 45 5782421, Telefax (31) 45 5740680
Internet: www.dsm.com
E-mail: media.relations@dsm.com

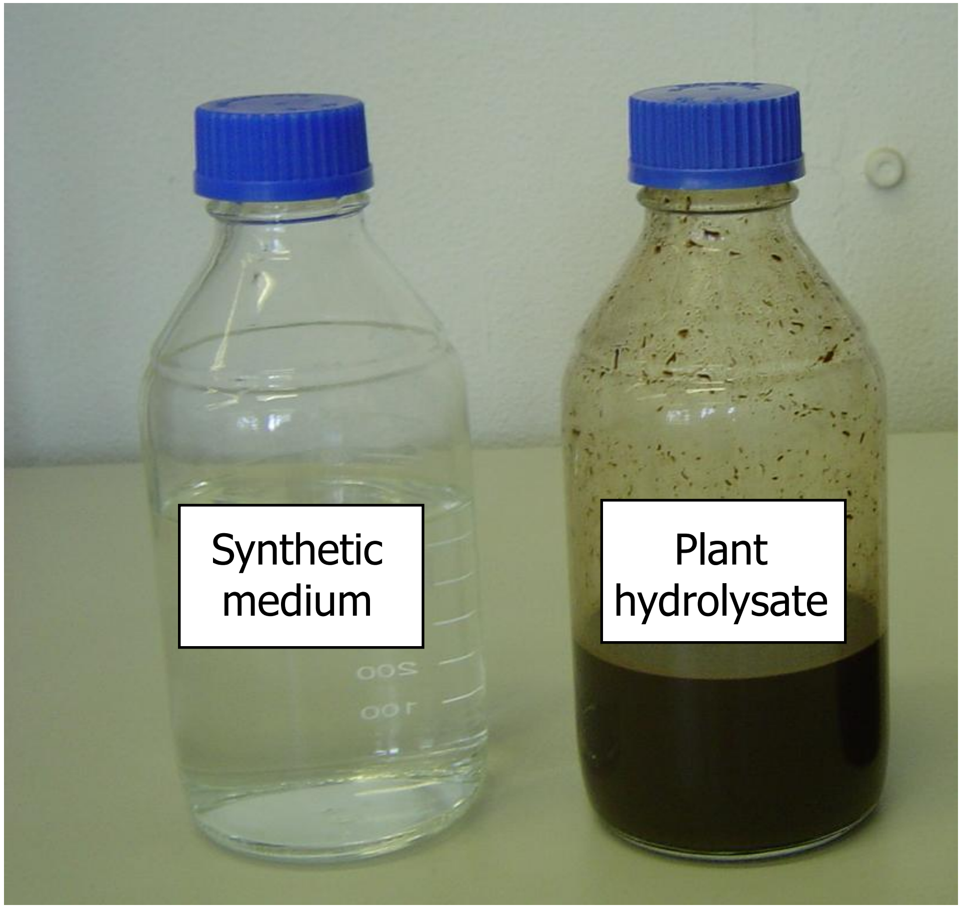
'All you can eat yeast'

A breakthrough in bioconversion technology is bringing second generation bio fuels a major step closer to commercial reality

From the Lab…

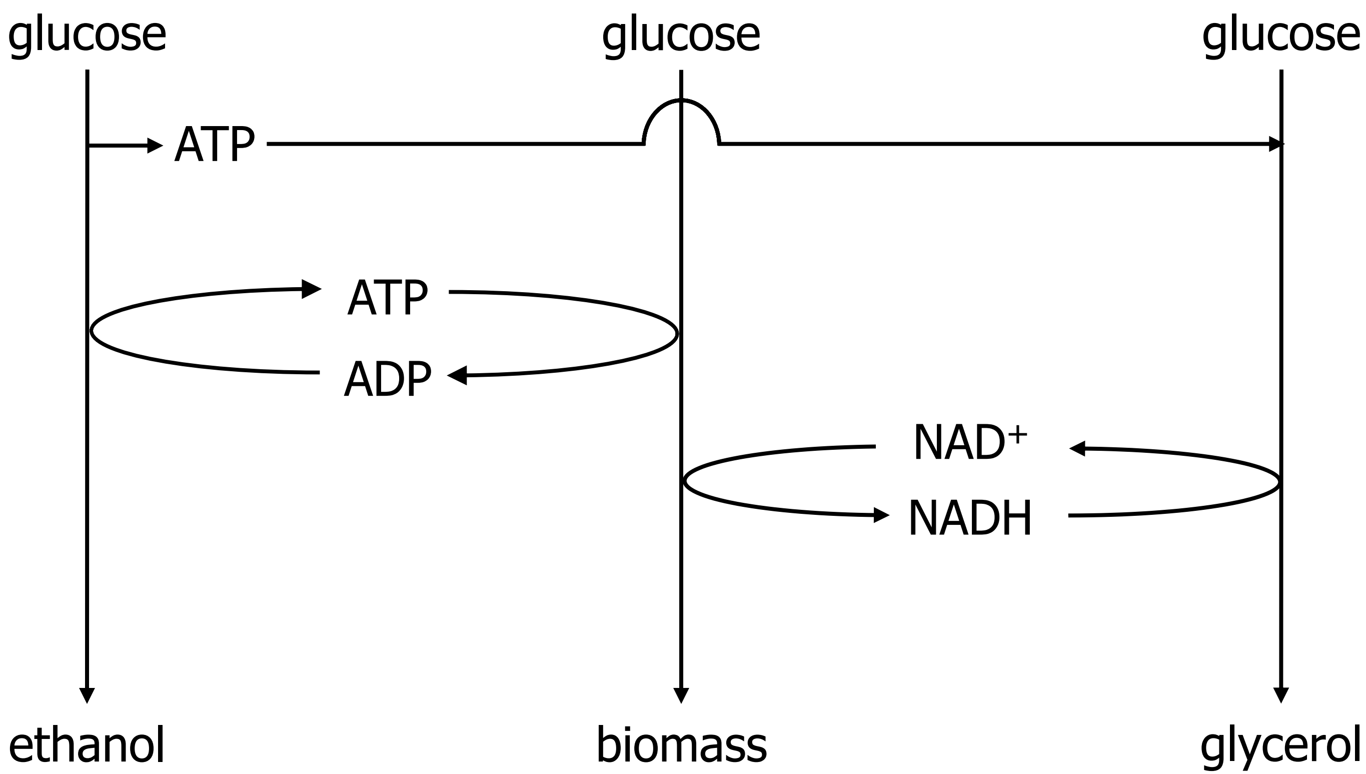


From the Lab to the Real World



Amongst others: acetic acid

How to turn a nuisance into a benefit?



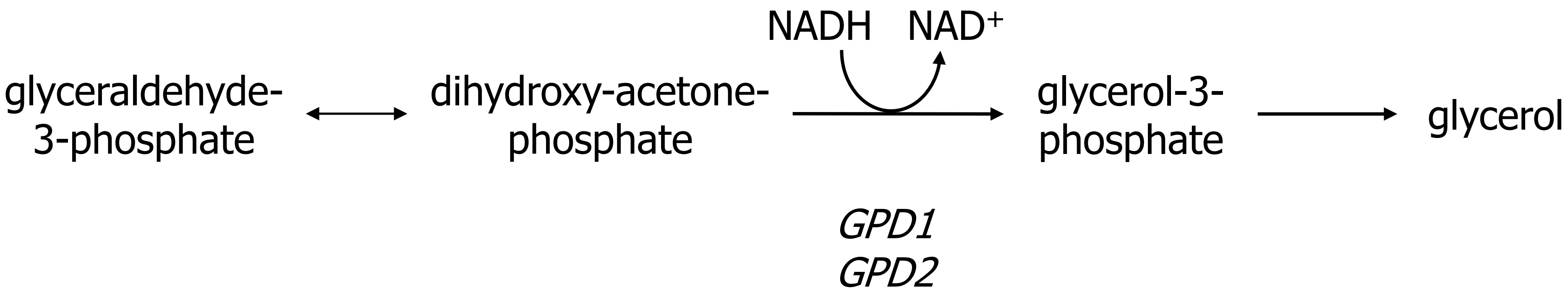
Guadalupe et al. 2009
Appl. Environ. Microbiol.

Glycerol formation

glyceraldehyde-3-phosphate ⟷ dihydroxy-acetone-phosphate —(NADH → NAD^+)→ glycerol-3-phosphate → glycerol

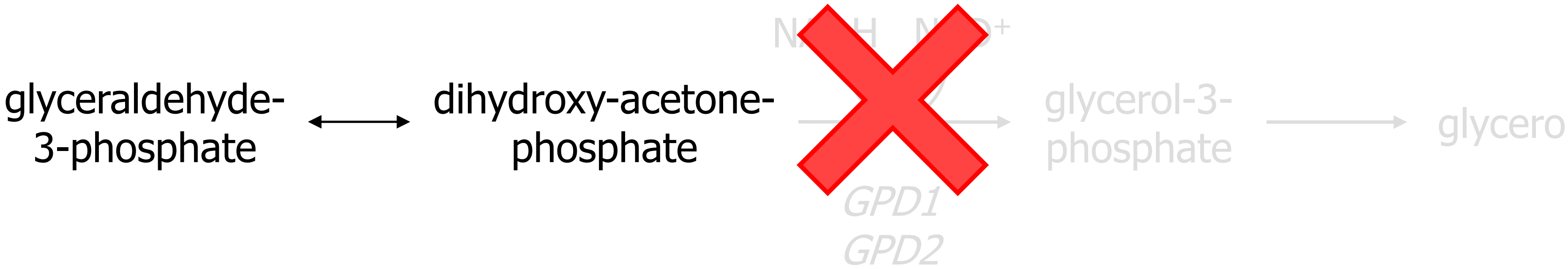
Guadalupe et al. 2009
Appl. Environ. Microbiol.

Glycerol formation

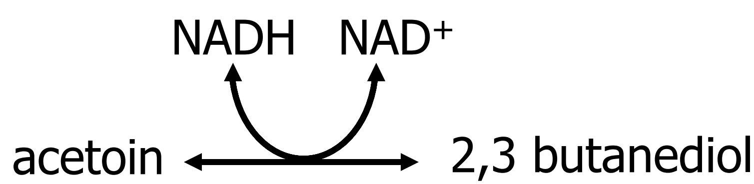


Guadalupe et al. 2009
Appl. Environ. Microbiol.

Glycerol formation



- Anaerobic growth only possible in presence of an **external** electron acceptor.



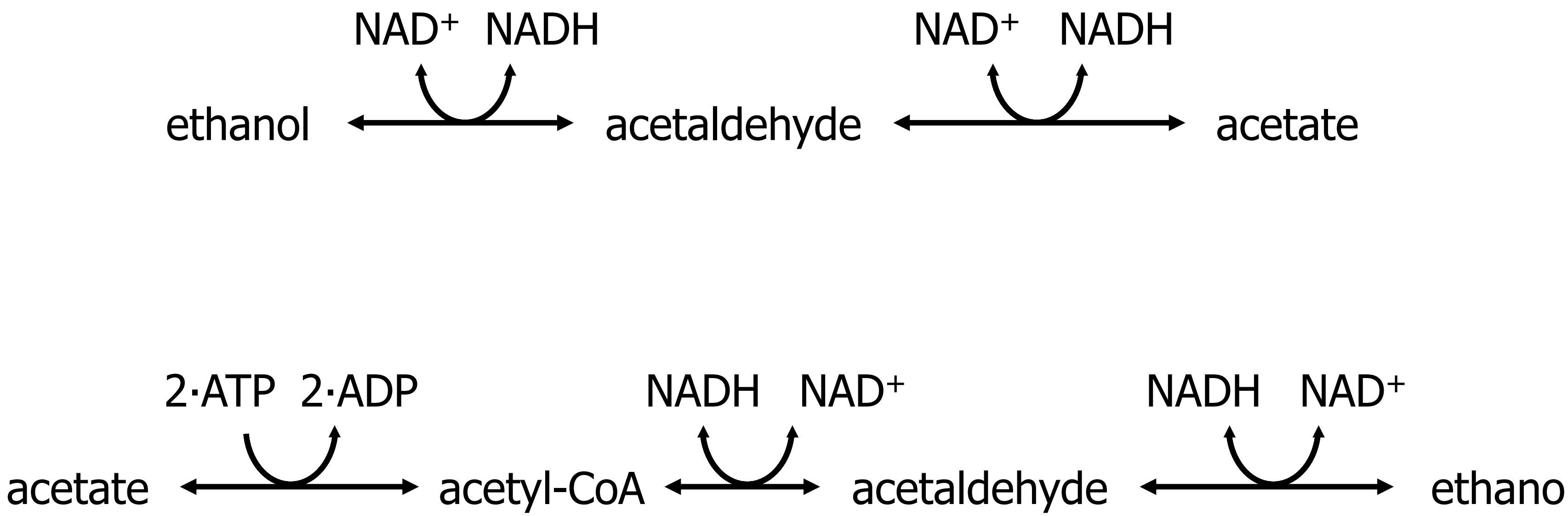
Normal yeast acetate metabolism

Guadalupe et al. 2009
Appl. Environ. Microbiol.

Acetate as electron acceptor?

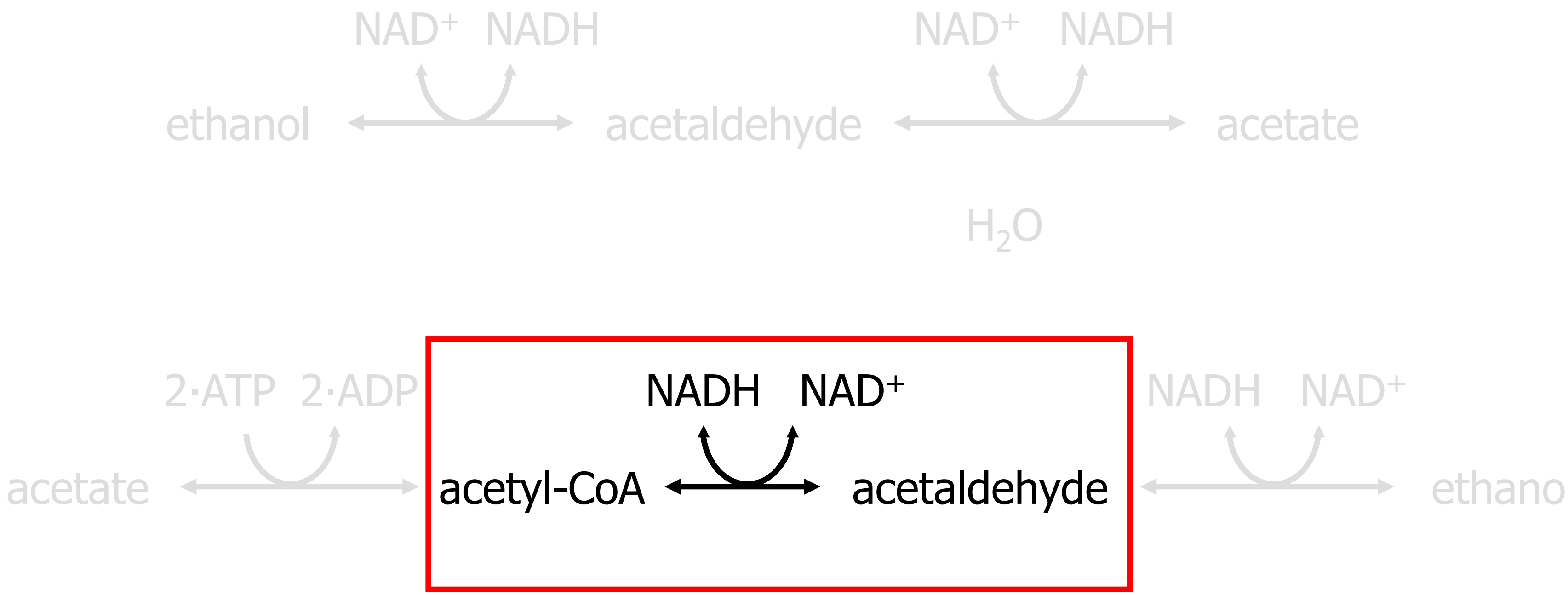
Guadalupe et al. 2009
Appl. Environ. Microbiol.

Acetate as electron acceptor?



Guadalupe et al. 2009
Appl. Environ. Microbiol.

Acetate as electron acceptor?

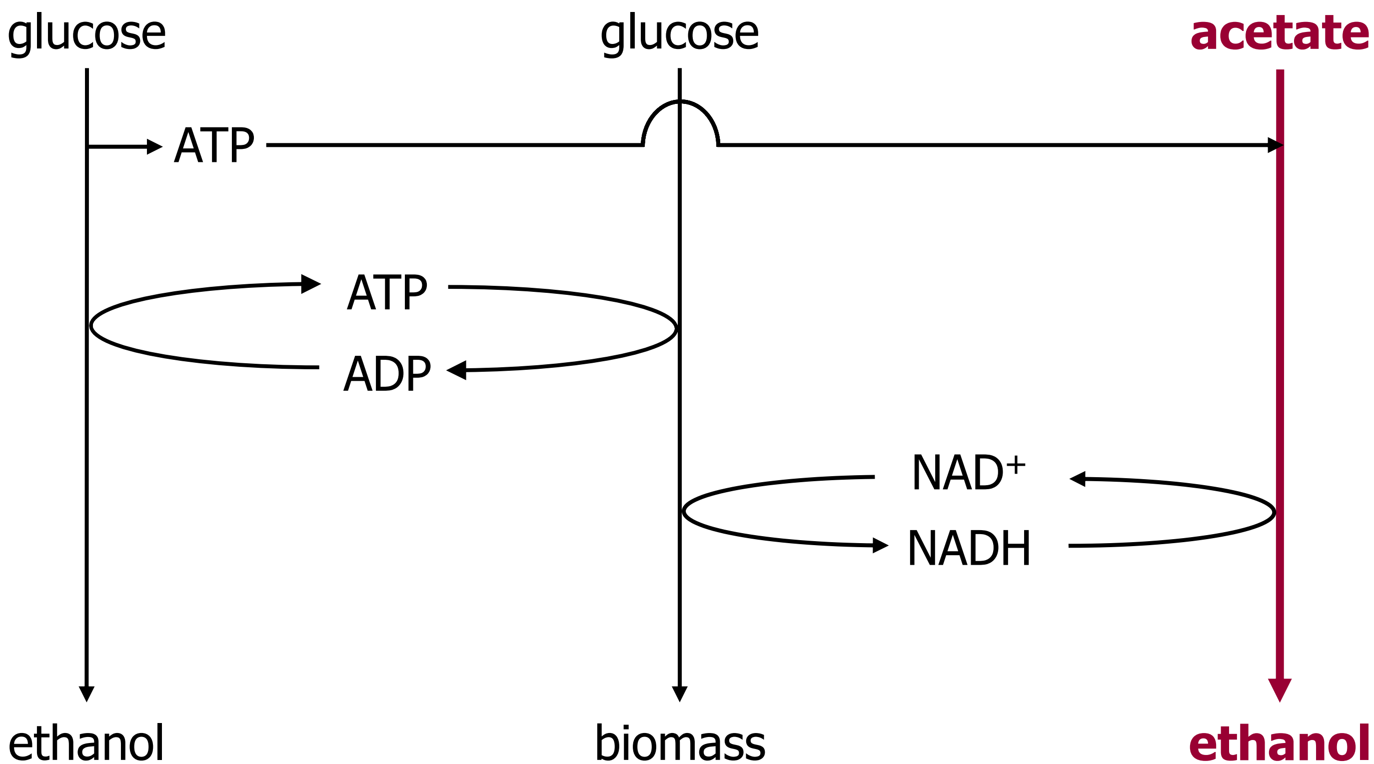


NAD$^+$ dependent (acetylating) acetaldehyde dehydrogenase

EC 1.2.1.10

Guadalupe et al. 2009
Appl. Environ. Microbiol.

Acetate as electron acceptor?



Guadalupe et al. 2009
Appl. Environ. Microbiol.

Strain construction

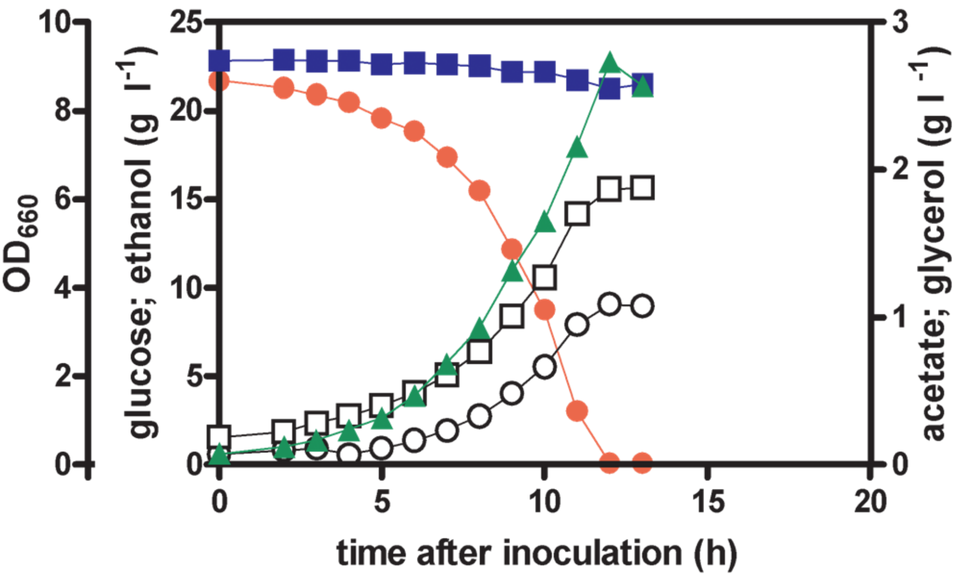
- Enzyme activities

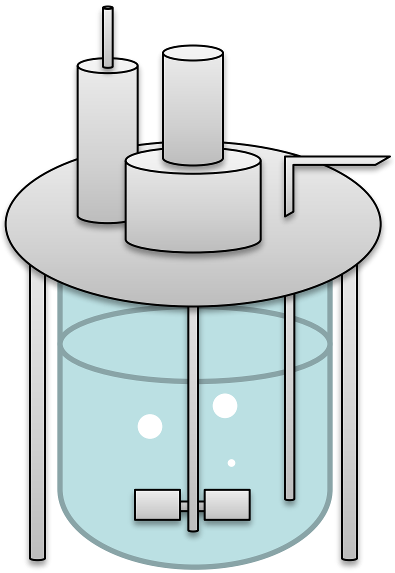
Yeast strain	IME076	IMZ132
Relevant genotype	*GPD1 GPD2*	*gpd1Δ gpd2Δ + mhpF*
Glycerol 3-phosphate dehydrogenase (μmol mg $protein^{-1}$ min^{-1})	0.034 ± 0.003	< 0.002
Acetaldehyde dehydrogenase (acetylating) (μmol mg $protein^{-1}$ min^{-1})	< 0.002	0.020 ± 0.004

Guadalupe et al. 2009
Appl. Environ. Microbiol.

Strain characterization in Batch

GPD1 GPD2





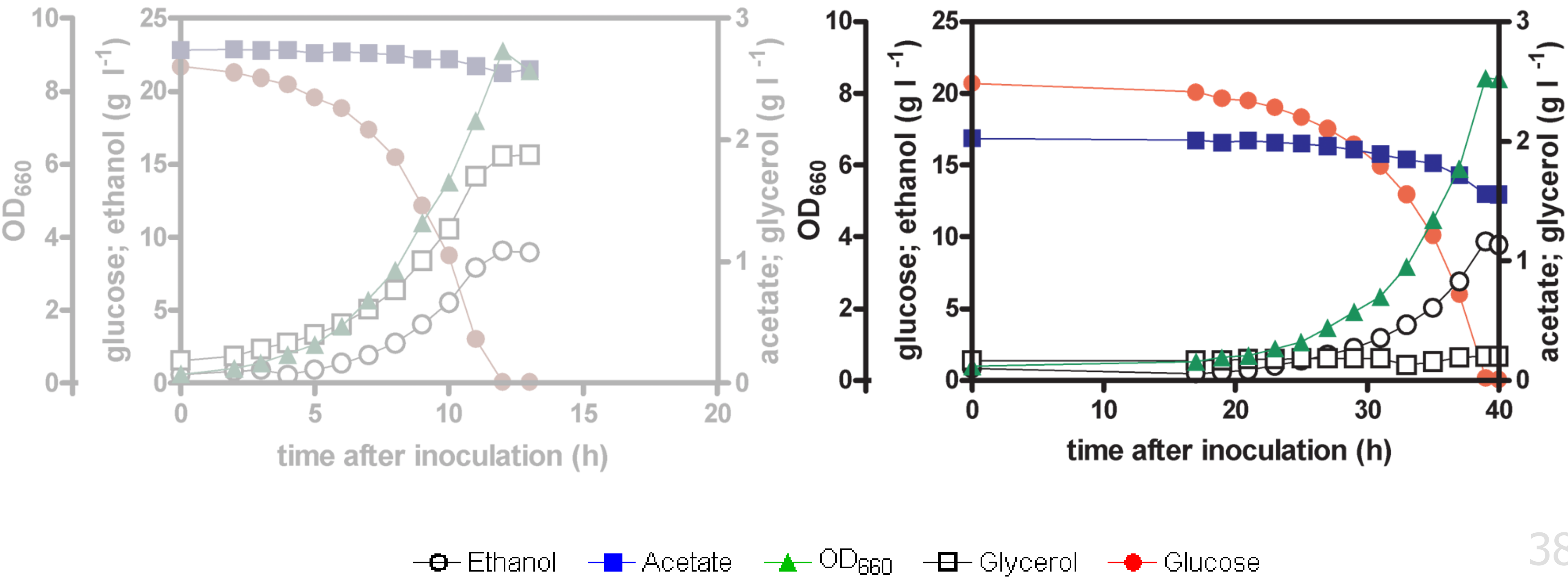
Ethanol Acetate OD_{660} Glycerol Glucose

Guadalupe et al. 2009
Appl. Environ. Microbiol.

Strain characterization in Batch

GPD1 GPD2

gpd1 Δ gpd2Δ + mhpF



Guadalupe et al. 2009
Appl. Environ. Microbiol.

Conclusions

- **Powerful combination in metabolic and evolutionary engineering**
- **Tailor-made evolution strategies are applicable to many other combinations of strains and substrate mixtures**
- **Reverse engineering phenotypes as important challenge**

Acknowledgements

Koninklijke Nedalco

BIRD ENGINEERING

stw

TATE & LYLE
CONSISTENTLY FIRST IN RENEWABLE INGREDIENTS

DSM

Netherlands Genomics Initiative

SenterNovem

Kluyver CENTRE | Kluyver Centre for Genomics of Industrial Fermentation

BE-Basic

http://www.be-basic.org/

Contributing Former IMB

Eleonora Bellissimi
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Marko Kuyper
Jeremiah Wright

Industrial Microbiology Delft

Marinka Almering
Thiago Basso
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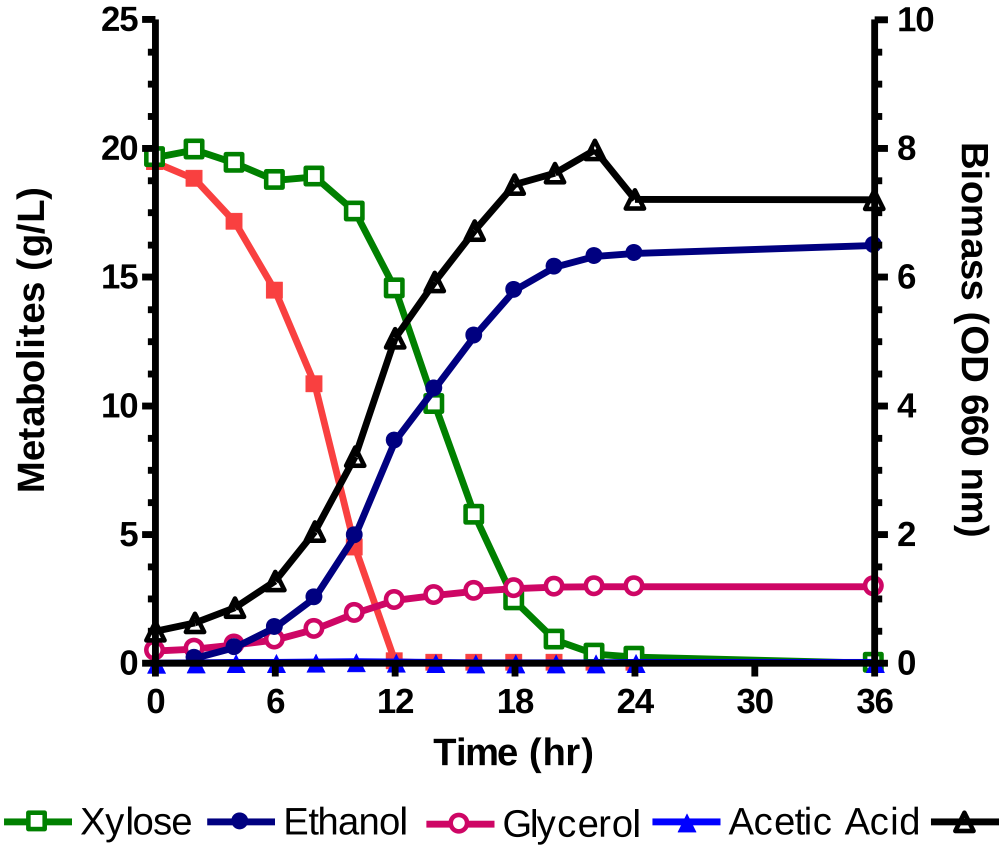
Irina Bolat
Lizanne Bosman
Marcel van den Broek
Barbara Crimi
Jean-Marc Daran
Pascale Daran-Lapujade
Victor Guadalupe
Marit Heblij
Eline Huisjes
Erik de Hulster
Yulia Ilina
Stefan de Kok
Frank Koopman
Barbara Kozak
Niels Kuijpers
Marijke Luttik
Filipa Mendes
Bart Oud
Jack Pronk
Daniel Ramos
Gabriele Romagnoli
Daniel Solis Escalante
Tânia Veiga
Han de Winde
Wouter Wisselink
Rintze Zelle

?

Low pH as such is not a problem for xylose-fermenting *S. cerevisiae*….



Fermentation of glucose-xylose mixture by *S. cerevisiae* RWB218

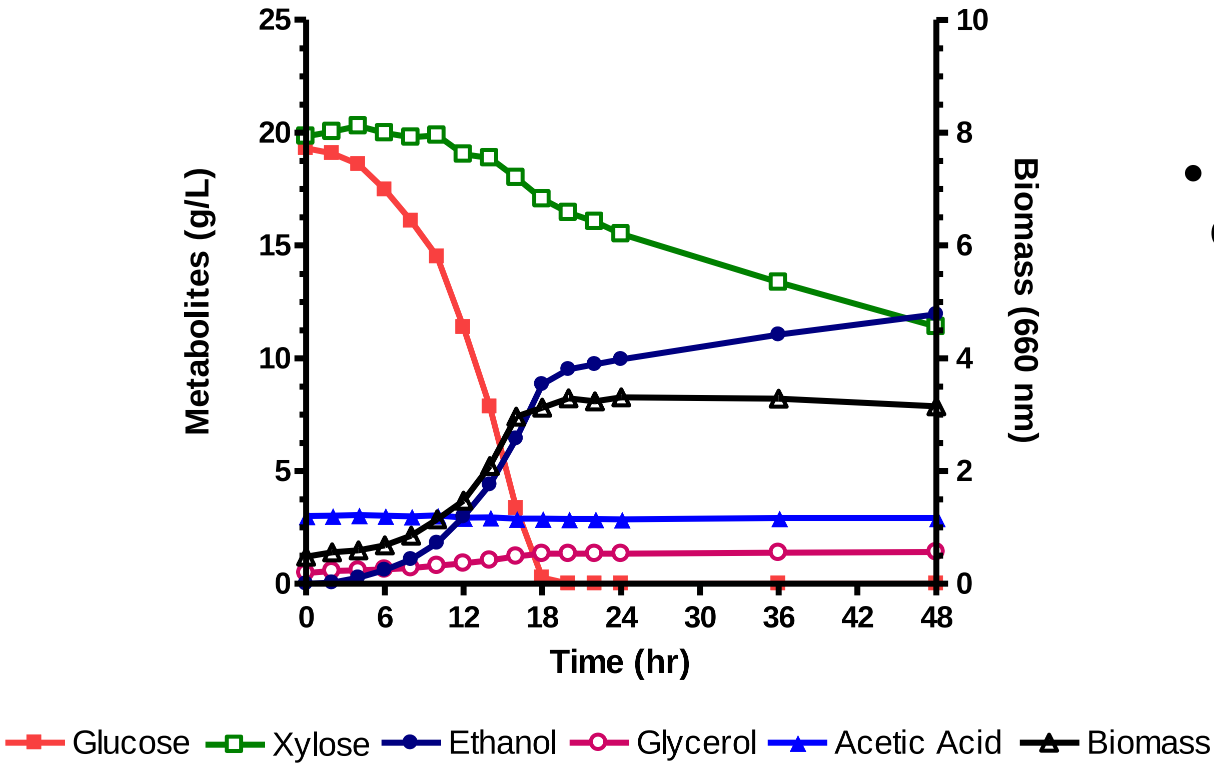
pH 3.5

Anaerobic batch culture, synthetic medium, 20 g.l^{-1} glucose and 20 g.l^{-1} xylose

Bellissimi et al. 2006
unpublished

TU Delft

....but acetic acid specifically inhibits xylose fermentation



- ethanol **yield** on xylose not affected

Fermentation of glucose-xylose mixture by *S. cerevisiae* RWB218

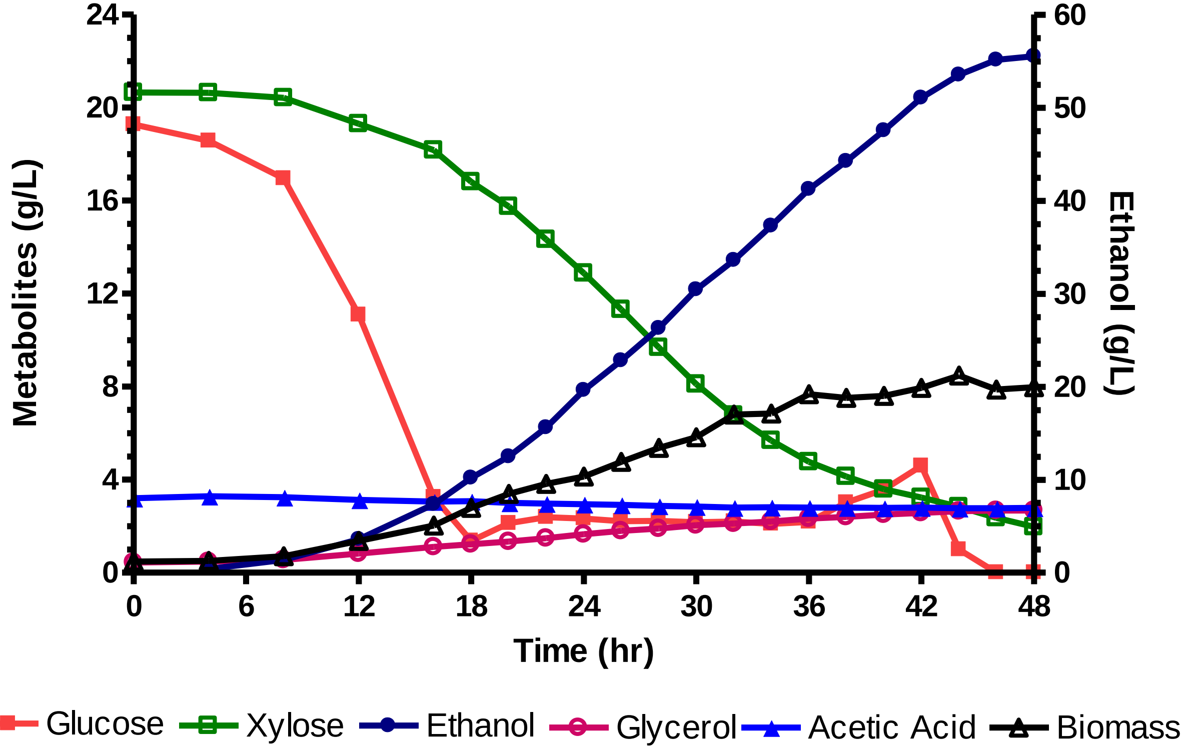
pH 3.5, 3 $g.l^{-1}$ acetic acid

Anaerobic batch culture, synthetic medium, 20 $g.l^{-1}$ glucose and 20 $g.l^{-1}$ xylose

Bellissimi et al. 2006 unpublished

TU Delft

Glucose co-feeding alleviates acetic acid inhibition of xylose fermentation (pH 3.5 + 3 $g.l^{-1}$ acetic acid)



Similar to simultaneous saccharification and fermentation (SSF)

Bellissimi et al. 2006
unpublished

TU Delft

The challenge continued: uronic acids?

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Other (%)			
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Grohmann & Bothast 1994
Lee *et al.* 1997
www.ecn.nl

A next challenge for metabolic engineering: pectine

+ Beetpulp

up to 30% pectine (galacturonic acid)

From the Dutch news:

11 June 2008

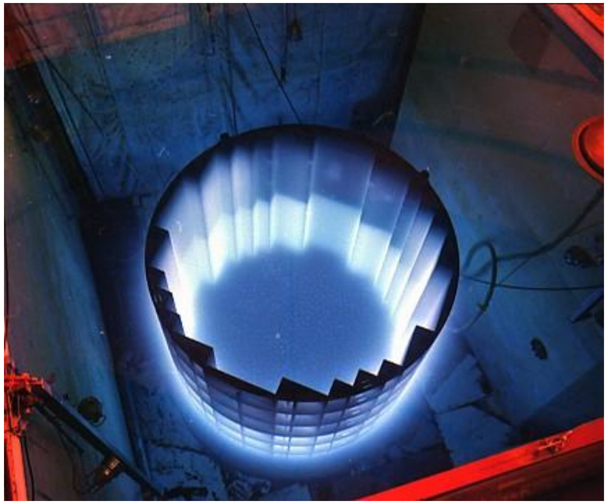
Donkeys back in the limelight in Turkey

Rising fuel prices are not bad for everyone: Donkeys are 'hot' again in Turkey. The market for donkeys is going through a serious surge in activity.

The prices of the animals skyrocketed from roughly 30 euros last year to between 60 and occasionally even 210 euros this year.

Mechanical agricultural equipment, the primary cause for the decline in popularity of the donkey over the past decades, are nowadays only used when totally unavoidable.. Due to the high fuel prices, a ride between the village and the field is much cheaper by donkey.

Alternative energy sources

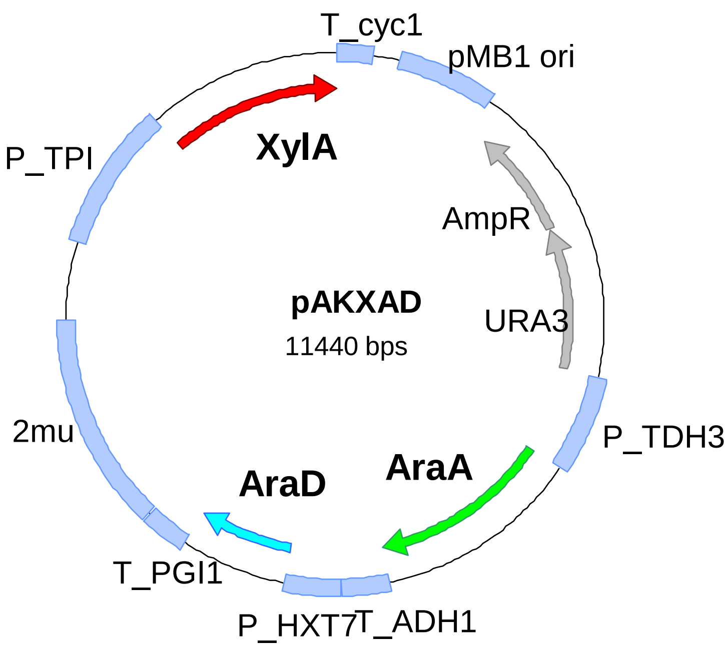


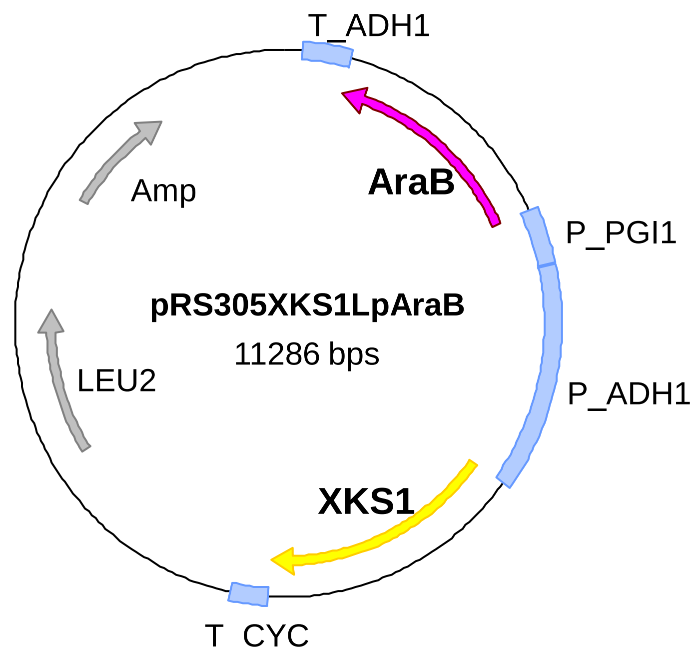
Arabinose fermentation by engineered *S. cerevisiae*

M.E. strategy	Ethanol productivity	
Fungal pathway		
P. stipitis XYL1 + XYL2, T. reesei lad1 + lxr1, S. cerevisiae XKS1	0.35 mg $g^{-1}h^{-1}$	Richard et al. (2003)
Bacterial pathway		
E. coli AraABD	-	Sedlak & Ho (2001)
B. subtilis AraA, E. coli AraBD ***+ evolution***	60-80 mg $g^{-1}h^{-1}$	Becker & Boles (2003)

- Bacterial pathway most promising (Becker & Boles 2003)
- Challenges: rate, anaerobicity, arabinitol formation

Strain construction





- **Host**:
  XylA-expressing *S. cerevisiae* strain optimized for xylose fermentation
- Expression of *AraA*, *AraB* and *AraD* from *Lactobacillus plantarum*

Wouter Wisselink *et al*. 2007

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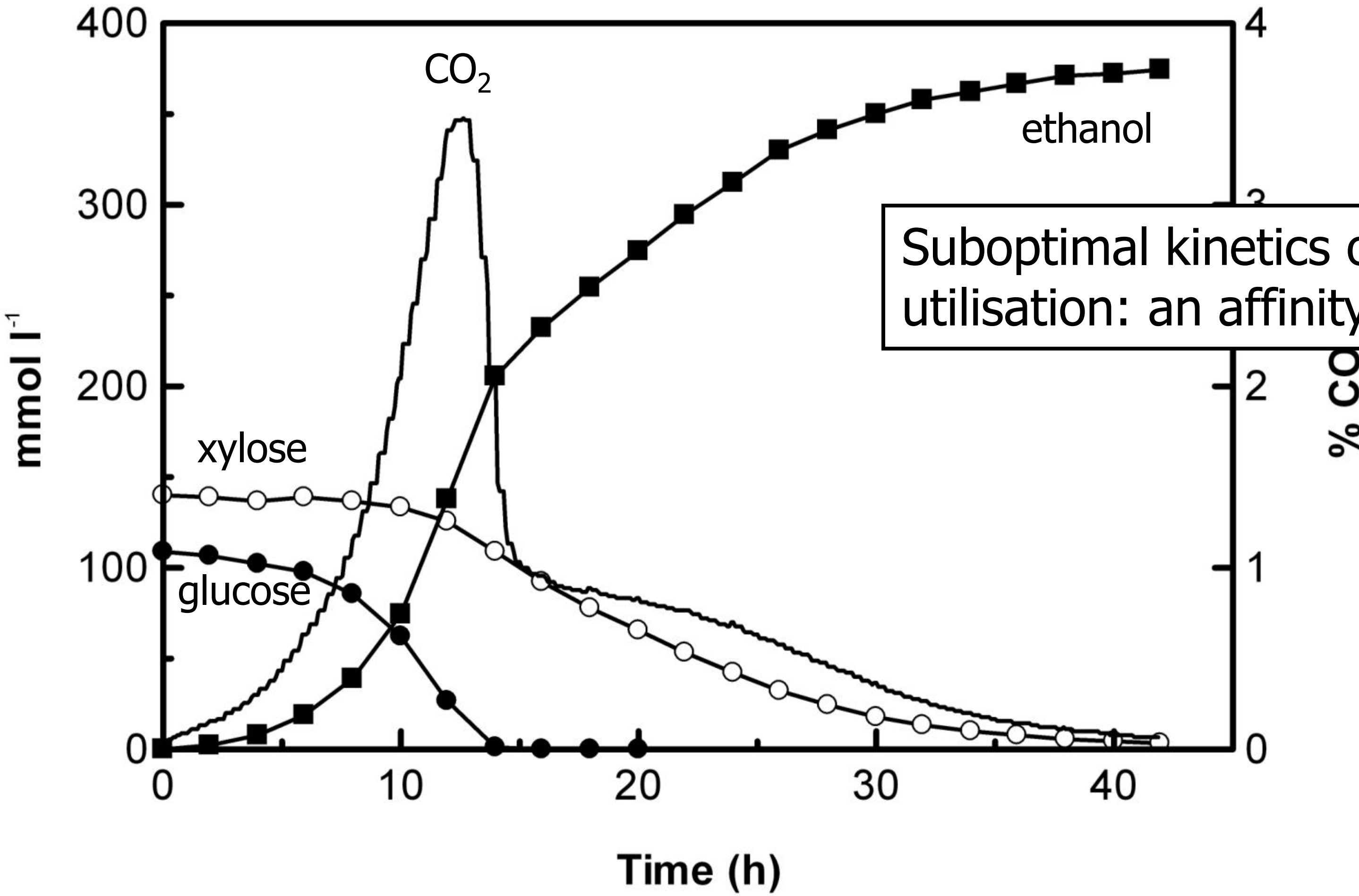
Sugar mixtures: glucose and xylose



Suboptimal kinetics of xylose utilisation: an affinity problem?

Anaerobic growth of *S. cerevisiae* RWB217
on 20 g/L glucose and 20 g/L xylose

Kuyper *et al*. 2005
FEMS Yeast Research **5**:399-409

Evolutionary engineering in chemostat

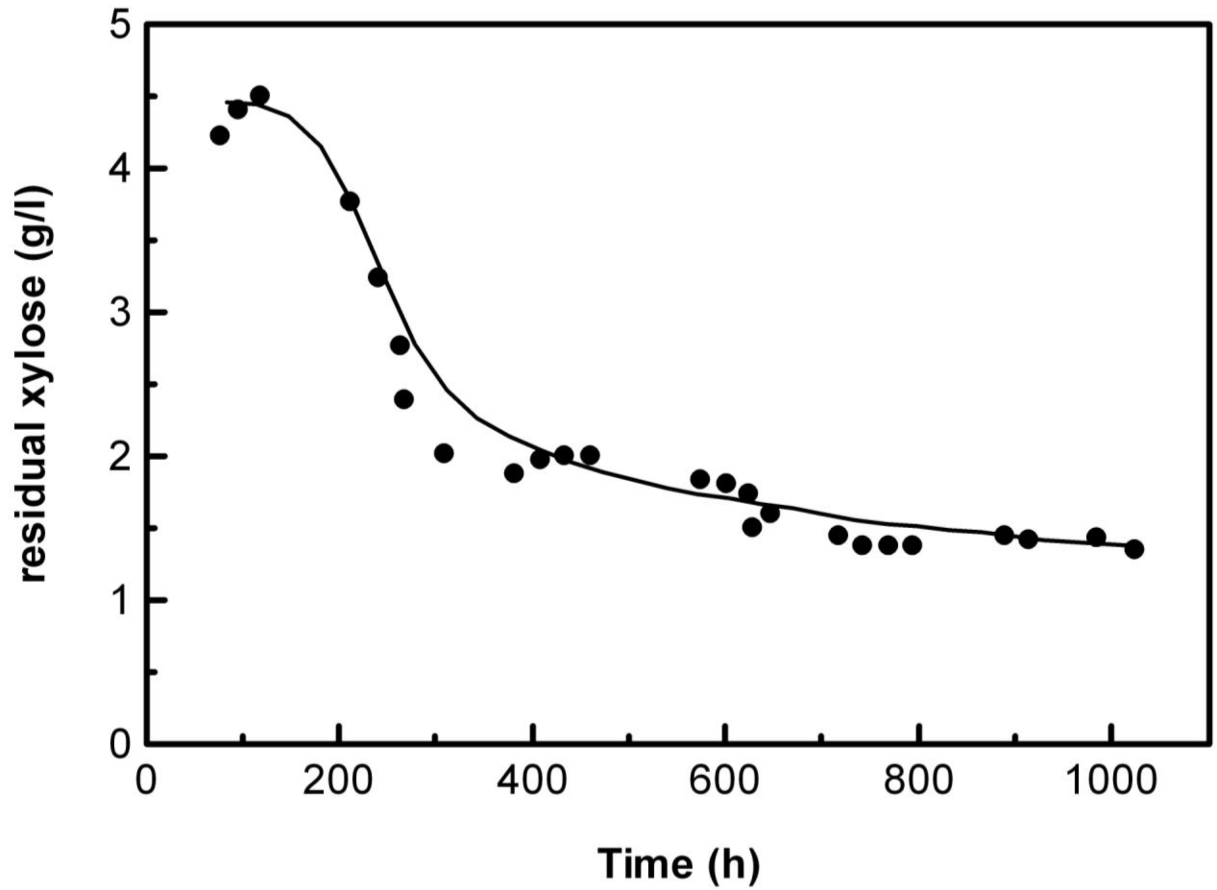
- Selection for improved affinities → decrease of residual sugar
  - Affinity = μ^{max} / K_s

Monod:

$$\mu = \mu_{max} \frac{C_S}{K_S + C_S}$$

  - Selection for mutant that grows faster at a lower C_s
  - Wash-out of strains that cannot grow at the set D with the lowered C_s

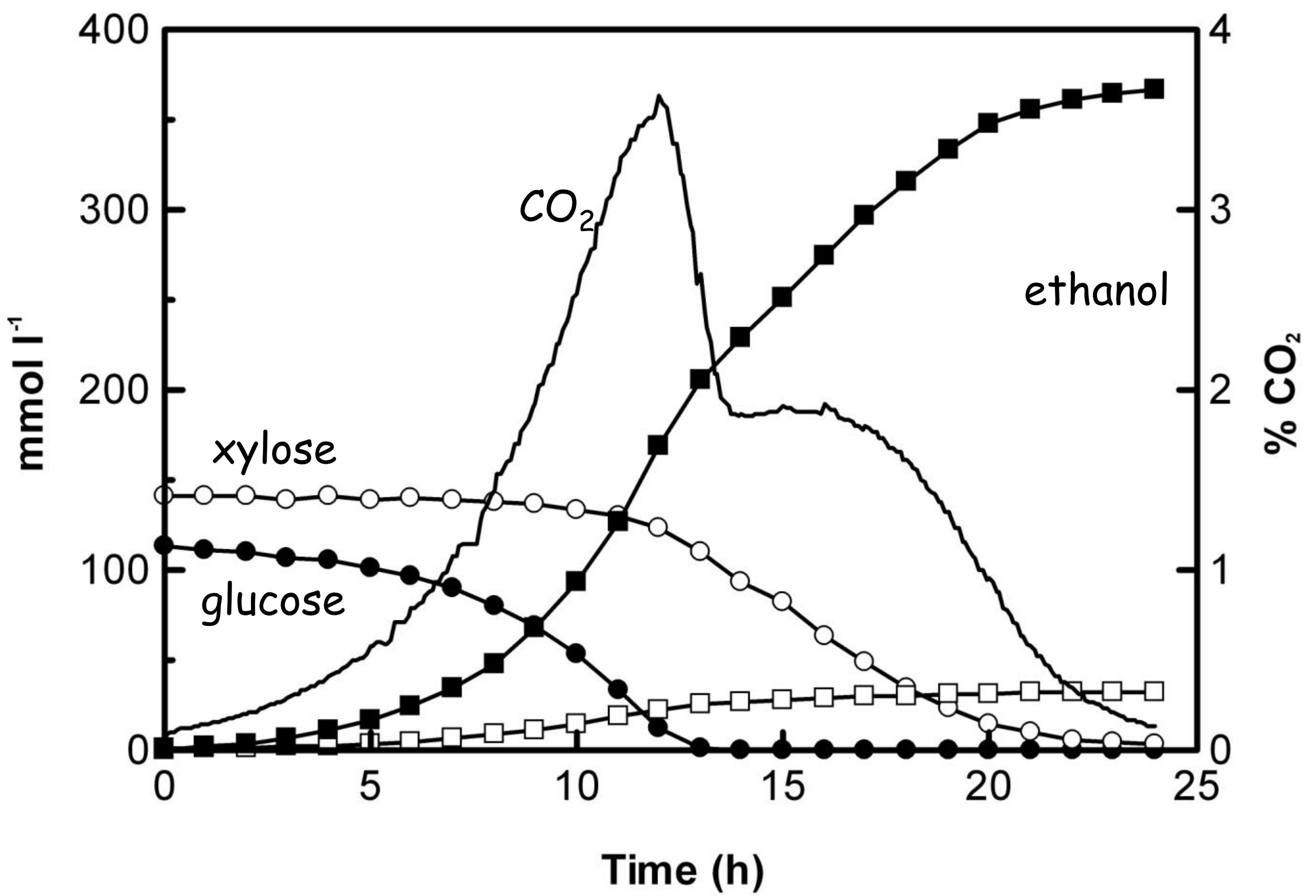
Selection for Improved Xylose Affinity



Long-term cultivation of RWB217 in anaerobic, **xylose-limited chemostat** (D = 0.06 h^{-1}): decrease of residual xylose concentration

Kuyper *et al*. 2005
FEMS Yeast Research **5**: 925-934

Anaerobic Fermentation of a glucose-xylose Mixture



Kuyper *et al*. 2005
FEMS Yeast Research **5**: 925-934

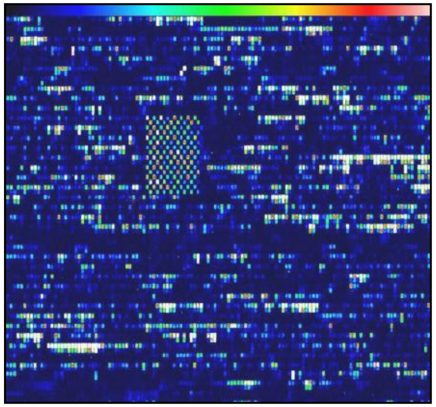
Transcriptome analysis

CEN.PK113-7D
Reference

→ RWB217
Genetically engineered

→ RWB218
Evolved

CEN.PK113-7D	RWB217	RWB218
Glucose	Glucose	Glucose
	Xylose ↔	Xylose



- triplicate chemostat cultures (anaerobic, C-limited, $D = 0.05\ h^{-1}$) for each strain/condition

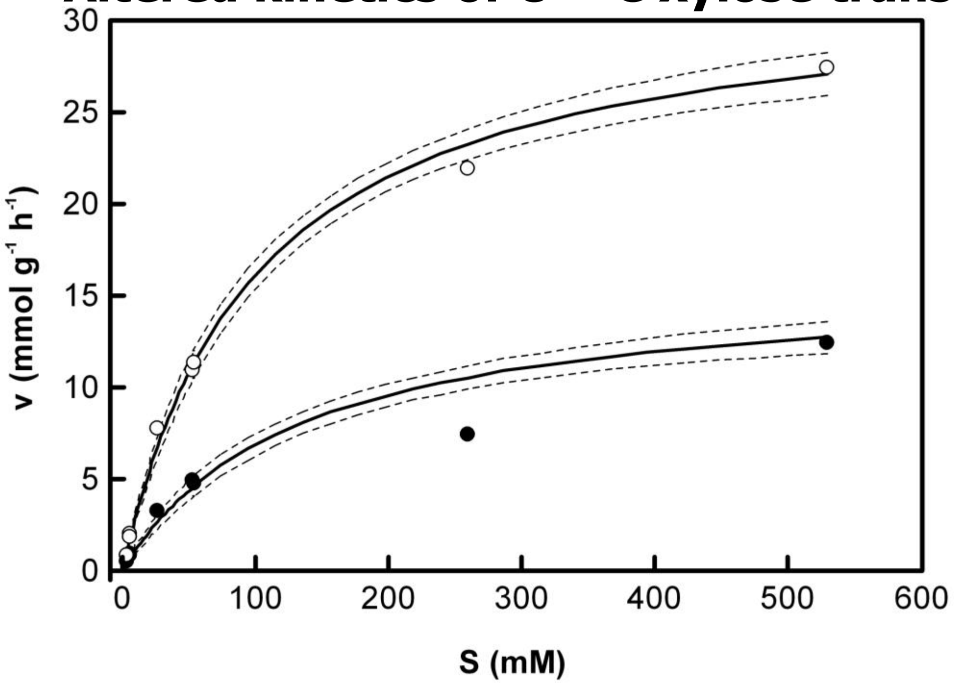
- Affymetrix GeneChips

Transcriptome Analysis (1)

Genes involved in hexose transport

Genes	Transcript Levels		Fold Change	*p-value*
	Parental strain RWB 217	Evolved strain RWB 218		
		3	6.44	**0.034**
		56	9.5	**0.025**
		8	-39	0.18
		0	8.4	**0.008**
		.3	-3.59	**0.002**
		9	-1.12	0.26
		41	1.07	0.44
		4	1.13	0.34
			1.0	-
		7	1.16	0.47
		1	-1.3	0.17
		27	-1.01	0.92
		1	-31	0.026
		7	1.01	0.58
RGT 2	36.6 7.80	55.1 8.51	1.39	0.08
SNF 3	29.6 3.66	25.2 3.46	-1.17	0.21

Altered kinetics of U-^{14}C xylose transport



Bellissimi *et al*. unpublished

Kluyver CENTRE

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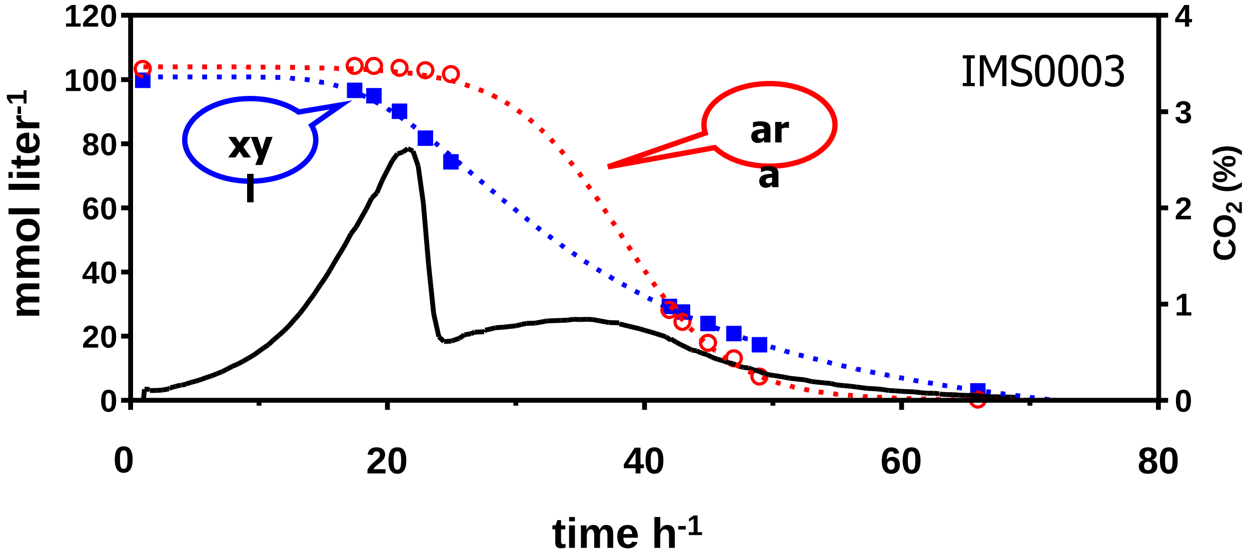
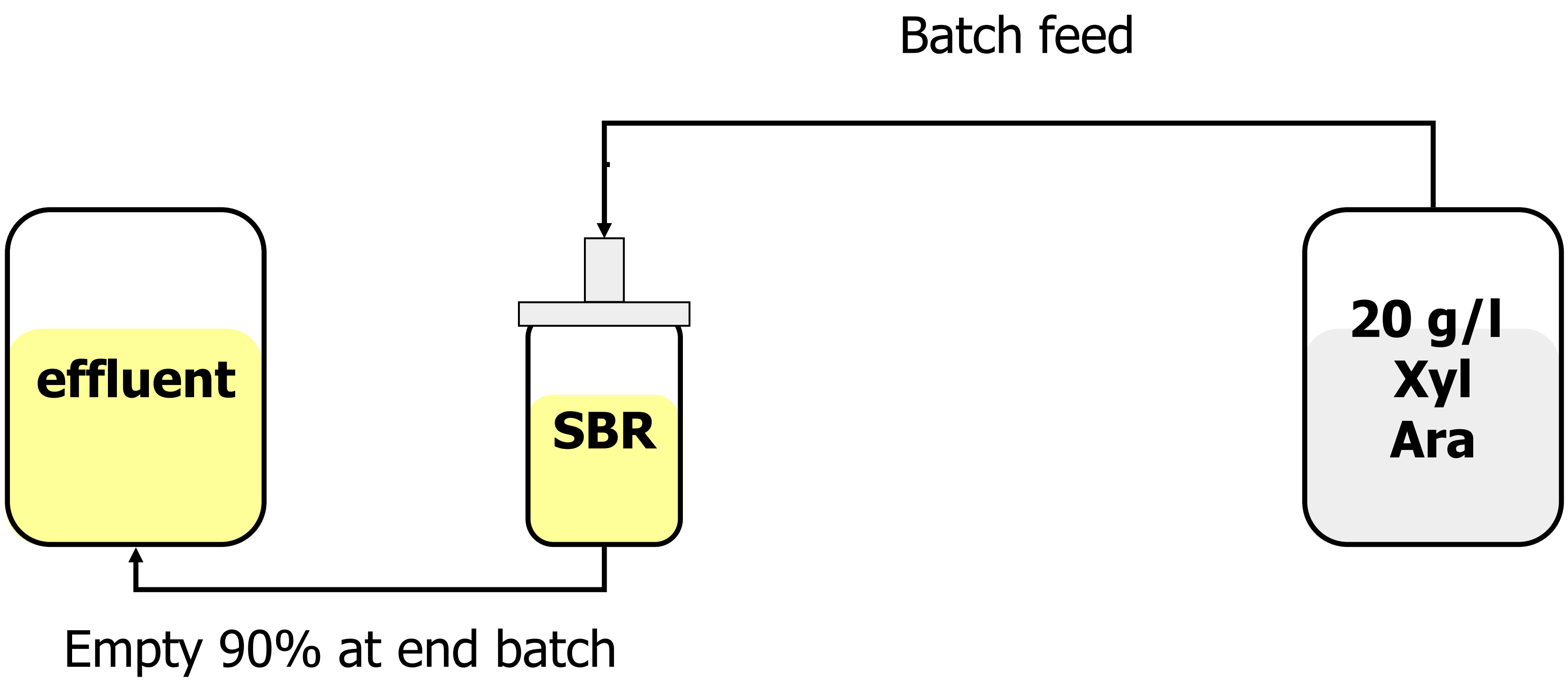
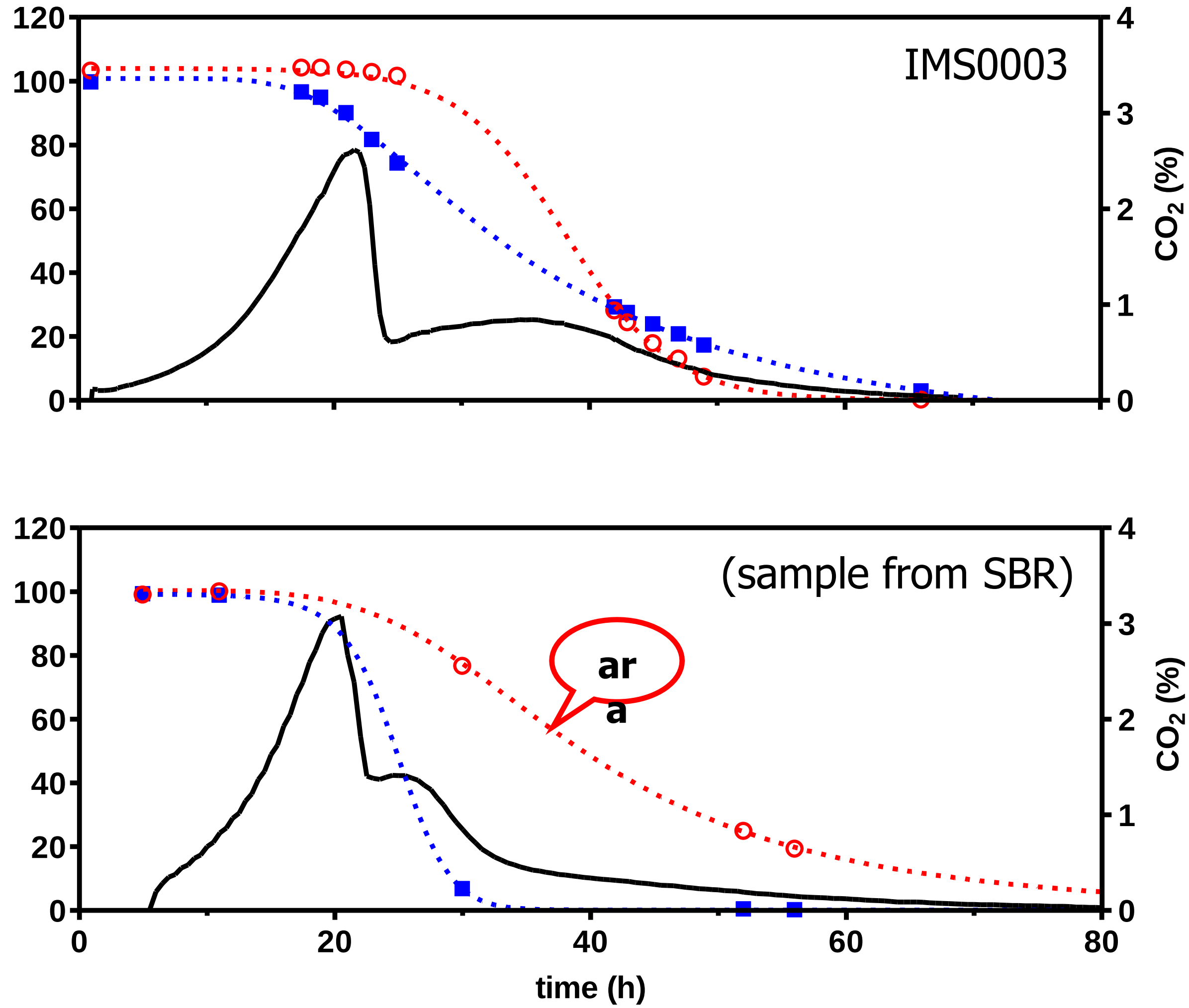
Anaerobic batch glc/xyl/ara 30/15/15 g l^{-1}



Selection with SBR (I)



Anaerobic batch glc/xyl/ara 30/15/15 g l^{-1}



B-Basic
Bio-based Sustainable Industrial Chemistry

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Counting the generations on the substrates

	Biomass g l^{-1}/generations on:		
Batch	**Glc**	**Xyl**	**Ara**
Xyl/ara 20/20 g l^{-1}		0.2 → 1.8 3.2	1.8 → 3.4 0.9

B-Basic Bio-based Sustainable Industrial Chemistry

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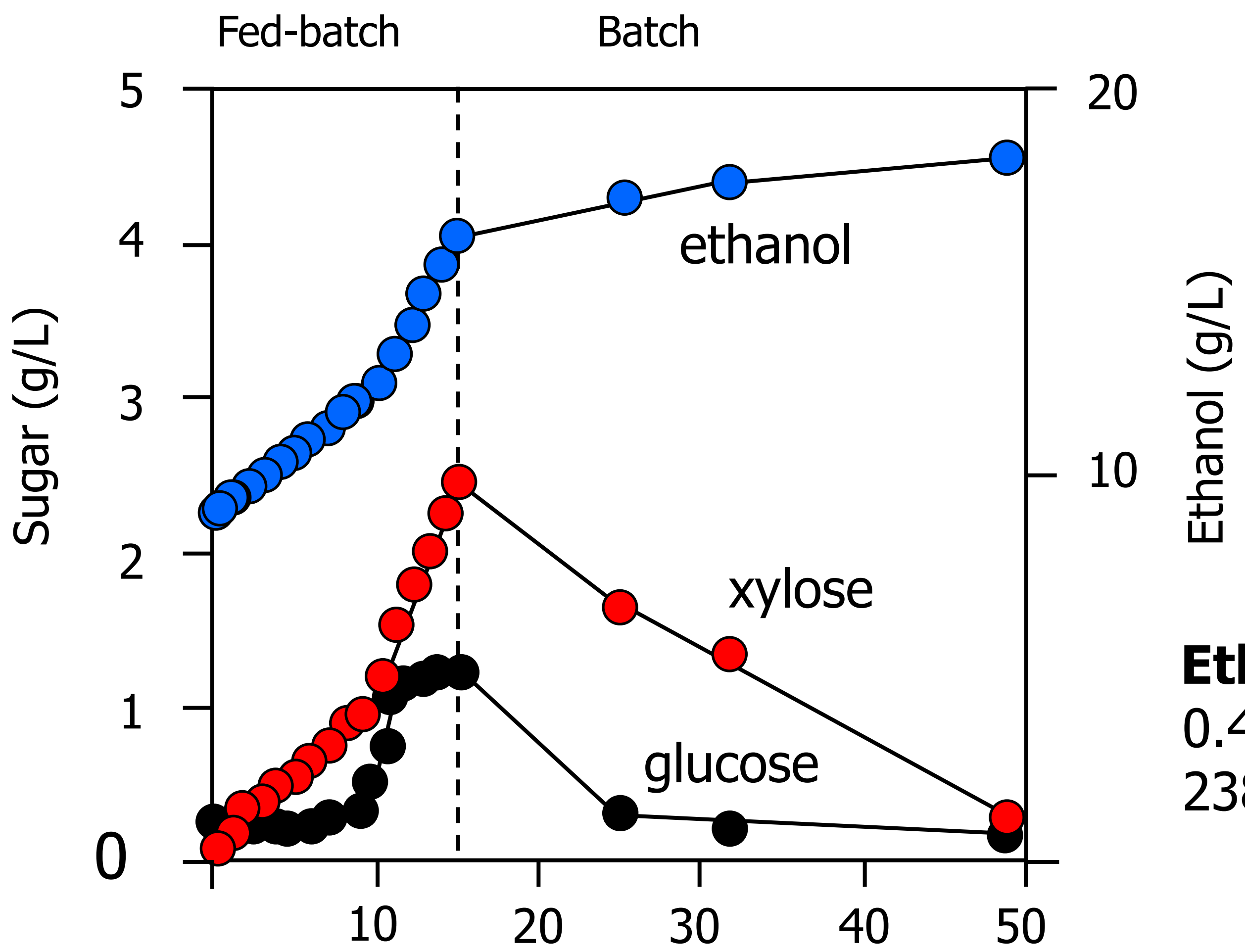
From the Lab to the Real World...

Ethanol Production from Wheat-Straw Hydrolysate
('academic' xylose-fermenting strain)



Ethanol yield:
0.47 g ethanol/g sugar
238 L ethanol/ton dry biomass

BE-Basic