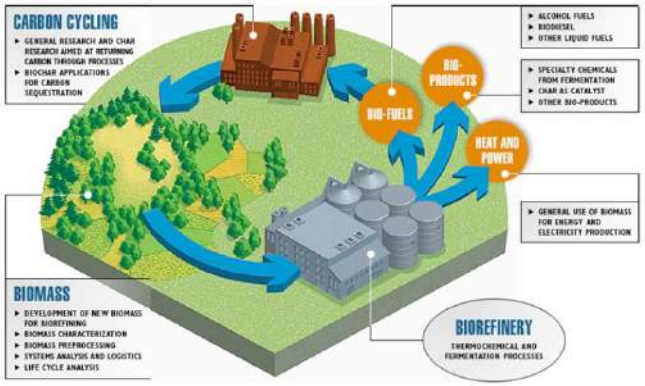
Source: biochar.org



UNICAMP

$$C_6H_{12}O_6 \longrightarrow 2\ C_2H_6O + 2\ CO_2$$

Conversion of sugars into ethanol: state-of-the-art and perspectives for 1G ethanol

Andreas K. Gombert
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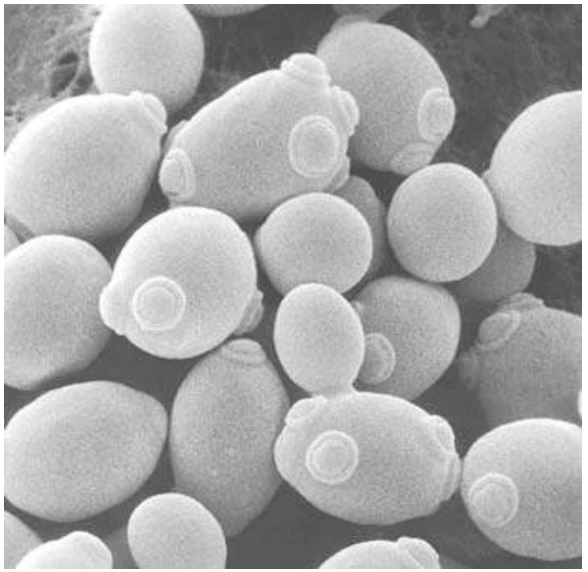
Faculty of Food Engineering
University of Campinas, Brazil
gombert@unicamp.br



Source: yourweeklymicrobe.blogspot

I st generation fuel ethanol

- From corn (USA): ~60 billion liters/year

mainly composed of starch

- From sugar-cane (Brazil): ~30 billion liters/year

mainly composed of sucrose

BRAZIL

- ~400 sugar-cane mills (no 2 of them are equal)
- Sugar-cane represents a bigger share (~18%) in the energy matrix than e.g. hydroelectricity (~16%)
- >40% of the energy matrix is renewable
- Producing 1 kJ in the form of ETH is cheaper than producing 1kJ in the form of gasoline
- Flex fuel motors dominate the light vehicle fleet
- 1G ETH is doing good (energy balance), but can be improved
- Brazil is introducing 2G ETH (but 1G won't disappear!)
- No clear governmental policy: decreased production in the recent years, hard-time for this business!

The current Brazilian biorefinery: 3 main products

Sugar cane

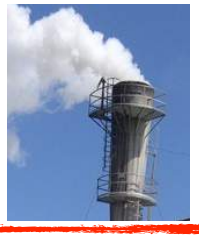
Sugar cane juice

Sugar

S. cerevisiae

Sugar cane bagasse

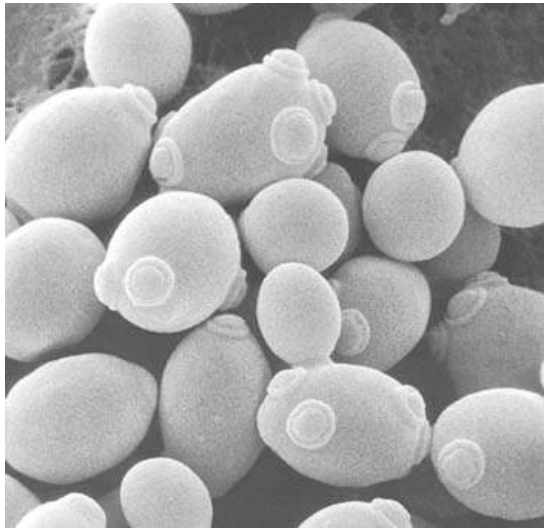
Molasses



Electricity

Ethanol

Saccharomyces cerevisiae

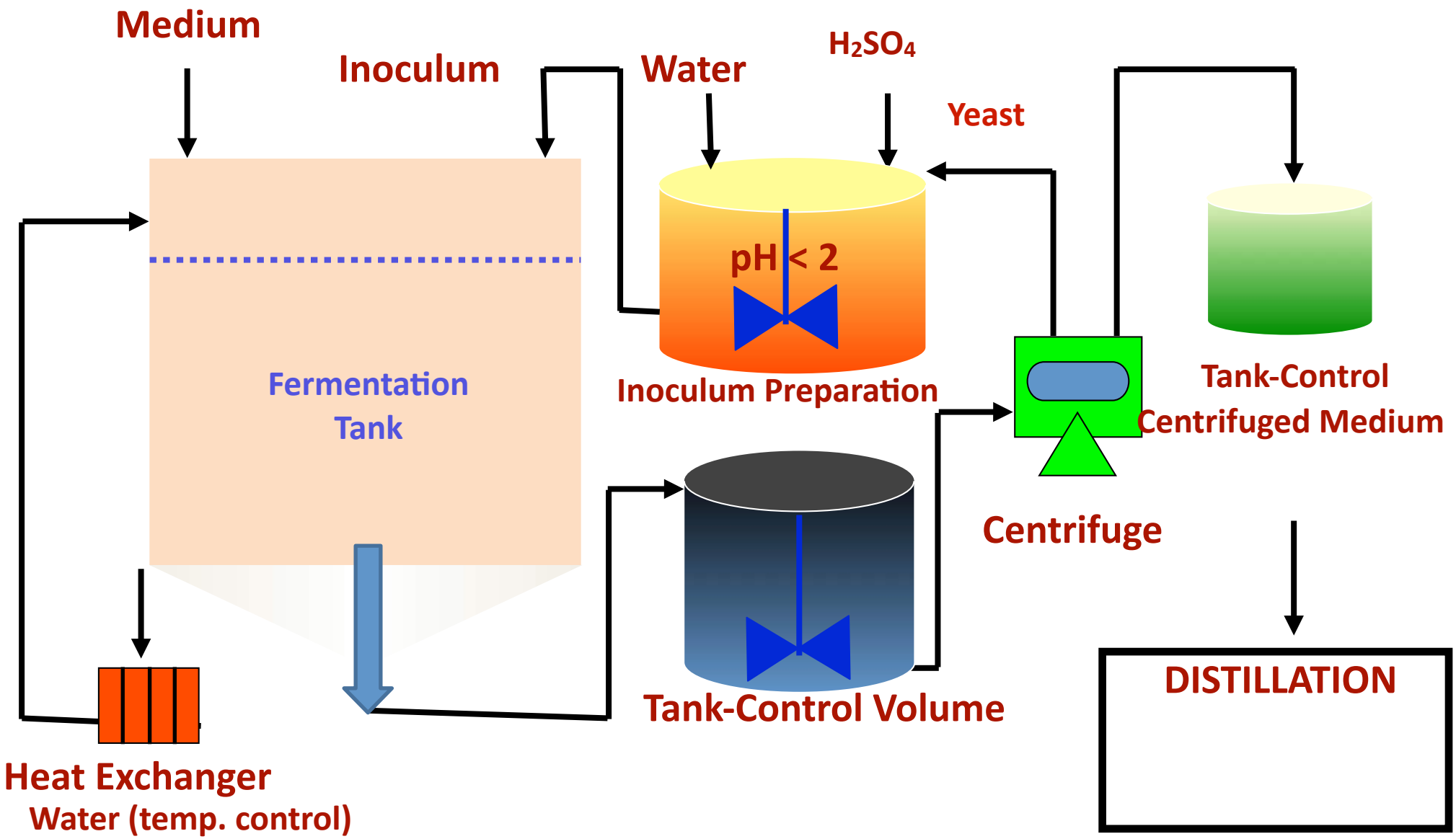


yourweeklymicrobe.blogspot

- =yeast (a unicellular eukaryotic microorganism)
- converts sugars into ethanol under anaerobiosis, with high yields and high final titers
- genetics, metabolism, physiology well known
- easy to genetically engineer
- robustness towards harsh industrial conditions
- simple nutritional requirements

FED BATCH FERMENTATION WITH Cell Recycle via CENTRIFUGATION

(MELLE-BOINOT System)



Important

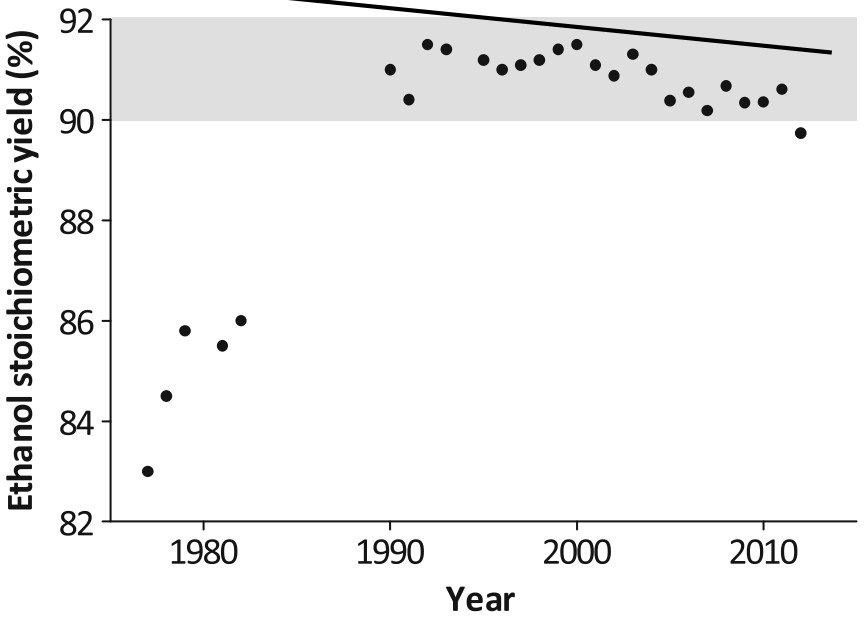
- The “Brazilian” process for producing ethanol from sugar-cane sucrose is carried out without complete asepsis

- Yeast cells are recycled and a drastic sulfuric acid treatment is carried out at the end of every cycle

- A high inoculum fraction and a low N-content in the medium favor ethanol production

- The industrial yield (g Ethanol/g sugar) has been stable for many years now (around 91%)

small increases in yield might make the difference between a profitable and a non-profitable business!



Della-Bianca et al Appl Microbiol Biotechnol 2013

- "Learning by doing" is finished.

- If we want to improve 1G ethanol yields, breakthrough technologies will be required (e.g. use of metabolically engineered yeast with improved traits).

Why is **yield** so important?

- Because ~70% of the final ethanol cost is due to raw material cost

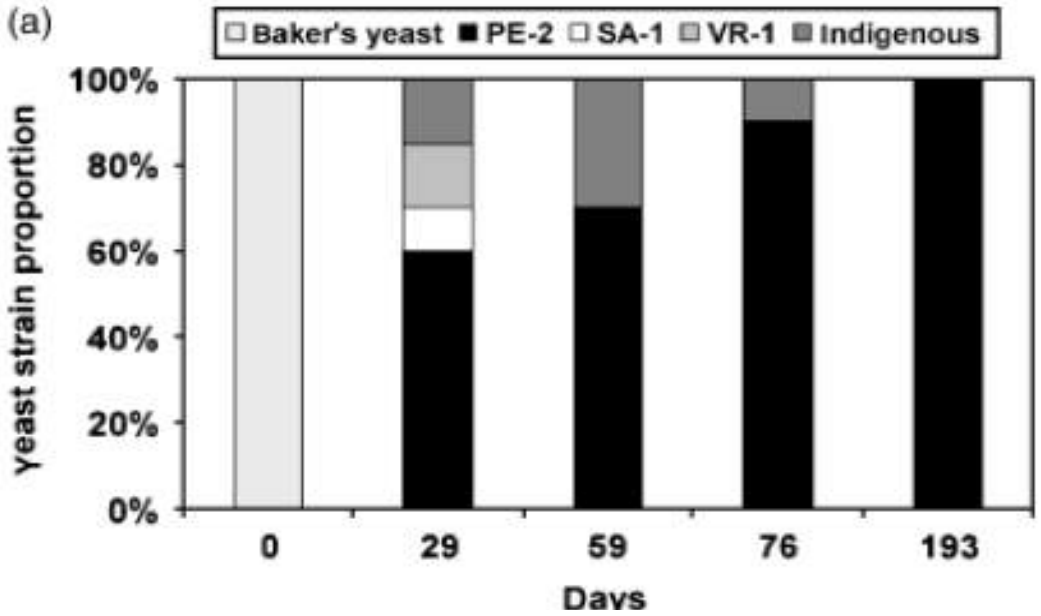
Yield = g ethanol/g sugar

Productivity = g ethanol/(L.h)

yeast population dynamics during a crushing season

open fermentation vat

http://www.biocane.com.br



Basso et al, (2008) FEMS Yeast Res

How do yeasts make ethanol?

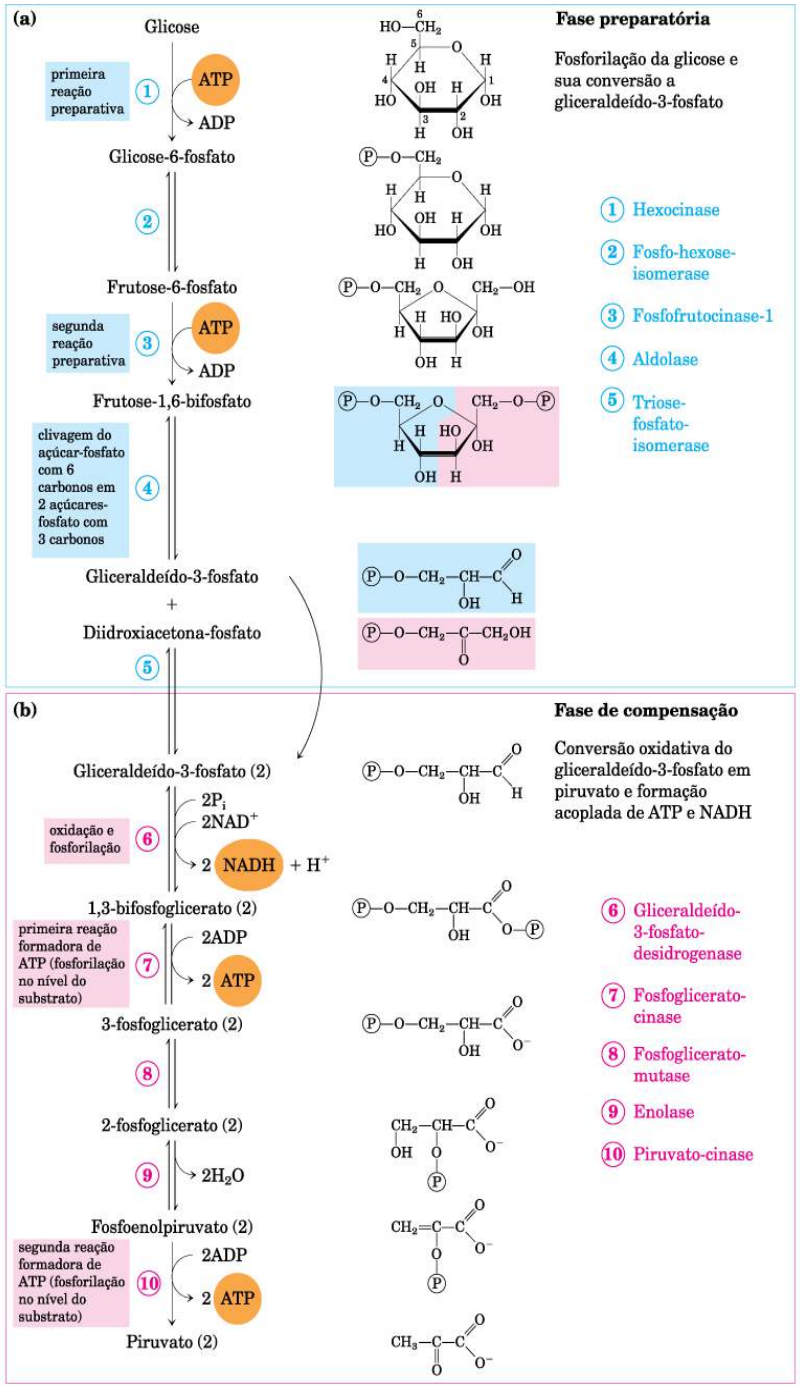


FIGURA 14-2 **As duas fases da glicólise.** Para cada molécula de glicose que passa pela fase preparatória **(a)**, duas moléculas de gliceraldeído-3-fosfato são formadas; as duas passam pela fase de compensação **(b)**. O piruvato é o produto final da segunda fase da glicólise. Para cada molécula de glicose, dois ATPs são consumidos na fase preparatória e quatro ATPs são produzidos na fase de compensação, dando um rendimento líquido de dois ATPs por molécula de glicose convertida em piruvato. As reações numeradas são catalisadas pelas enzimas listadas à direita e também correspondem aos títulos numerados discutidos no texto. Lembre-se que cada grupo fosforil, representado aqui como Ⓟ, possui duas cargas negativas ($—PO_3^{2-}$).

Embden-Meyerhof-Parnas (EMP) pathway

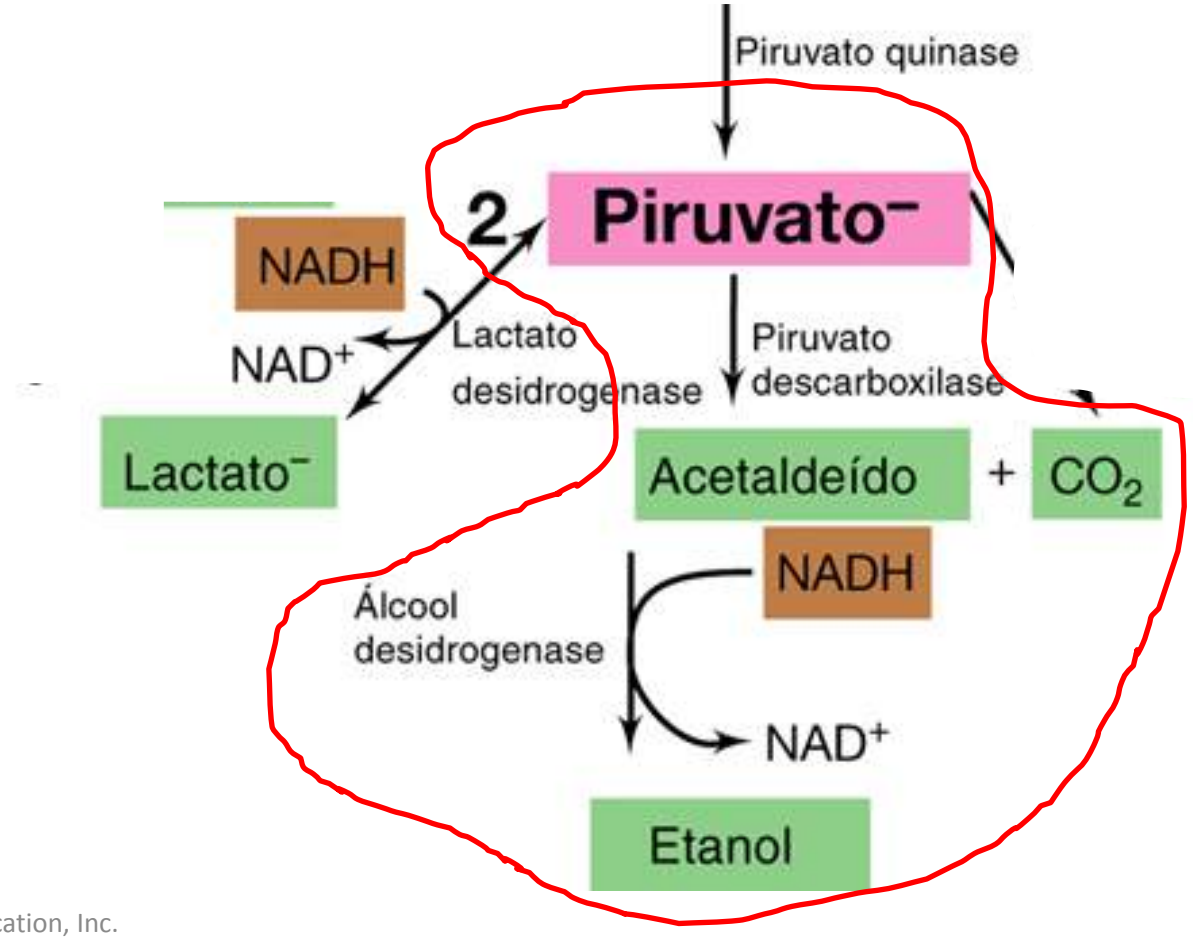
OR

classical glycolysis

Yields 2 ATP/hexose

Reduces NAD^+ to NADH + H^+

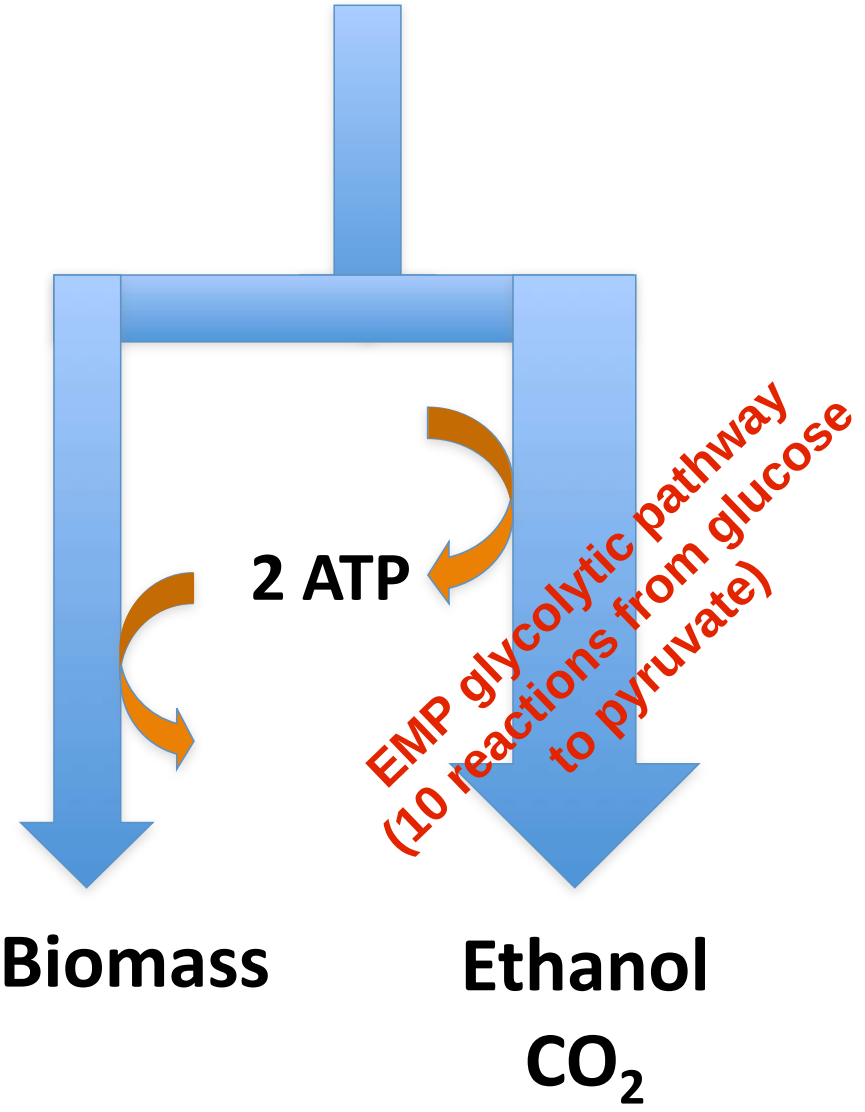
Ethanolic fermentation
(yeast)
Reoxidizes NADH + H^+ to NAD^+



Why do yeasts make ethanol?

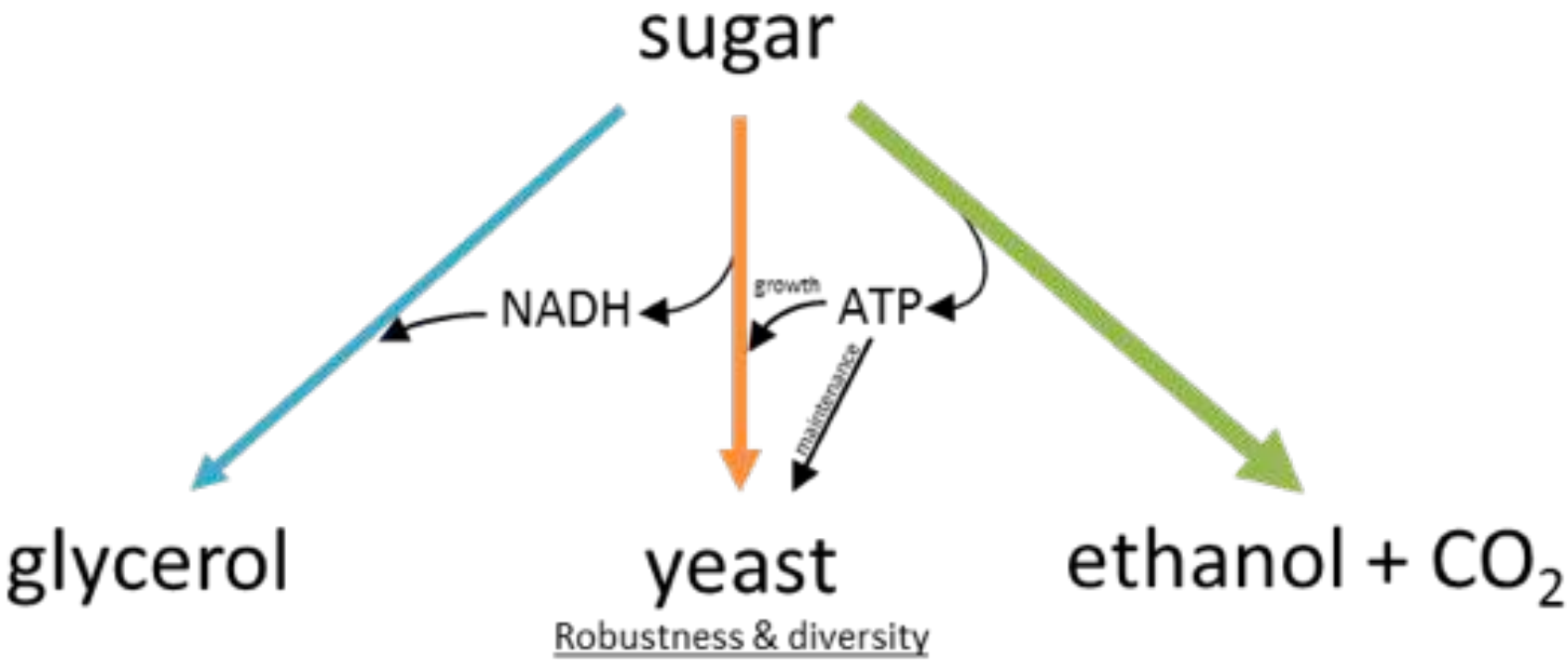
It is a cell's dream to become two cells. François Jacob.

Fuel ethanol production: The conflict of interests!



$C_6H_{12}O_6$ —> 2 C_2H_6O + 2 CO_2

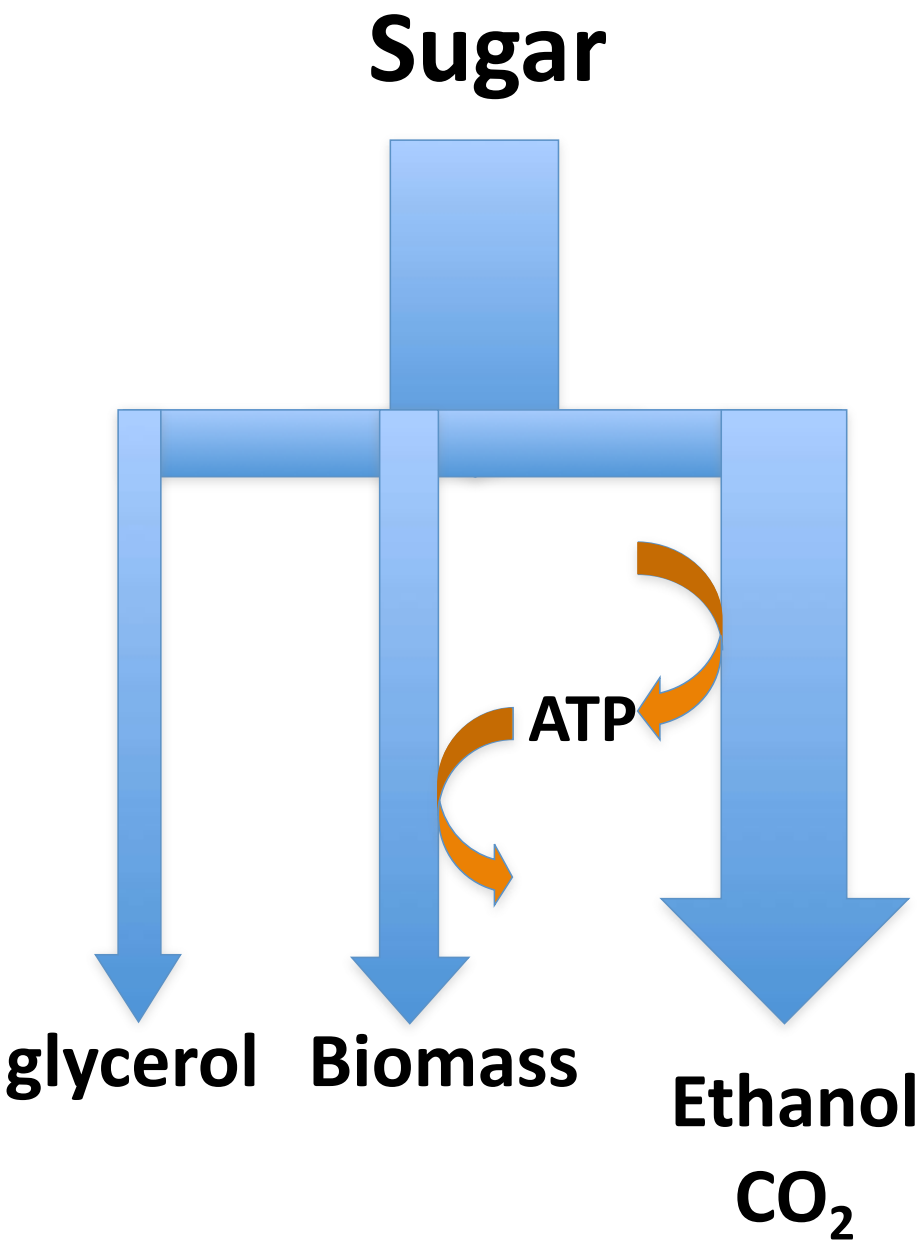
- This is a redox neutral conversion.
- But…



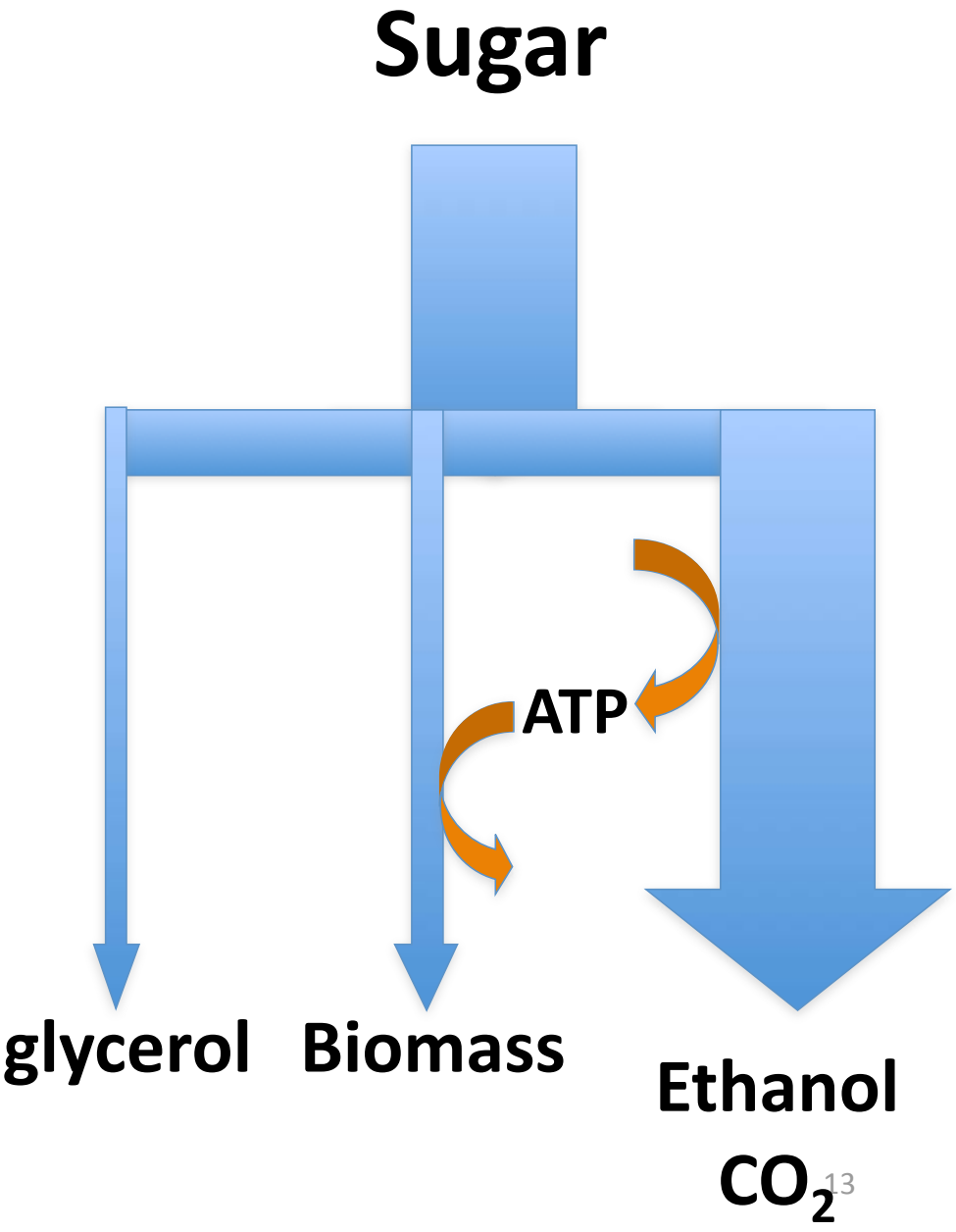
Strategies aiming at increasing ethanol yields on sugar include:

- Decreasing glycerol and/or biomass formation
- Decreasing free-energy conservation
- Improving yeast robustness towards process stressors (e.g. high ethanol concentrations, low pH, high T, etc.)
- Improving the process (e.g. using bacteriocins or other strategies to fight contamination, thereby decreasing sugar loss)

Wild-type strain



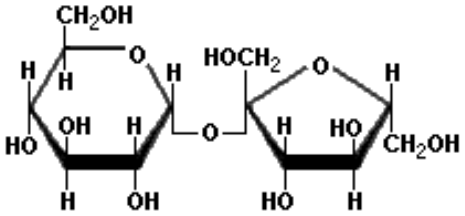
Engineered strain



EXAMPLE 1:

Improving ethanol formation in *S. cerevisiae* by decreasing free-energy conservation

Energetics of SUCROSE metabolism in *Saccharomyces cerevisiae*



Sucrose

Extracellular Invertase

facilitated diffusion

Glucose + Fructose

Glc + Fru

4 ATP

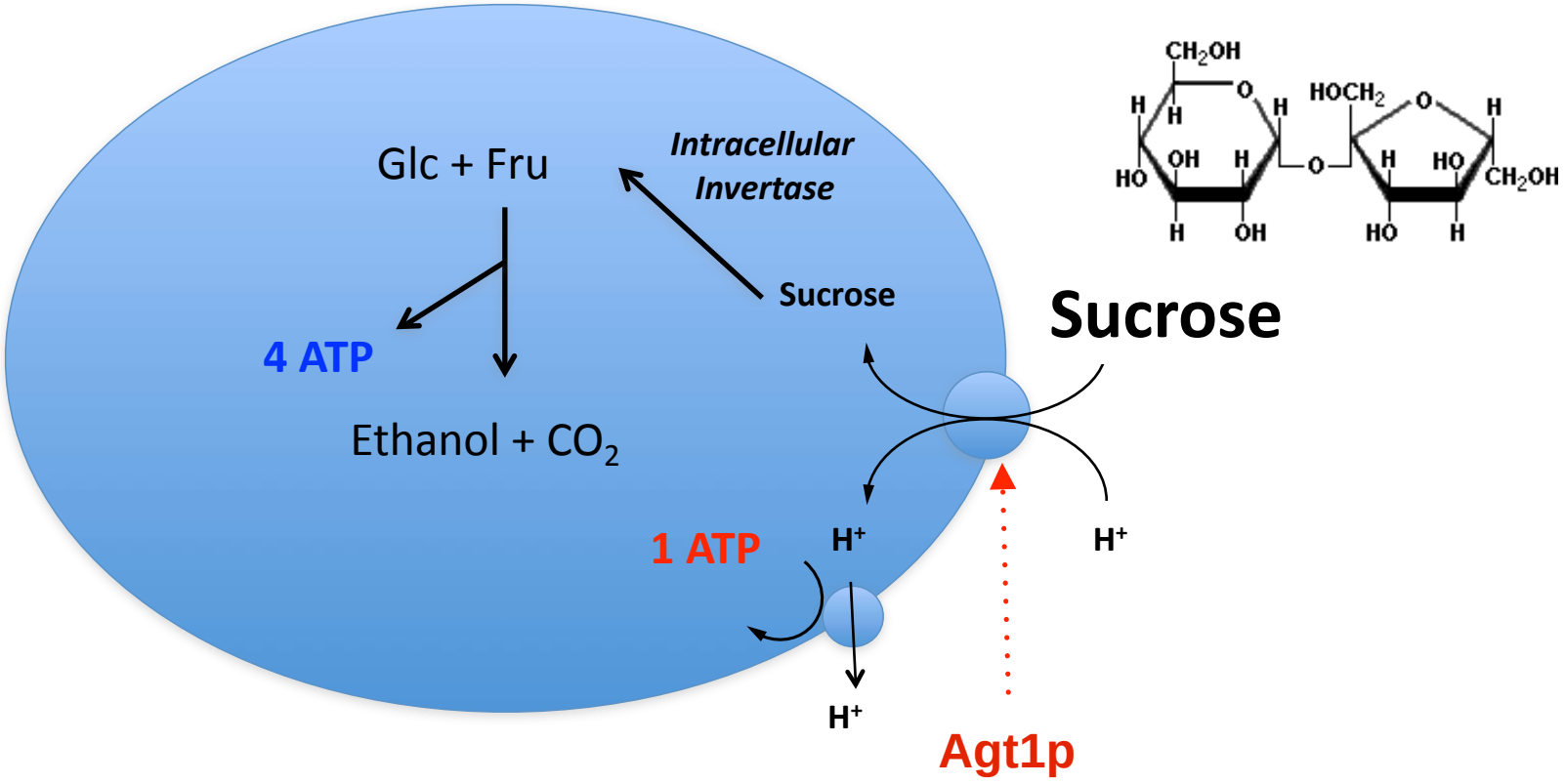
Ethanol + CO_2

4 ATP for each Sucrose

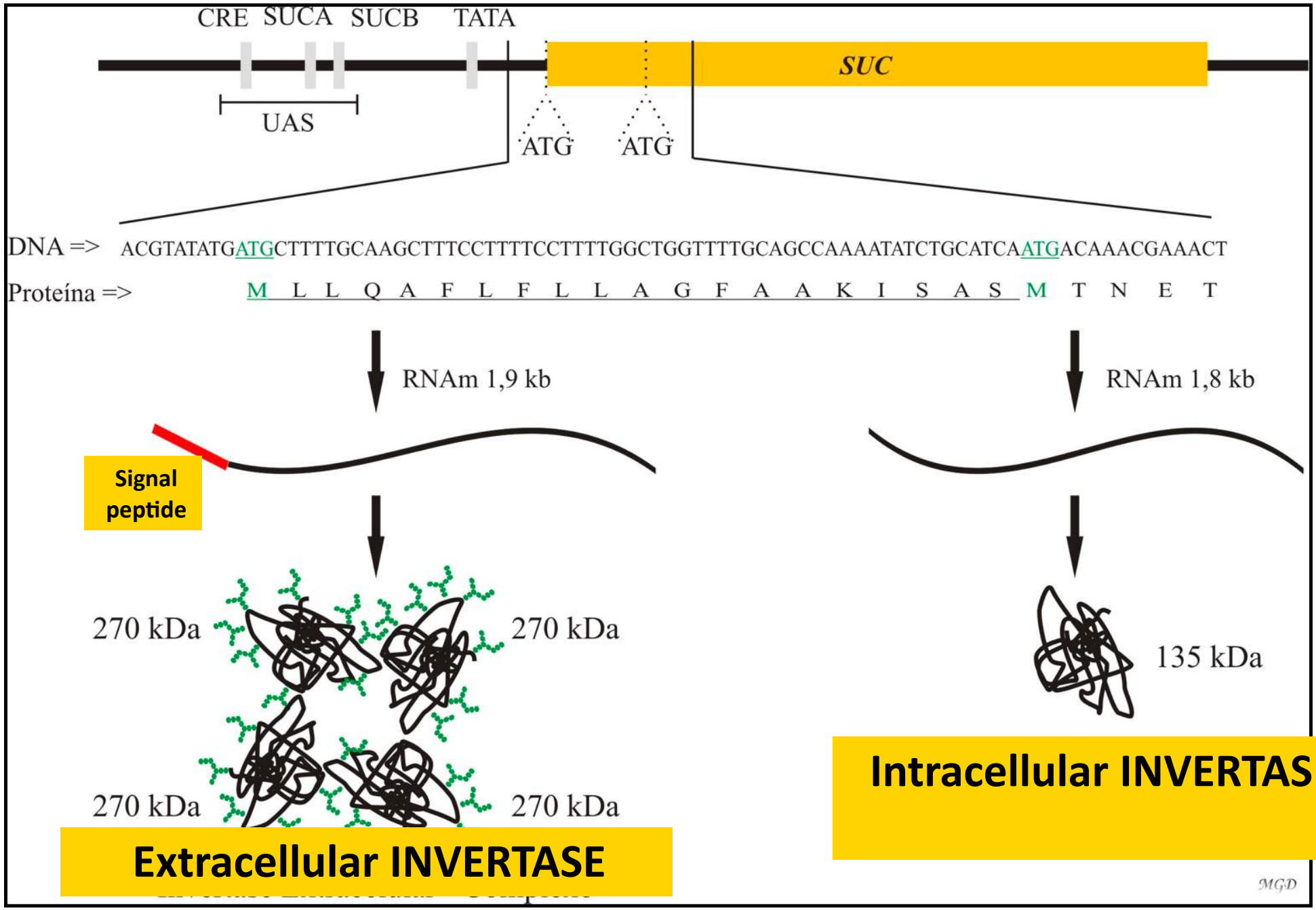
Modulating the energetics of SUCROSE metabolism in *Saccharomyces cerevisiae*

Stambuk et al. ***INPI PI 0901254-0. (2009)***

4 ATP - 1 ATP = 3 ATP for each Sucrose



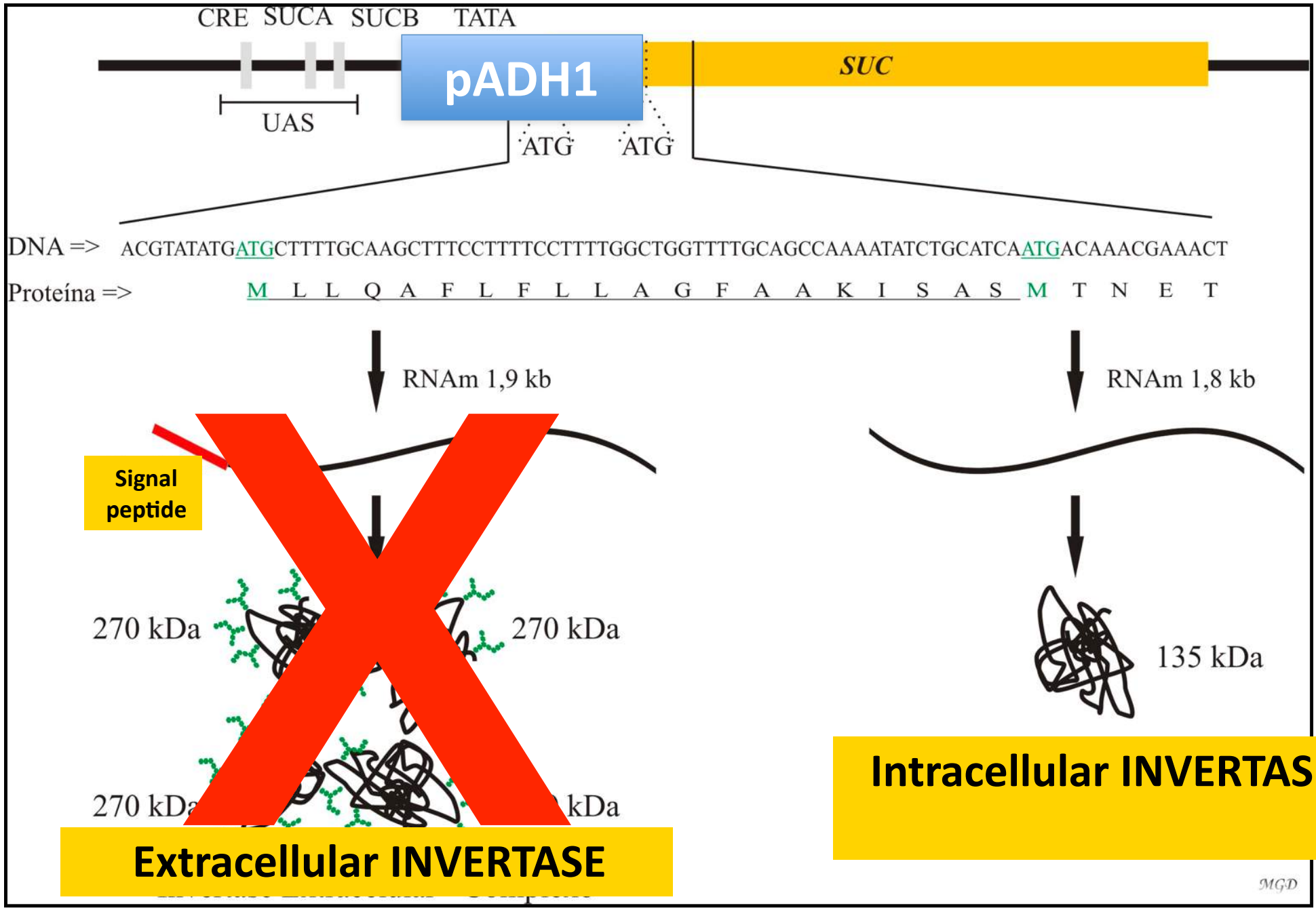
Carlson & Botstein. ***Cell*** *28 (1982)*



99% **1%**

Stambuk et al. **INPI PI 0901254-0. (2009)**

Carlson & Botstein. **Cell** 28 (1982)



0%

100%

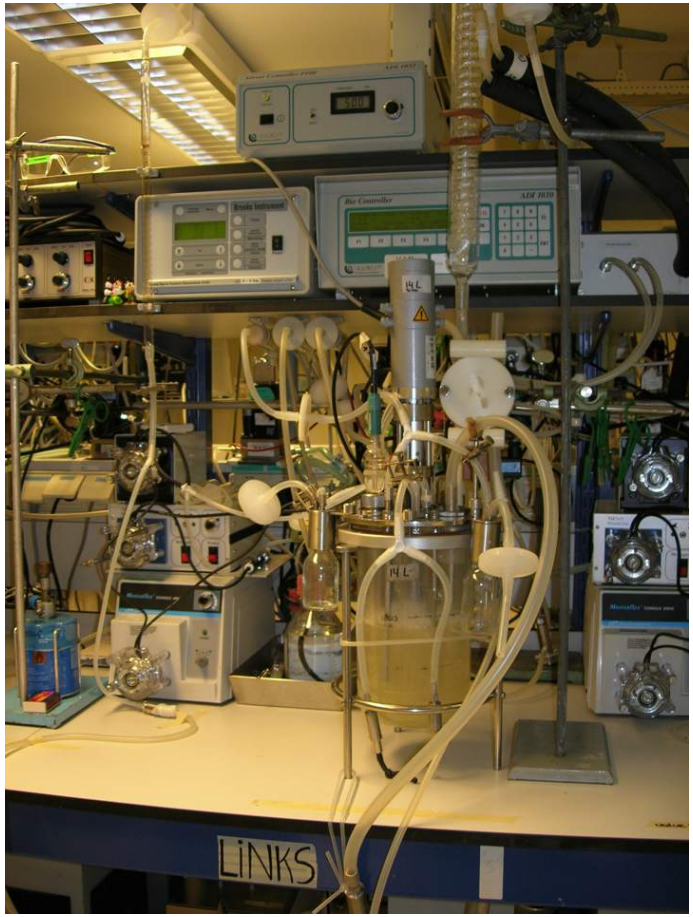
Anaerobic sucrose-limited chemostat D = 0.10 h^{-1}

Parameters	Strains		Change	
	SUC2	i*SUC2*	Observed	Theoretical
Y	0.094 ± 0.001	0.088 ± 0.001	- 6%	- 25 %
Y (g.g glc eq.	0.378 ± 0.001	0.395 ± 0.007	+ 4%	+ 8 %
Residual sugar (g.l	0.05 (glc) 0.11 (fru) 0.00 (suc)	0.09 (glc) 0.16 (fru) 1.79 (suc)	---	---

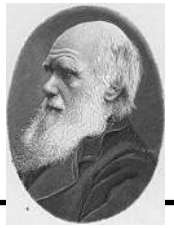
Low affinity for sucrose

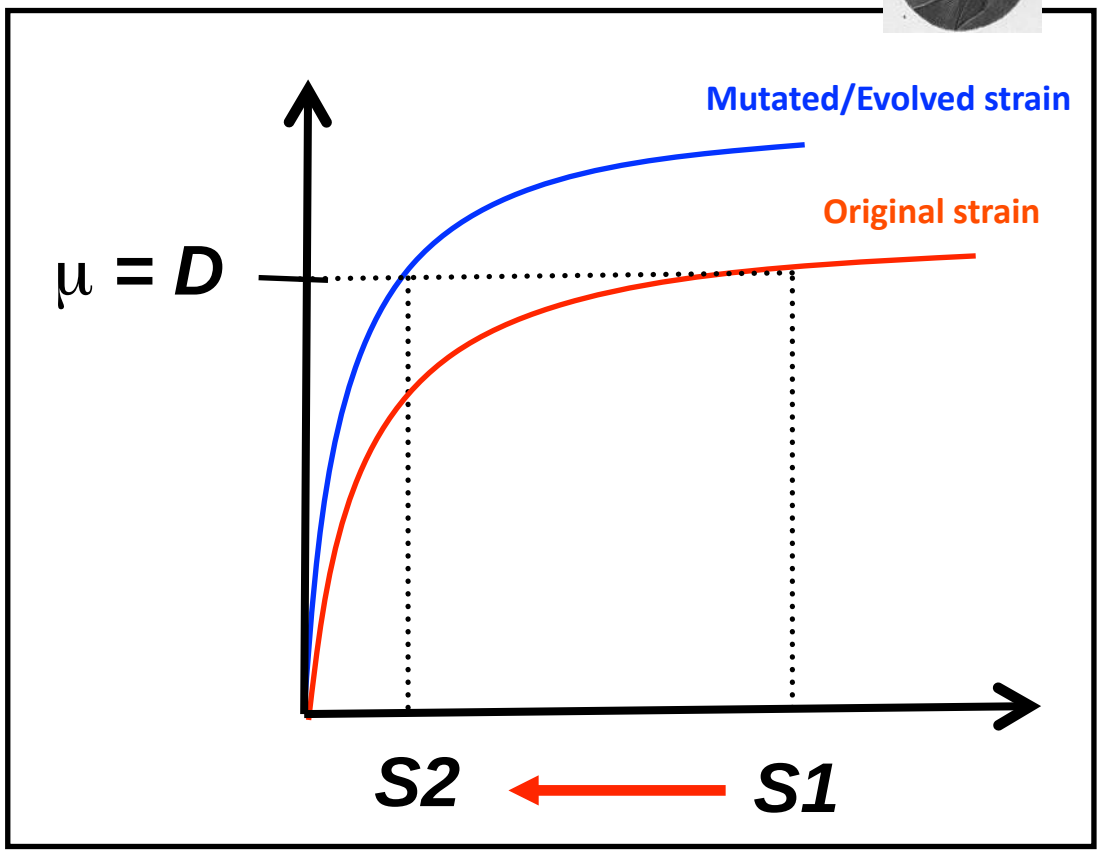
Evolutionary Engineering: Chemostat as a tool to increase affinity

Selective pressure for an IMPROVED AFFINITY/CAPACITY for the growth-limiting substrate (sucrose)

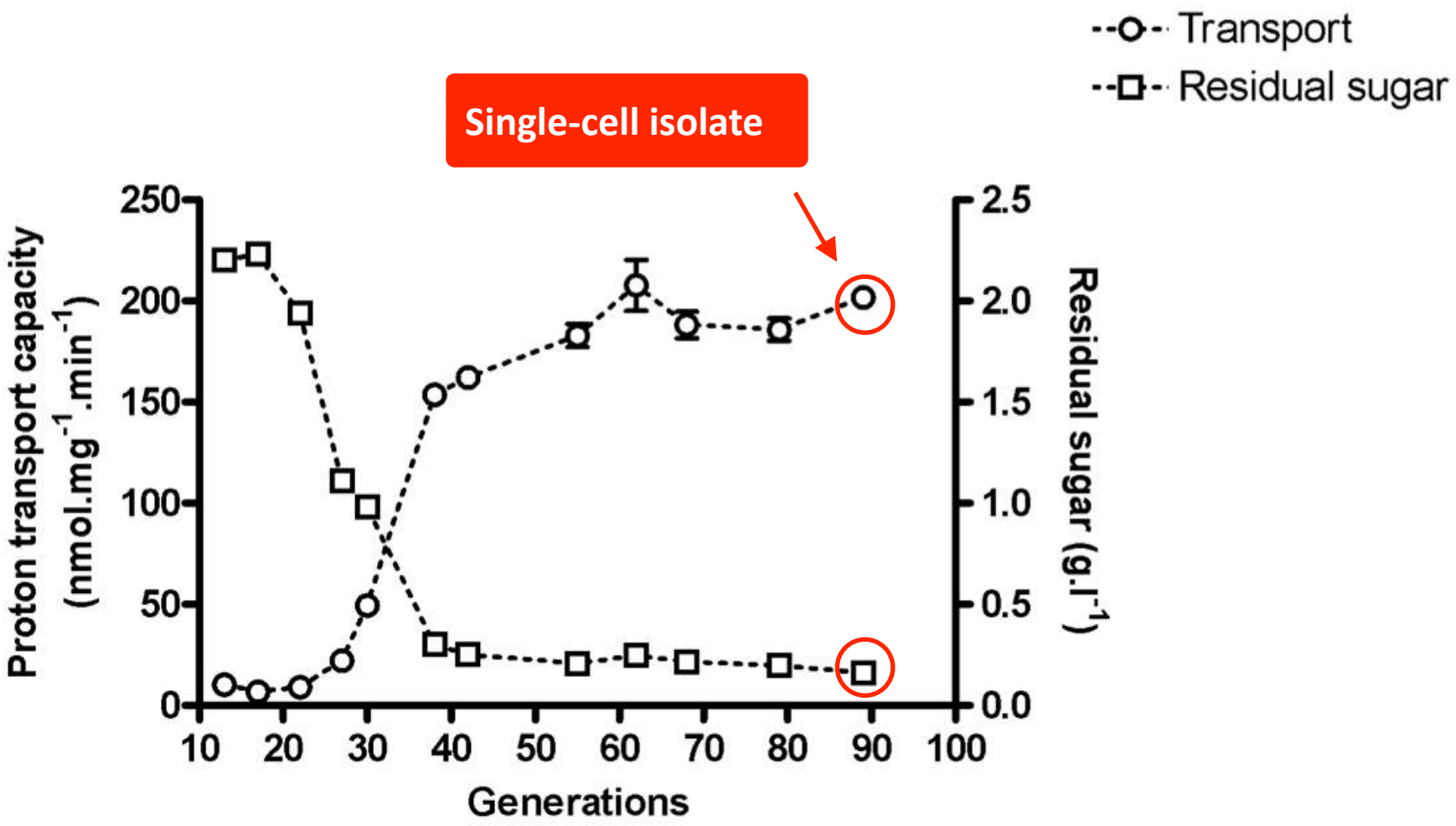


Chemostat setup





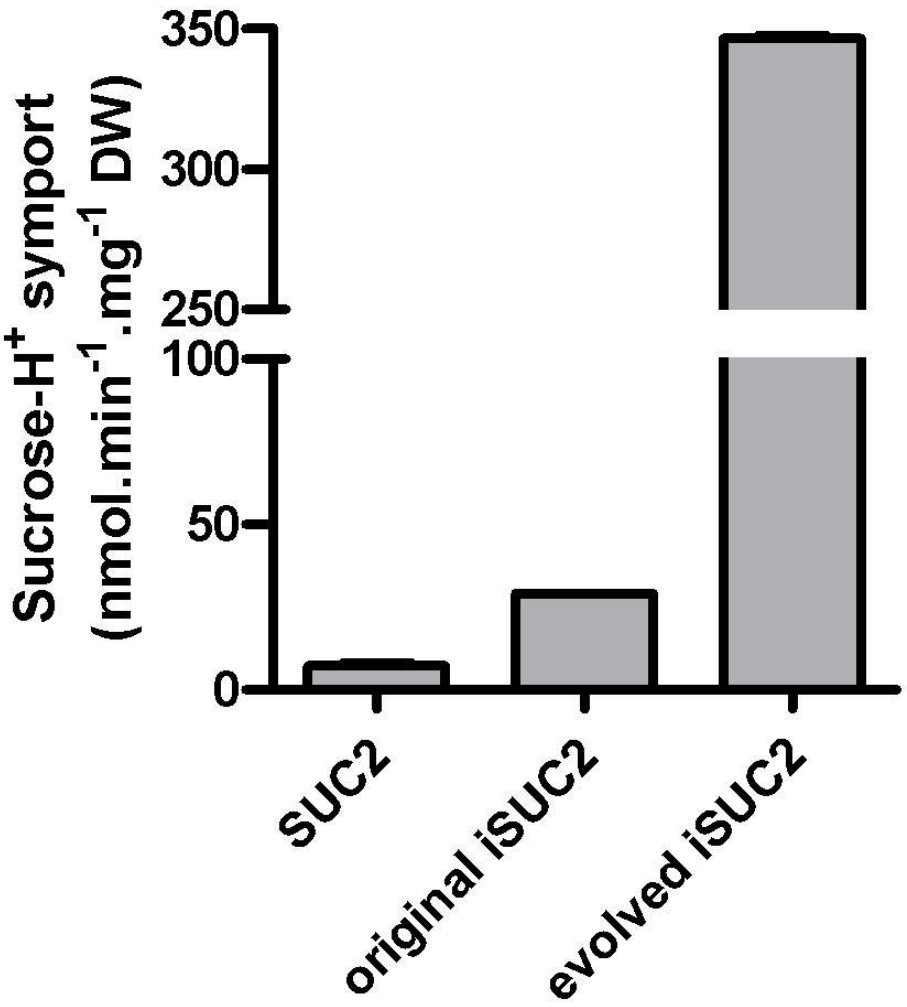
Evolutionary Engineering
(Long-term sucrose limited chemostat culture)



Anaerobic sucrose-limited chemostat
$D = 0.10\ h^{-1}$

Parameters	Strains			Change	
	SUC2	i*SUC2* original	i*SUC2* evolved	Observed	Theoretical
$Y_{X/S}$ (g.g glc eq.	0.094 ± 0.001	0.088 ± 0.001	0.066 ± 0.002	- 29 %	- 25 %
$Y_{Ethanol/S}$ (g.g glc eq.	0.378 ± 0.001	0.395 ± 0.007	0.421 ± 0.006	+ 11 %	+ 9 %
Residual sugars (g.l	0.05 (glc) 0.11 (fru) 0.00 (suc)	0.09 (glc) 0.16 (fru) 1.79 (suc)	< 0.01 (glc) 0.03 (fru) 0.08 (suc)	---	---

What changed? Transport capacity (Vmax)



ROLE OF AGT1

- A duplication of *AGT1* was found in the evolved iSUC2 strain
- Double-deletion of *AGT1* in the iSUC2 evolved strain restored the reference strain phenotype, but...
- Insertion of an additional copy of *AGT1* in the reference strain did not lead to the evolved iSUC2 phenotype

Conclusion

Relocation of sucrose metabolism in yeast, by a combination of metabolic and evolutionary engineering, resulted in an 11 % increase in the ethanol yield on sucrose

Basso et al (2011) Metabolic Engineering 13:694

this work was performed under a collaboration with Boris Stambuk (UFSC) and Ton van Maris and Jack Pronk (TU Delft)

EXAMPLE 2:

Improving ethanol formation in *S. cerevisiae* by introducing CO_2 fixation

Guadalupe-Medina *et al. Biotechnology for Biofuels* 2013, **6**:125
http://www.biotechnologyforbiofuels.com/content/6/1/125

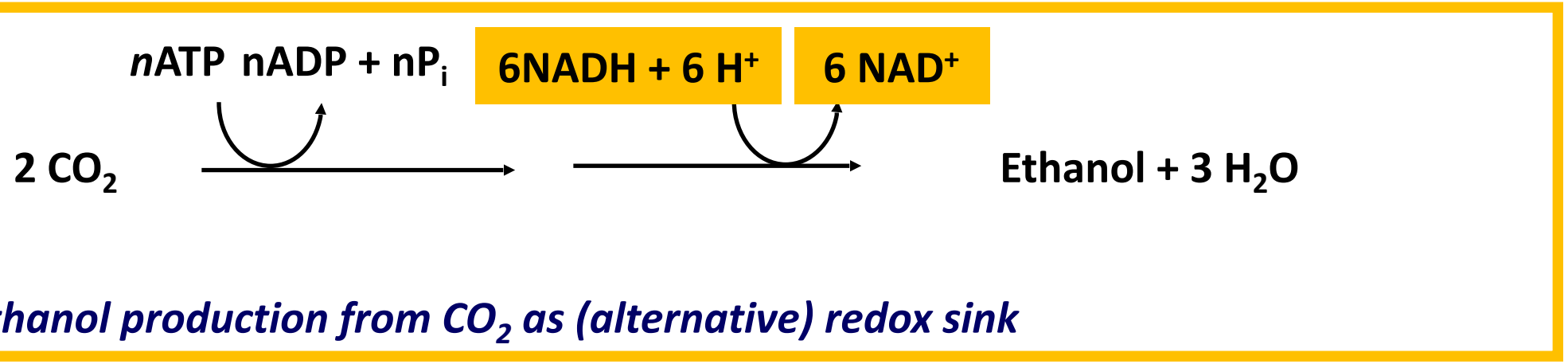
Biotechnology for Biofuels

RESEARCH Open Access

Carbon dioxide fixation by Calvin-Cycle enzymes improves ethanol yield in yeast

Víctor Guadalupe-Medina[1,2], H Wouter Wisselink[1,2], Marijke AH Luttik[1,2], Erik de Hulster[1,2], Jean-Marc Daran[1,2], Jack T Pronk[1,2] and Antonius JA van Maris[1,2*]

Can *S. cerevisiae* be engineered to reduce CO_2 to ethanol?

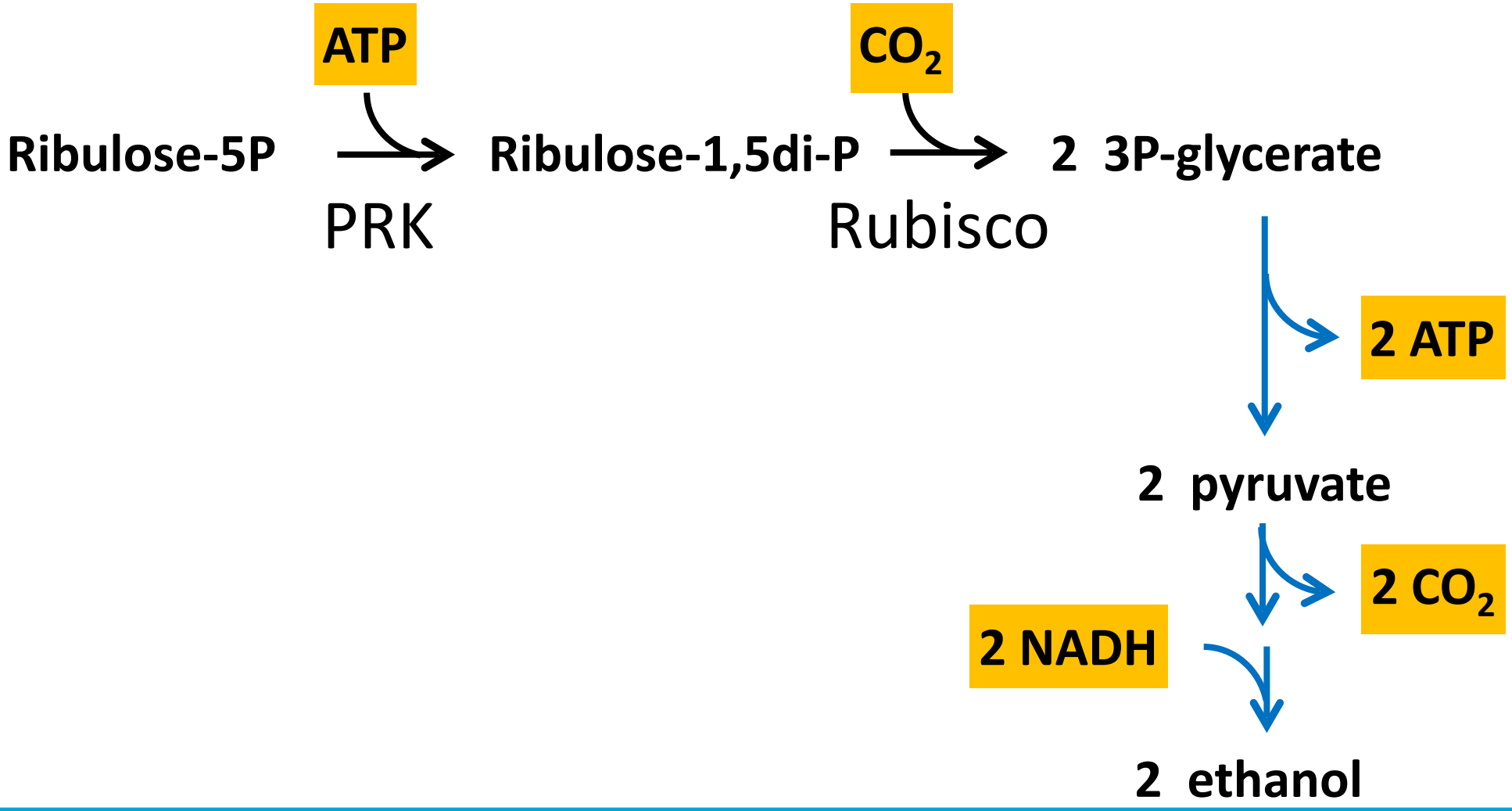


slide kindly provided by Ton van Maris and Jack Pronk (TU Delft)

TU Delft

Phosphoribulokinase (PRK) and ribulose-1,5-bisphosphate carboxylase (Rubisco)

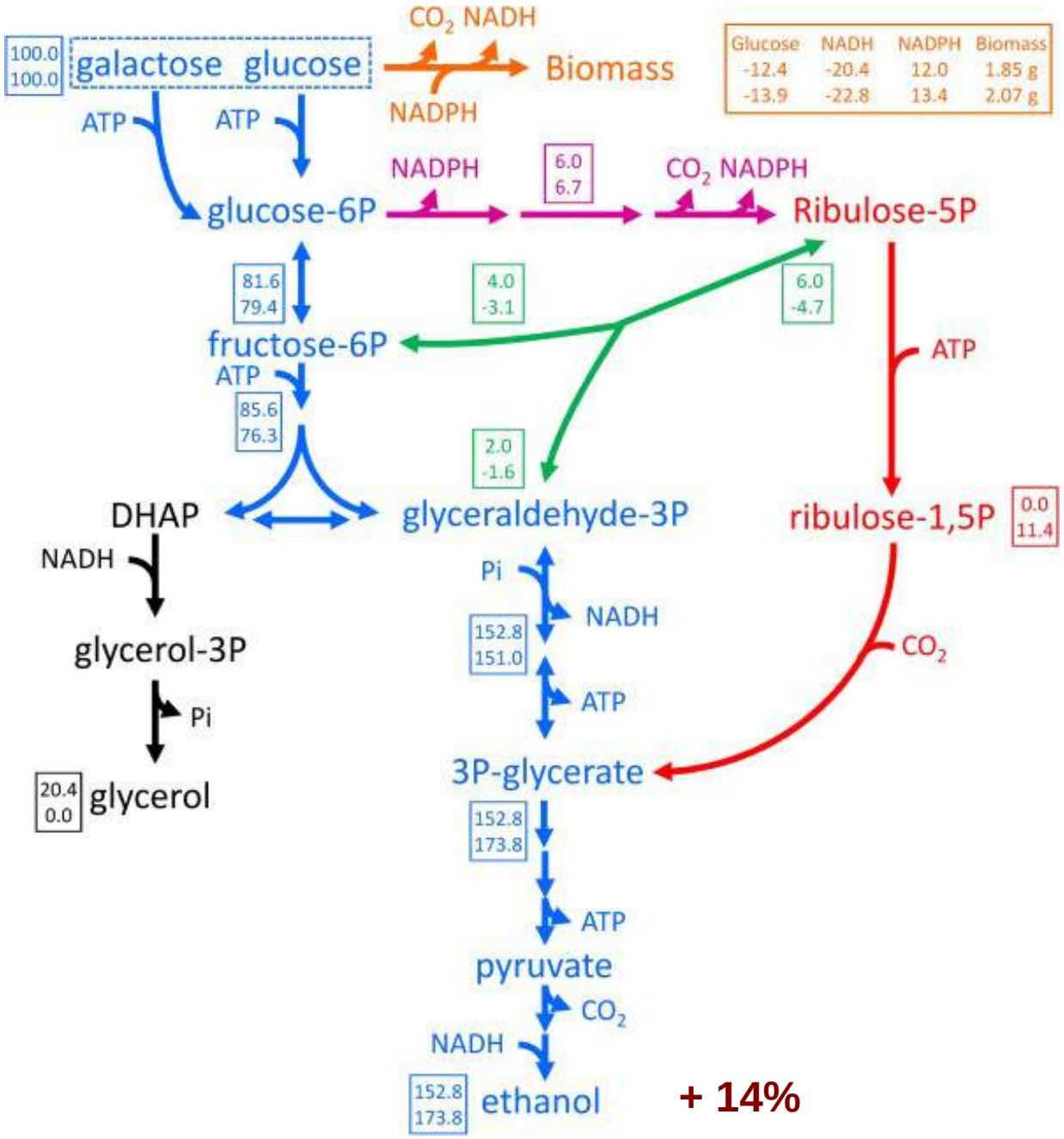
key enzymes in the Calvin cycle for autotrophic CO_2 fixation



Victor Guadalupe et al. (2013)
Biotechnology for Biofuels – 6:125

slide kindly provided by Ton van Maris and Jack Pronk (TU Delft)

TU Delft

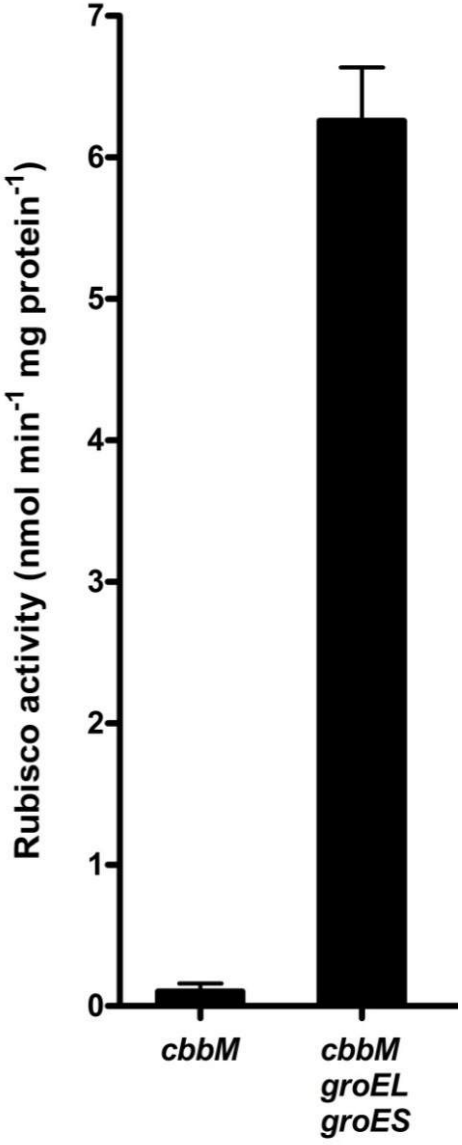


Victor Guadalupe et al. (2013)
Biotechnology for Biofuels – 6:125

slide kindly provided by Ton van Maris and Jack Pronk (TU Delft)

TU Delft

Functional expression of Rubisco and PRK in *S. cerevisiae*



Rubisco

Form II Rubisco from *Thiobacillus denitrificans* (cbbM)

Coexpression of chaperones (*E. coli* groEL/groES) essential

PRK

PRK gene from spinach
expressed from *GAL1* promoter

High enzyme activities upon induction with galactose

slide kindly provided by Ton van Maris and Jack Pronk (TU Delft)

Ribulose-1,5-bisphosphate-dependent fixation of $^{14}CO_2$ by cell extracts of *S. cerevisiae* strains

Victor Guadalupe et al. (2013)
Biotechnology for Biofuels – 6:125

Rubisco and PRK-expressing *S. cerevisiae*

Product yields in anaerobic, sugar-limited chemostat cultures

($D = 0.05\ h^{-1}$, N_2-sparged, equimolar glucose/galactose feed)

Relevant genotype	reference	cbbM, PRK, groEL/ ES
Biomass yield on sugar ($g\ g^{-1}$	0.083 ± 0.000	0.093 ± 0.000
Glycerol yield on sugar (mol mol	**0.14 ± 0.00**	**0.04 ± 0.00**
Ethanol yield on sugar (mol mol Corrected for evaporation	1.56 ± 0.03	1.73 ± 0.01

70 % reduction of glycerol production

Rubisco/PRK competes with native glycerol pathway

Victor Guadalupe et al. (2013)
Biotechnology for Biofuels – 6:125

TU Delft

Rubisco and PRK-expressing *S. cerevisiae*

Product yields in anaerobic, sugar-limited chemostat cultures

(D = 0.05 h^{-1}, 10 % CO_2, equimolar glucose/galactose feed)

Relevant genotype	reference	cbbM, PRK, groEL/ ES
Biomass yield on sugar ($g\ g^{-1}$	0.084 ± 0.000	0.095 ± 0.000
Glycerol yield on sugar (mol mol	**0.12 ± 0.00**	**0.01 ± 0.00**
Ethanol yield on sugar (mol mol Corrected for evaporation	**1.56 ± 0.02**	**1.73 ± 0.01**

> 90 % reduction of glycerol production

11 % increase of ethanol yield

Victor Guadalupe et al. (2013)
Biotechnology for Biofuels – 6:125

TU Delft

Strategies aiming at increasing ethanol yields on sugar include:

- Decreasing glycerol and/or biomass formation
- Decreasing free-energy conservation
- Improving yeast robustness towards process stressors (e.g. high ethanol concentrations, low pH, high T, etc.)
- Improving the process (e.g. using bacteriocins or other strategies to fight contamination, thereby decreasing sugar loss)
- 1G ethanol processes will not be substituted by 2G processes!

Thanks for your attention!

Andreas K. Gombert
gombert@unicamp.br

Visit our lab's webpage:

http://www.fea.unicamp.br/leb

Visit the Ph.D. program in Bioenergy's webpage:

http://genfis40.esalq.usp.br/pg_bio/

There does not exist a category of science to which one can give the name applied science. There are sciences and the applications of science, bound together as the fruit of the tree which bears it.
Louis Pasteur (1822-1895).