



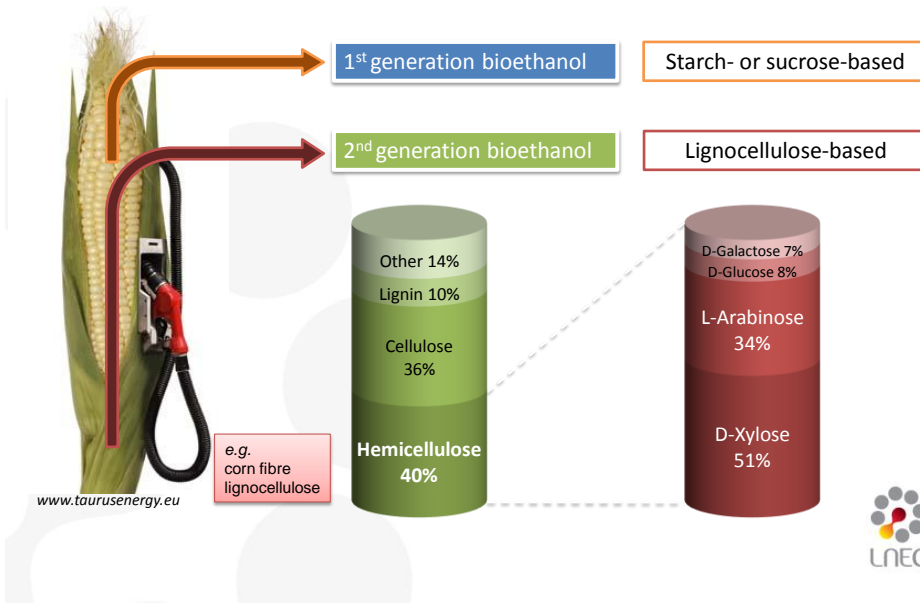
## SECOND GENERATION BIOETHANOL: THE CHALLENGES OF PENTOSE FERMENTATION

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Laboratório Nacional de Energia e Geologia (LNEG)



### 1G vs 2G Bioethanol



### Hemicellulose composition



Raw material	Xyl	Ara	Man	Gal	Rha	UA	AcG
<b>Softwoods</b>							
Douglas fir	6.0	3.0	-	3.7	-	-	-
Pine	5.3-10.6	2.0-4.2	5.6-13.3	1.9-3.8	-	2.5-6.0	1.2-1.9
Spruce	5.3-10.2	1.0-1.2	9.4-15.0	1.9-4.3	0.3	1.8-5.8	1.2-2.4
<b>Hardwoods</b>							
Aspen	18-27.3	0.7-4.0	0.9-2.4	0.6-1.5	0.5	4.8-5.9	4.3
Birch	18.5-24.9	0.3-0.5	1.8-3.2	0.7-1.3	0.6	3.6-6.3	3.7-3.9
Black locust	16.7-18.4	0.4-0.5	1.1-2.2	0.8	-	4.7	2.7-3.8
Eucalypt	14-19.1	0.6-1	1-2.0	1-1.9	0.3-1	2	3-3.6
Maple	18.1-19.4	0.8-1	1.3-3.3	1.0	-	4.9	3.6-3.9
Oak	21.7	1.0	2.3	1.9	-	3	3.5
Poplar	17.7-21.2	0.9-1.4	3.3-3.5	1.1	-	2.3-3.7	0.5-3.9
Sweet gum	19.9	0.5	0.4	0.3	-	2.6	2.3
Sycamore	18.5	0.7	1.0	-	-	-	3.6
Willow	11.7-17.0	2.1	1.8-3.3	1.6-2.3	-	-	-
<b>Agricultural and agro-industrial materials</b>							
Almond shells	34.3	2.5	1.9	0.6	-	-	-
Barley straw	15	4.0	-	-	-	-	-
Brewery's spent grain	15	8	0	1	0	2	0.8
Cardoon	26.0	2.5	3.7	1.4	0.9	-	-
Corn cobs	28-35.3	3.2-5.0	-	1-1.2	1	3	1.9-3.8
Corn fibre	21.6	11.4	-	4.4	-	-	-
Corn stalks	25.7	4.1	<3.0	<2.5	-	-	-
Corn stover	14.8-25.2	2-3.6	0.3-0.4	0.8-2.2	-	-	1.7-1.9
Olive stones	2.0-3.7	1.1-1.2	0.2-0.3	0.5-0.7	0.3-0.5	1.2-2.2	-
Rice husks	17.7	1.9	-	1.8	-	-	1.62
Rice straw	14.8-23	2.7-4.5	1.8	0.4	-	-	-
Sugar cane bagasse	20.5-25.6	2.3-6.3	0.5-0.6	1.6	-	-	-
Wheat bran	16	9	0	1	0	2	0.4
Wheat straw	19.2-21.0	2.4-3.8	0-0.8	1.7-2.4	-	-	-

Non-glycosidic units.

Expressed as g/100 g of dry material.

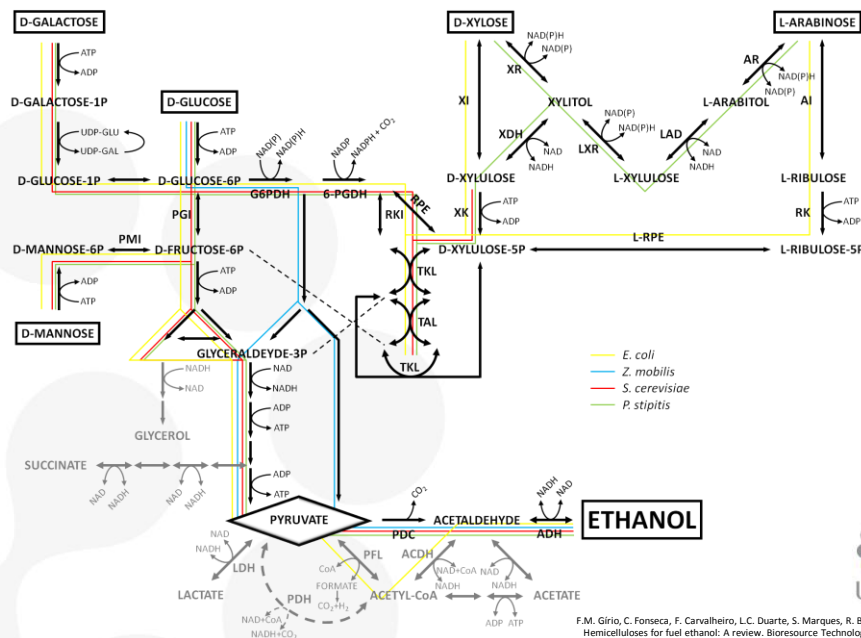
The percentages of oses were, in some cases, calculated from the corresponding "polymers".

Xyl, xylose; Ara, arabinose; Man, mannose; Gal, galactose; Rha, rhamnase; UA, uronic acids; AcG, acetyl groups.



F.M. Girio, C. Fonseca, F. Carvalho, L.C. Duarte, S. Marques, R. Bogel-Lukasik, 2010. Hemicelluloses for fuel ethanol: A review. *Bioresource Technology* 101, 4775-4800.

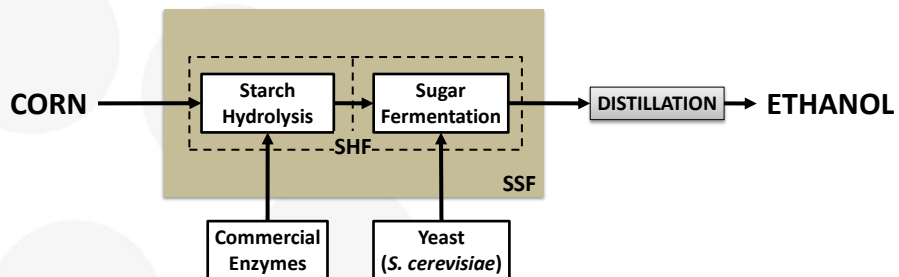
### Pathways of hemicellulose-derived monosaccharides to ethanol



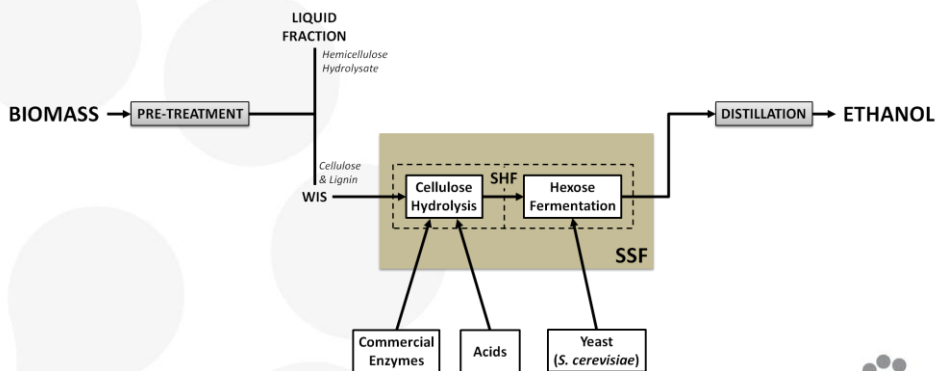
F.M. Girio, C. Fonseca, F. Carvalho, L.C. Duarte, S. Marques, R. Bogel-Lukasik, 2010. Hemicelluloses for fuel ethanol: A review. *Bioresource Technology* 101, 4775-4800.



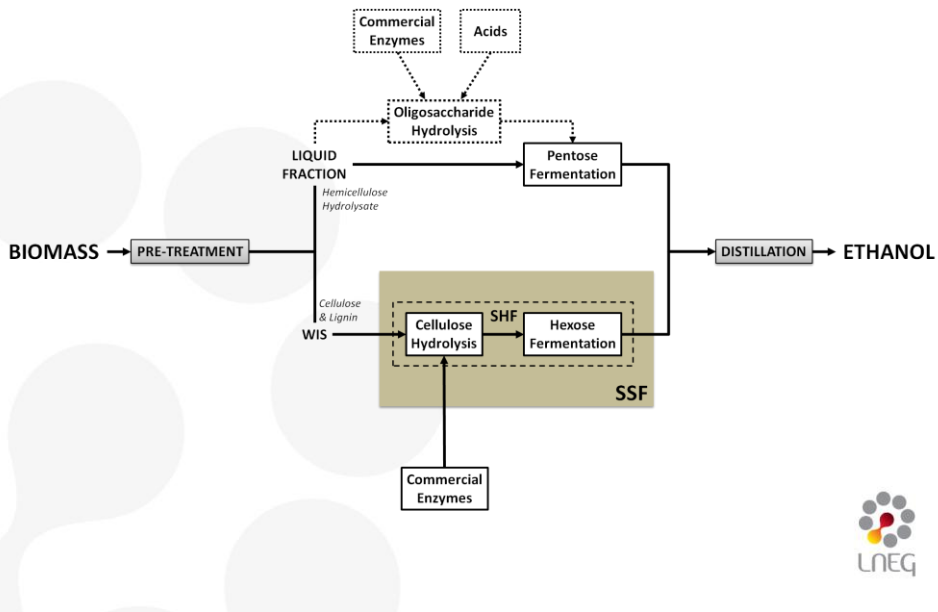
## 1G Bioethanol Bioprocesses

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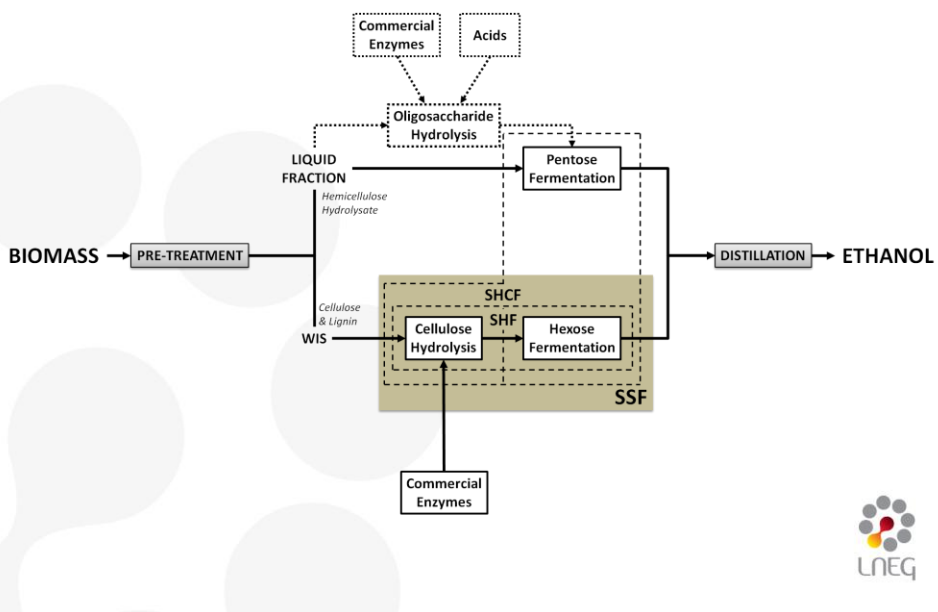
## 2G Bioethanol Bioprocesses

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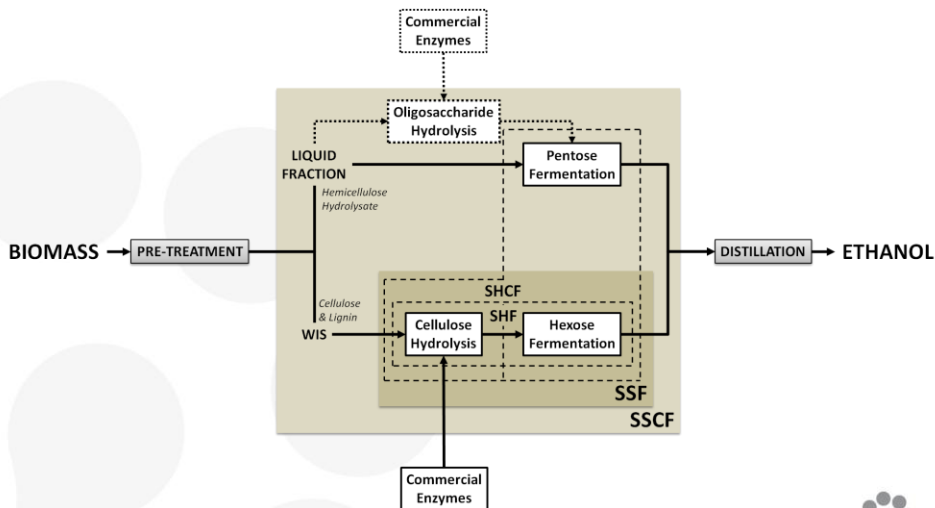
## 2G Bioethanol Bioprocesses

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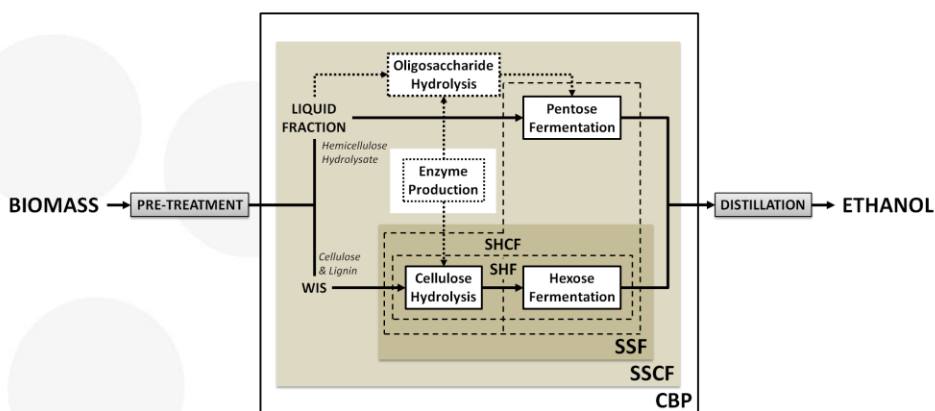
## 2G Bioethanol Bioprocesses

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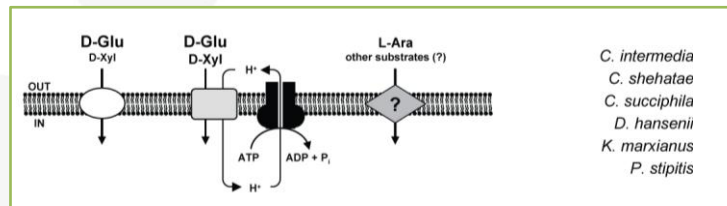
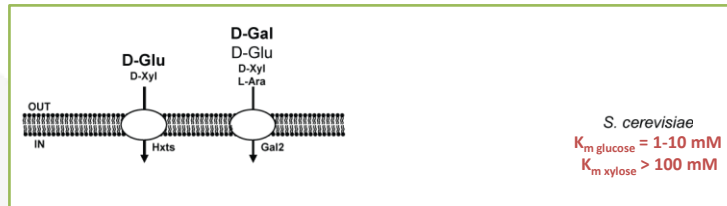
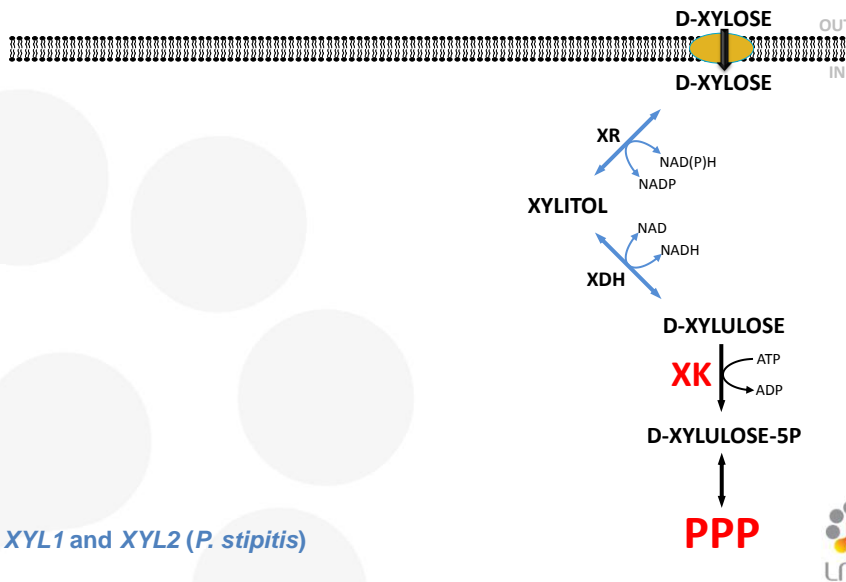
2G Bioethanol Bioprocesses



2G Bioethanol Bioprocesses

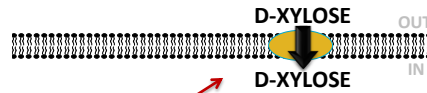
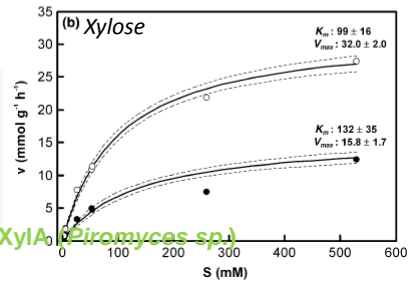
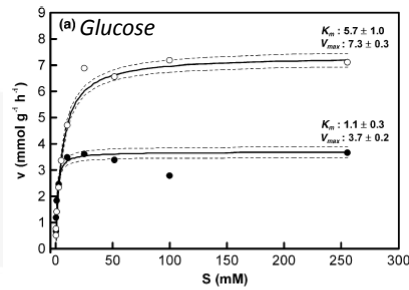




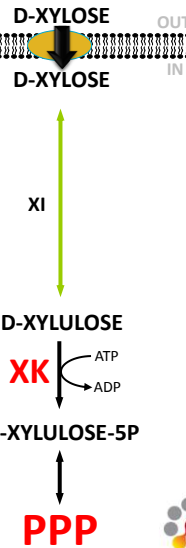
Sugar transport in *S. cerevisiae* and other yeasts2010  
CIENCIAEngineering *S. cerevisiae* for pentose fermentation2010  
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Karhumaa et al. 2005. Yeast 22, 359-368.

Engineering *S. cerevisiae* for pentose fermentation

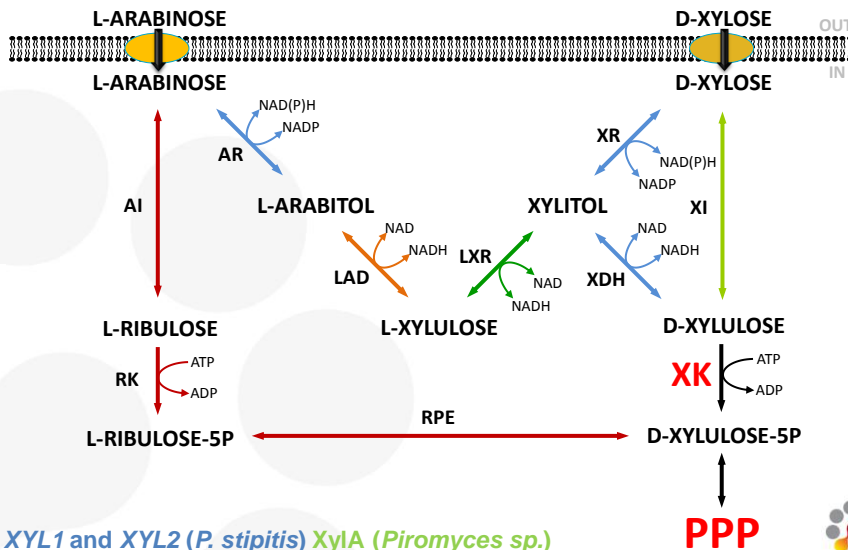


Evolutionary engineering of mix-sugar utilization



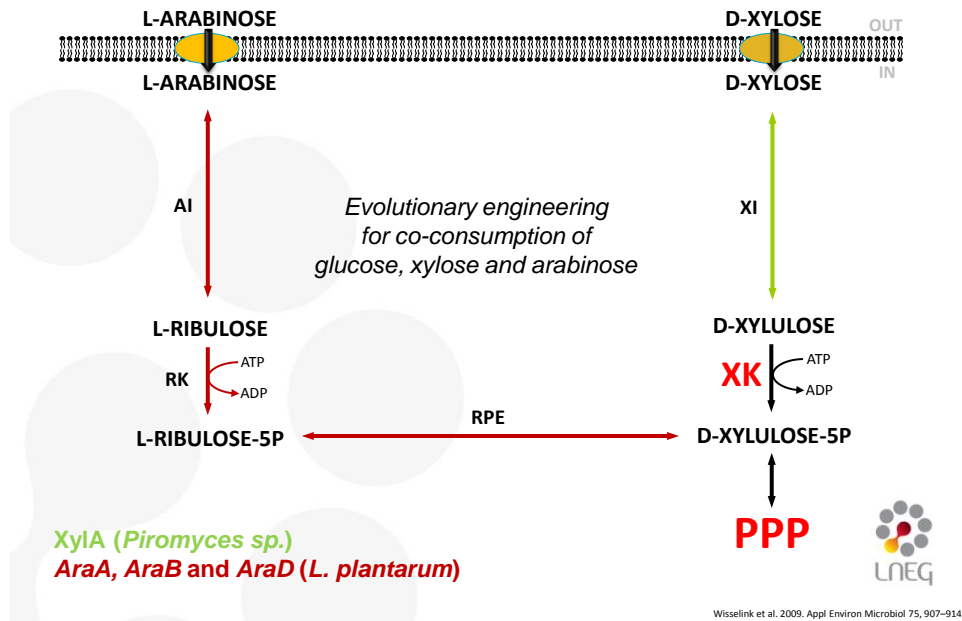
Kuyper et al. 2005. FEMS Yeast Res 5, 925-934.

Engineering *S. cerevisiae* for pentose fermentation



*XYL1* and *XYL2* (*P. stipitis*) *XylA* (*Piromyces* sp.)  
*AraA* (*B. subtilis*), *AraB* (*E. coli*) and *AraD* (*E. coli*)  
*LAD1* (*T. reesei*) *ALX1* (*A. monospora*)



Engineering *S. cerevisiae* for pentose fermentation2010  
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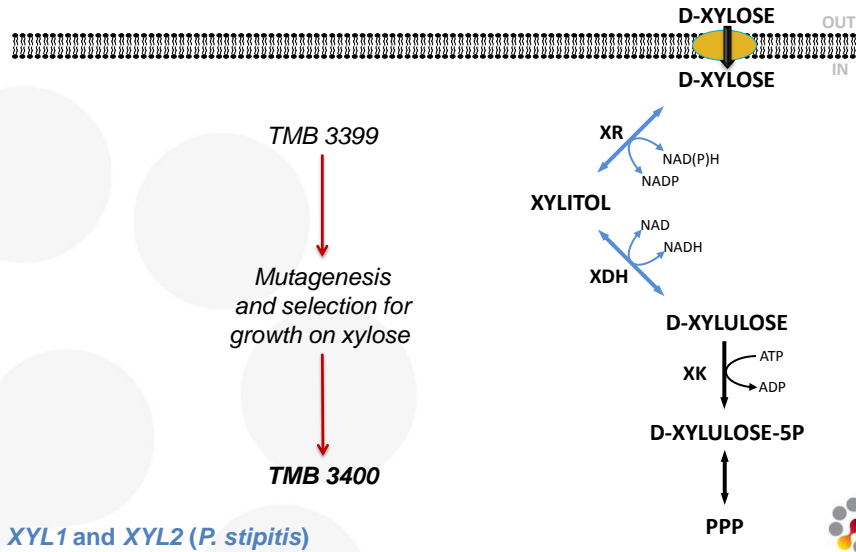
## XR/XDH vs XI and lab vs industrial strains

Table 2: Results of anaerobic batch fermentation of xylose (50 g/l) by strains TMB 3057, TMB 3066 and TMB 3400.

Strain	Relevant genotype	Xylose consumed in 100 h	Final ethanol concentration	Specific xylose consumption rate	Specific ethanol productivity	Yield		
						Ethanol	Xylitol	Glycerol
		g l <sup>-1</sup>	g l <sup>-1</sup>	g xylose g cells <sup>-1</sup> h <sup>-1</sup>	g ethanol g cells <sup>-1</sup> h <sup>-1</sup>	g g consumed xylose <sup>-1</sup>	g g consumed xylose <sup>-1</sup>	g g consumed xylose <sup>-1</sup>
TMB 3057	XR-XDH, plasmid	39.6 ± 3.4	13.3 ± 1.7	0.13 ± 0.04	0.04 ± 0.01	0.33 ± 0.02	0.22 ± 0.03	0.11 ± 0.02
TMB 3066	XI, plasmid	16.8 ± 3.8	7.3 ± 2.1	0.05 ± 0.02	0.02 ± 0.01	0.43 ± 0.03	0.04 ± 0.02	0.07 ± 0.02
TMB 3400	XR-XDH, integrated	36.5 ± 10.8	12.1 ± 2.5	0.06 ± 0.02	0.02 ± 0.01	0.34 ± 0.03	0.29 ± 0.07	0.04 ± 0.01

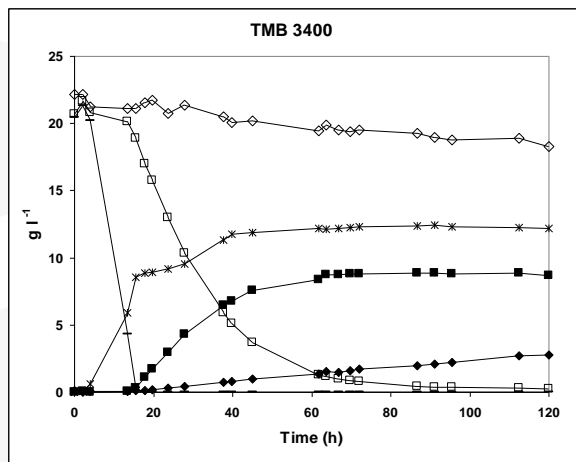
LNEG

Karhumaa et al. 2007. Microb Cell Fact 6, 5.

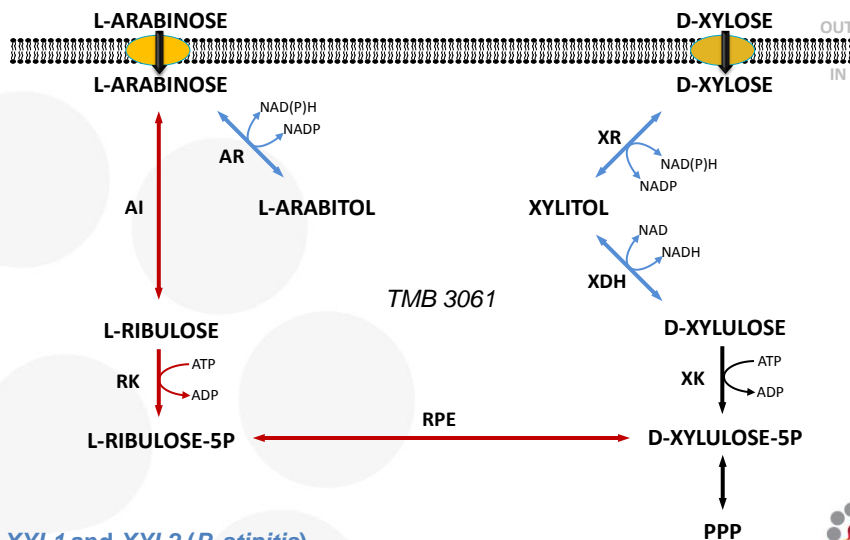
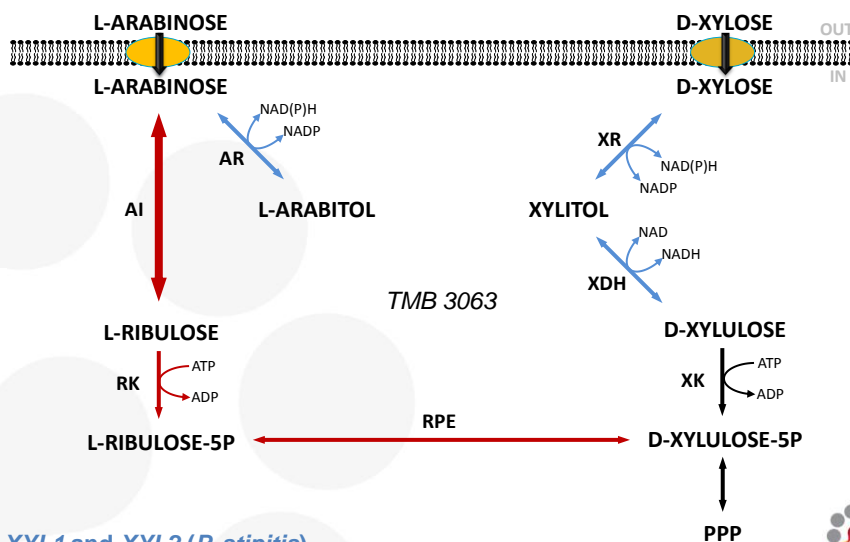


Wahlbom et al. 2003. FEMS Yeast Res 3, 319-326.

Anaerobic fermentation (glucose, xylose and arabinose - 20 g/L each)



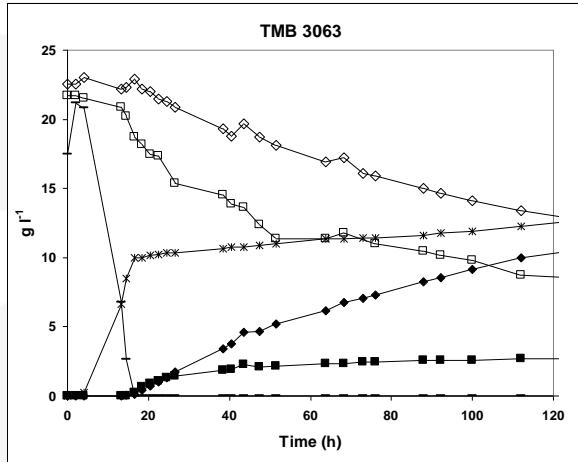
Garcia Sanchez R., K. Karhumaa, C. Fonseca, V. Sanchez Nogue, J.R. Almeida, C.U. Larsson, O. Bengtsson, M. Bettiga, B. Hahn-Hägerdal and M.F. Gorwa-Grauslund (2010) Improved xylose and arabinose utilization by an industrial recombinant *Saccharomyces cerevisiae* strain using evolutionary engineering. *Biotechnol Biofuels* 3:13.

*XYL1* and *XYL2* (*P. stipitis*)*AraA* (*B. subtilis*), *AraB* (*E. coli*) and *AraD* (*E. coli*)*XYL1* and *XYL2* (*P. stipitis*)*AraA* (*B. subtilis*), *AraB* (*E. coli*) and *AraD* (*E. coli*)

Engineering industrial *S. cerevisiae* for pentose fermentation

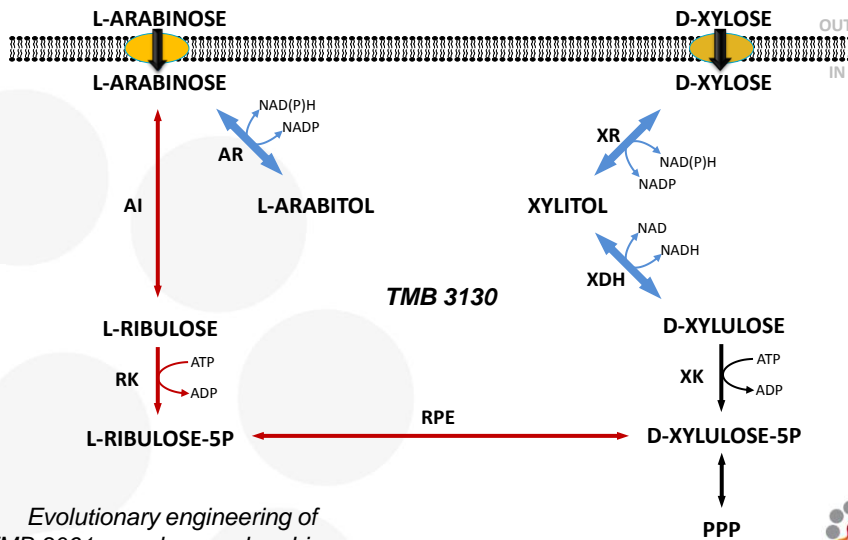


Anaerobic fermentation (glucose, xylose and arabinose - 20 g/L each)



García Sánchez R., K. Karhumaa, C. Fonseca, V. Sánchez Nogue, J.R. Almeida, C.U. Larsson, O. Bengtsson, M. Bettiga, B. Hahn-Hägerdal and M.F. Gorwa-Grauslund (2010) Improved xylose and arabinose utilization by an industrial recombinant *Saccharomyces cerevisiae* strain using evolutionary engineering. *Biotechnol Biofuels* 3:13.

Engineering industrial *S. cerevisiae* for pentose fermentation



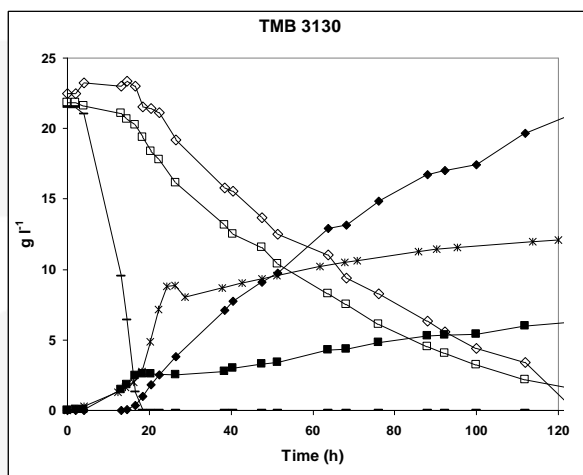
Evolutionary engineering of TMB 3061 on xylose and arabinose



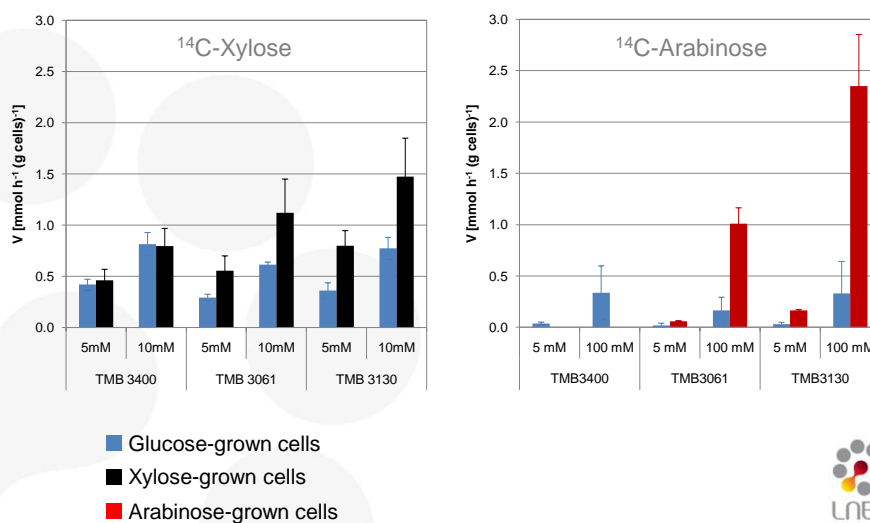
García Sánchez R., K. Karhumaa, C. Fonseca, V. Sánchez Nogue, J.R. Almeida, C.U. Larsson, O. Bengtsson, M. Bettiga, B. Hahn-Hägerdal and M.F. Gorwa-Grauslund (2010) Improved xylose and arabinose utilization by an industrial recombinant *Saccharomyces cerevisiae* strain using evolutionary engineering. *Biotechnol Biofuels* 3:13.

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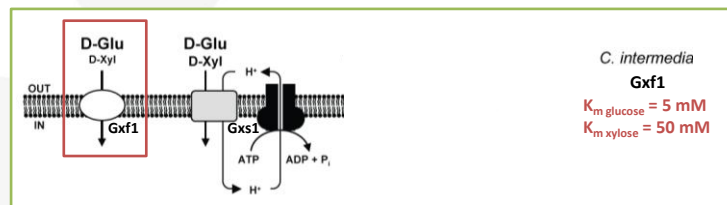
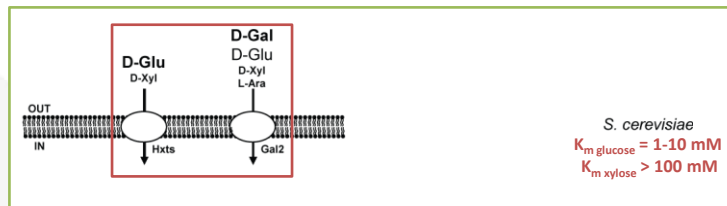
Anaerobic fermentation (glucose, xylose and arabinose - 20 g/L each)



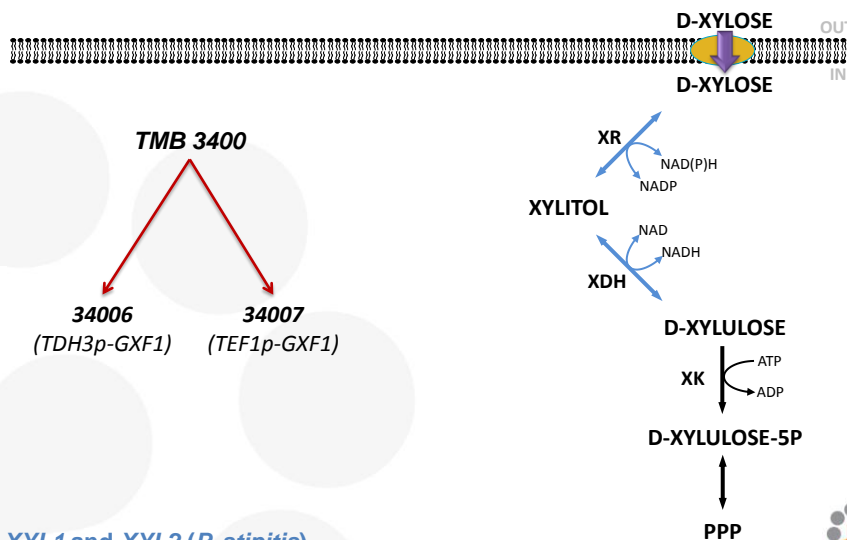
García Sánchez R., K. Karhumaa, C. Fonseca, V. Sánchez Nogué, J.R. Almeida, C.U. Larsson, O. Bengtsson, M. Bettiga, B. Hahn-Hägerdal and M.F. Gorwa-Grauslund (2010) Improved xylose and arabinose utilization by an industrial recombinant *Saccharomyces cerevisiae* strain using evolutionary engineering. *Biotechnol Biofuels* 3:13.

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CIENCIA $^{14}\text{C}$ -Xylose and  $^{14}\text{C}$ -Arabinose uptake in industrial strains

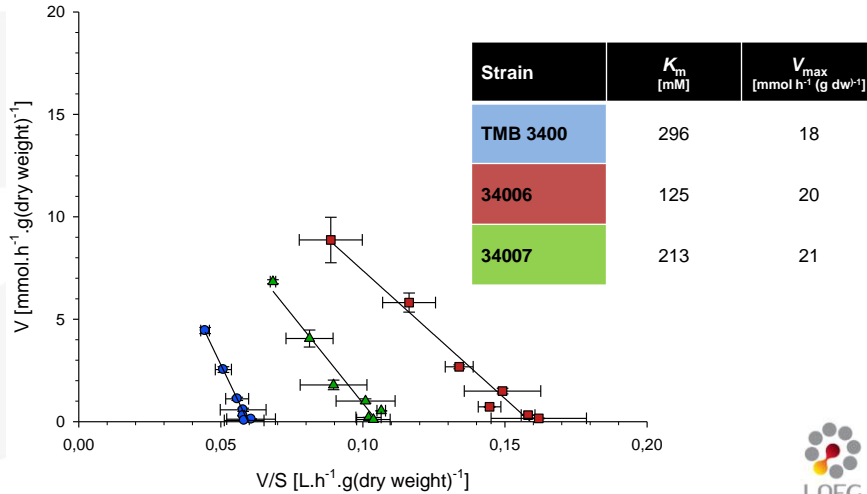
García Sánchez R., K. Karhumaa, C. Fonseca, V. Sánchez Nogué, J.R. Almeida, C.U. Larsson, O. Bengtsson, M. Bettiga, B. Hahn-Hägerdal and M.F. Gorwa-Grauslund (2010) Improved xylose and arabinose utilization by an industrial recombinant *Saccharomyces cerevisiae* strain using evolutionary engineering. *Biotechnol Biofuels* 3:13.

Glucose/xylose facilitator from *C. intermedia*2010  
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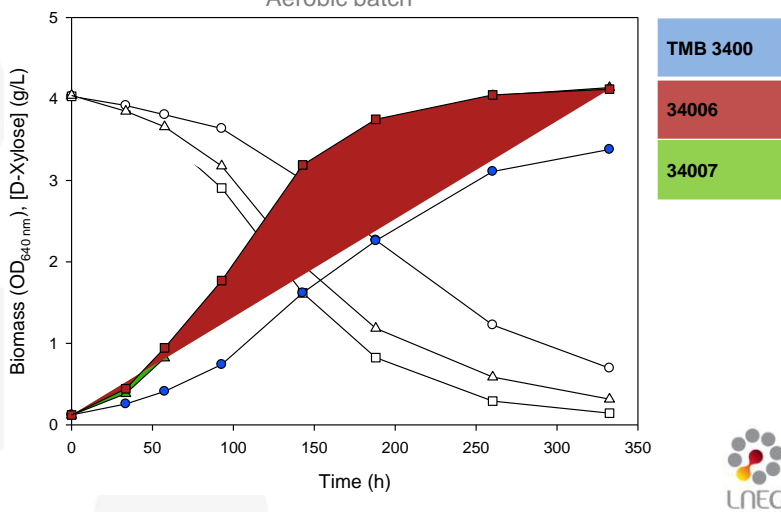
Gárdonyi et al. 2003. FEMS Yeast Res 3, 45-52.  
 Leandro et al. 2006. Biochem J 395, 543-549.

Engineering industrial *S. cerevisiae* for xylose transport2010  
CIENCIA

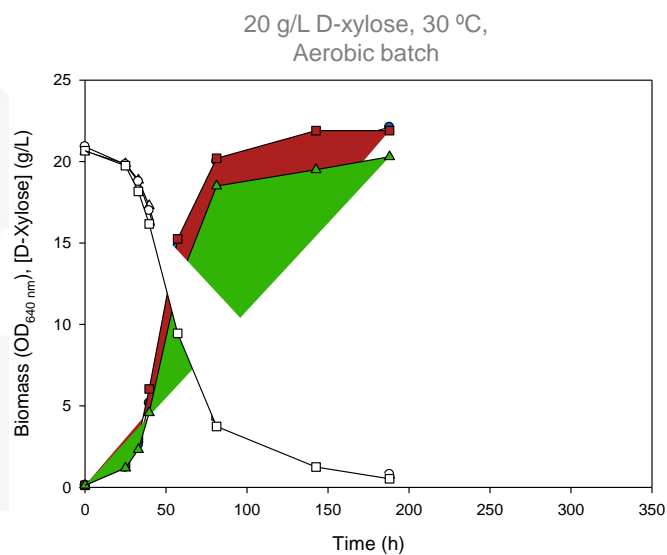
Fonseca C., K. Olofsson, D. Runquist, L.L. Fonseca, B. Hahn-Hägerdal, G. Lidén. Submitted.

D-[U-<sup>14</sup>C]Xylose uptake

Fonseca C., K. Olofsson, D. Runquist, L.L. Fonseca, B. Hahn-Hägerdal, G. Lidén. Submitted.

4 g/L D-xylose, 30 °C,  
Aerobic batch

Fonseca C., K. Olofsson, D. Runquist, L.L. Fonseca, B. Hahn-Hägerdal, G. Lidén. Submitted.



Fonseca C., K. Olofsson, D. Runquist, L.L. Fonseca, B. Hahn-Hägerdal, G. Lidén. Submitted.

Determination initial growth rates (h<sup>-1</sup>)

Strain	Initial D-xylose concentration (g/L)		
	4	20	40
TMB3400	0.022 ± 0.003	0.092 ± 0.004	0.082 ± 0.001
34006	0.039 ± 0.000	0.101 ± 0.001	0.085 ± 0.001
34007	0.038 ± 0.001	0.086 ± 0.007	0.080 ± 0.002



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CIENCIA20 g/L D-xylose (20X) or 20 g/L D-xylose + 20 g/L D-glucose (20X20G), 30 °C  
Anaerobic batch

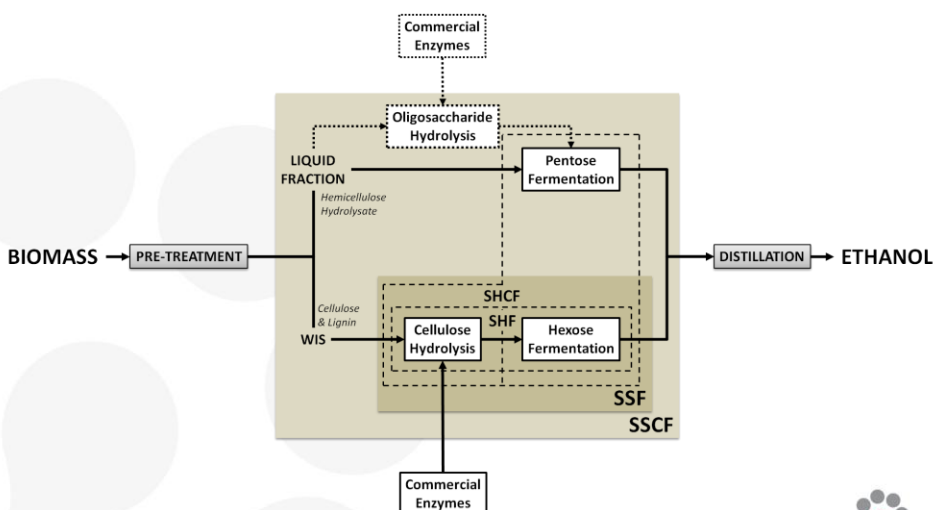
Strain (Condition)	$Y_{X/S}$ (g <sub>dw</sub> /g)	$Y_{XOH}^a$ (g/g)	$Y_{Gly}$ (g/g)	$Y_{Ac}$ (g/g)	$Y_{EtOH}$ (g/g)	$q_{Xyl}^b$ (g/h/g <sub>dw</sub> )	$q_{EtOH}^b$ (g/h/g <sub>dw</sub> )
<b>20X</b>							
TMB3400	0.03 ± 0.00	0.30 ± 0.03	0.07 ± 0.00	0.03 ± 0.00	0.27 ± 0.04	-0.22 ± 0.03	0.10 ± 0.01
34006	0.02 ± 0.01	0.32 ± 0.02	0.06 ± 0.00	0.02 ± 0.00	0.28 ± 0.01	-0.23 ± 0.04	0.11 ± 0.00
34007	0.02 ± 0.01	0.30 ± 0.02	0.06 ± 0.00	0.02 ± 0.01	0.28 ± 0.02	-0.24 ± 0.04	0.11 ± 0.00
<b>20X20G</b>							
TMB3400	0.04 ± 0.00	0.20 ± 0.00	0.14 ± 0.01	0.02 ± 0.00	0.34 ± 0.01	-0.28 ± 0.09	0.06 ± 0.02
34006	0.04 ± 0.00	0.24 ± 0.03	0.14 ± 0.01	0.02 ± 0.00	0.34 ± 0.00	-0.29 ± 0.07	0.07 ± 0.01
34007	0.05 ± 0.00	0.24 ± 0.00	0.11 ± 0.01	0.02 ± 0.00	0.34 ± 0.01	-0.29 ± 0.08	0.07 ± 0.01

$Y_{X/S}$  biomass yield,  $Y_{XOH}$  xylitol yield,  $Y_{Gly}$  glycerol yield,  $Y_{Ac}$  acetate yield,  $Y_{EtOH}$  ethanol yield,  $q_{Xyl}$  xylose specific consumption rate,  $q_{Xyl}$  specific xylose consumption rate,  $q_{EtOH}$  specific ethanol productivity.  
<sup>a</sup> In parenthesis is xylitol yield calculated only from xylose.  
<sup>b</sup> Specific xylose consumption and ethanol productivity determined when glucose is depleted in 20X20G.



Fonseca C., K. Olofsson, D. Runquist, L.L. Fonseca, B. Hahn-Hägerdal, G. Lidén. Submitted.

## SSCF of steam-pretreated wheat straw

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## SSCF of steam-pretreated wheat straw

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## Composition of the steam-pretreated wheat straw

Content in solid fraction (% of)			Concentration in the liquid fraction (g/L)				
Glucan	Xylan	Lignin	Glucose*	Xylose*	Acetic acid	HMF	Furfural
54.4	3.1	32.8	6.7	38.8	4.9	0.5	3.4

\*Both monomeric and oligomeric form is included.

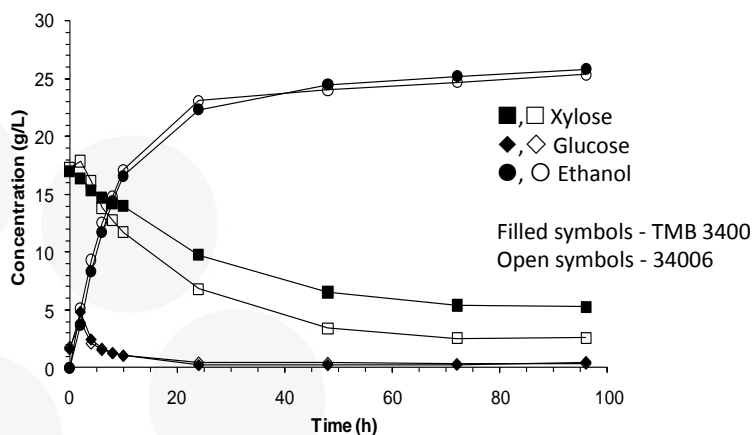
## SSCF conditions

- 7% WIS
- pH 5.0
- Enzymes - Xylanase XL & Novozyme 188
- Initial yeast concentration of 4 g(dry weight)/L (TMB 3400 & 34006)
- 34°C
- 96 h



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## SSCF of steam-pretreated wheat straw

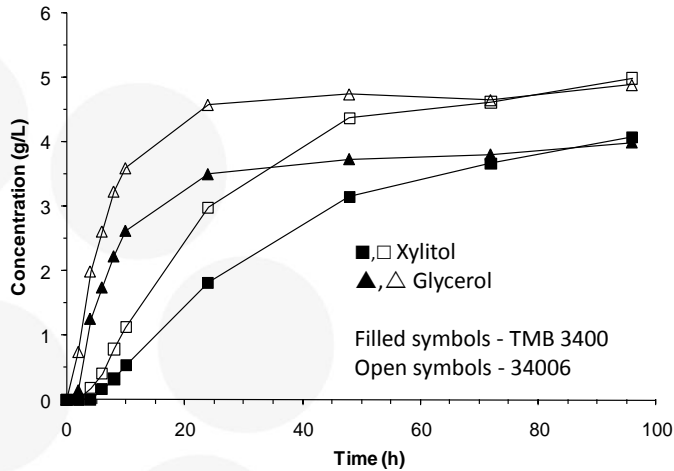
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	Final ethanol conc. (g/L)	Ethanol yield (g/g)	Ethanol yield (%)	Xylose uptake (%)
TMB3400	25.8	0.38	75	76.0
34006	25.4	0.38	75	88.3



Fonseca C., K. Olofsson, D. Runquist, L.L. Fonseca, B. Hahn-Hägerdal, G. Lidén. Submitted.

## SSCF of steam-pretreated wheat straw

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## Final Remarks

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- The initial catabolic reactions control pentose metabolism, therefore requiring further improvement through:
  - New enzyme activities (isomerases, NADH-dependent reductases)
  - Protein engineering (towards optimization of co-factor specificity)
  - Metabolic engineering towards cofactor balancing
  - Identification of specific and/or high affinity transporters
- Adaptation to inhibitors present in lignocellulosic hydrolysates
- Production of optimized CBP systems (for each lignocellulosic material)
- Optimization of SSCF and CBP processes
- Integrated second generation bioethanol biorefinery



## Aknowledgements

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FCT  
FACULDADE DE  
CIÊNCIAS E TECNOLOGIA  
UNIVERSIDADE NOVA DE LISBOA  
**CREM**  
Centro de Recursos Microbiológicos

- Prof. Isabel Spencer-Martins
- EU Project "New Improvements for Lignocellulosic Ethanol" (NILE), FP6 – 19882



**ITQB**

- Luís L. Fonseca and Prof. Helena Santos (ITQB/UNL)
- *Fundação para a Ciência e a Tecnologia* (SFRH/BPD/47082/2008)



- Rosa Garcia-Sanchez, David Runquist, Maurizio Bettiga, Prof. Marie-Françoise Gorwa-Grauslund and Prof. Bärbel Hahn-Hägerdal (Applied Microbiology, Lund University, Sweden)
- Kim Olofsson and Prof. Gunnar Lidén (Chemical Engineering, Lund University, Sweden)



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