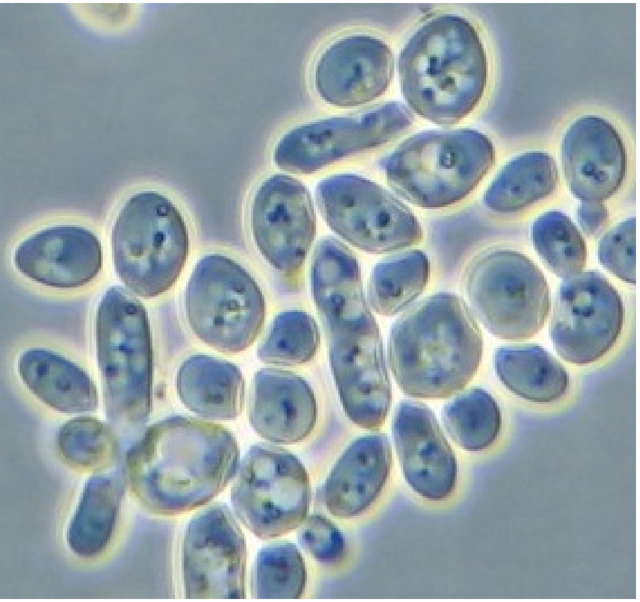

Development of industrial yeast strains for economically sustainable production of advanced biofuels

NovelYeast

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New Delhi
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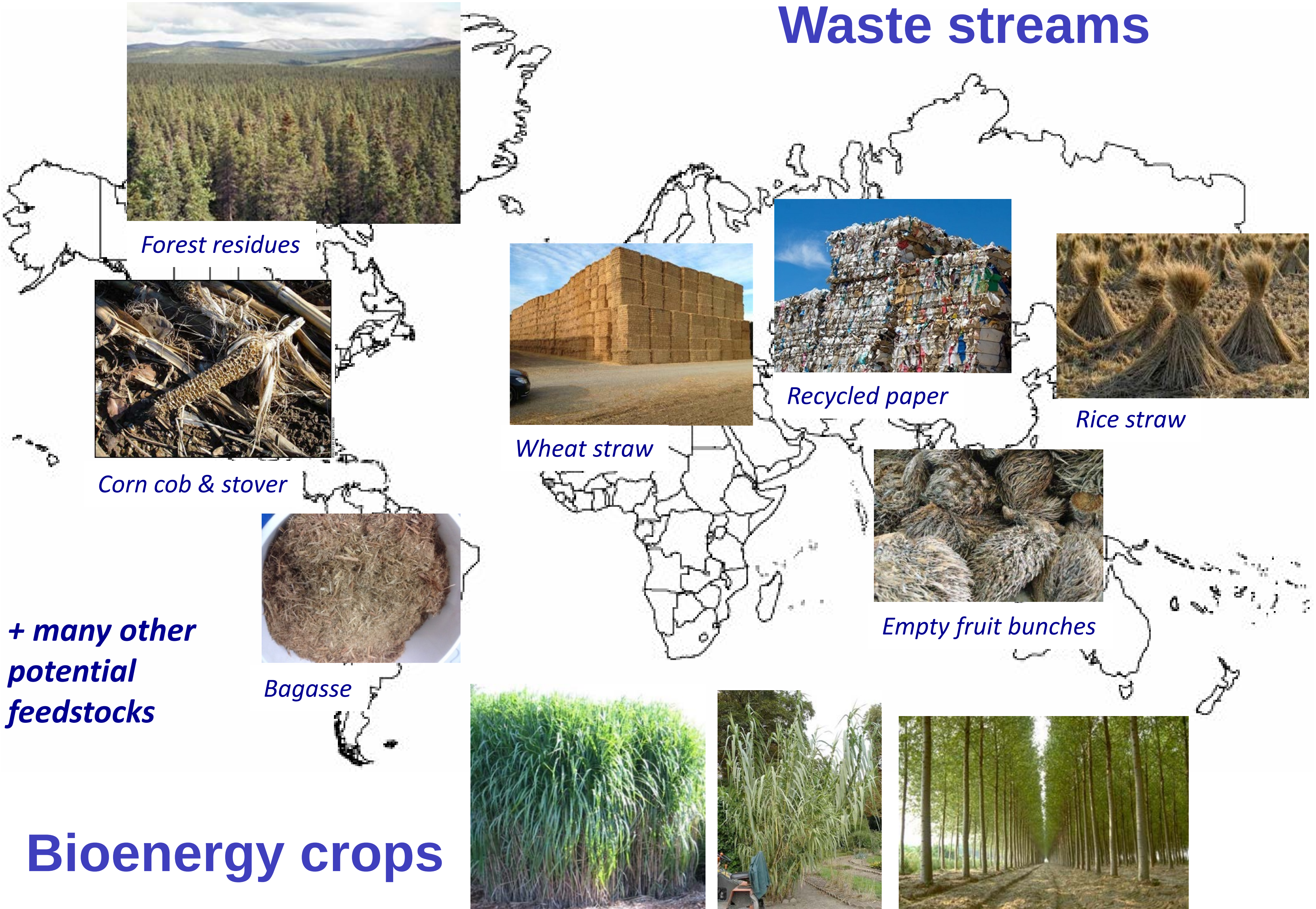
2G bioethanol and bio-chemicals production

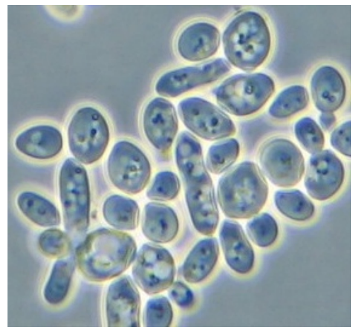


1G and 2G bioethanol production

- Sugar

1G YEAST

- 1G Bioethanol



- Biomass

2G YEAST

- 2G Bioethanol

Main challenges

- Efficient xylose fermentation
- High inhibitor tolerance

Demonstration plant in upstart

- Renasci NV: demonstration plant in harbor of Ostend, Belgium; fully operational in Q2 2020
- Recycling of Municipal Solid Waste: all fractions recycled/valorised; 120,000 ton/year

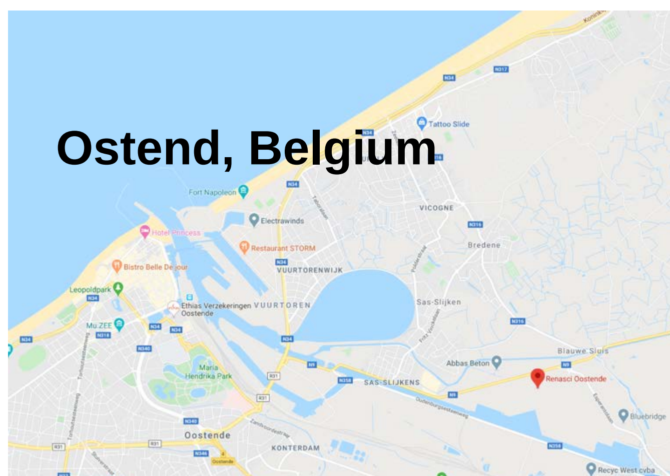
Fermentation unit in design

Organic fraction: mainly paper/cardboard (35,000 ton/year)
→ ± 5 million L ethanol/isobutanol
- Conversion to ethanol, established (fed-batch, partial SSF, 8-10% v/v)
- Conversion to isobutanol, 2G isobutanol strain under development

NovelYeast

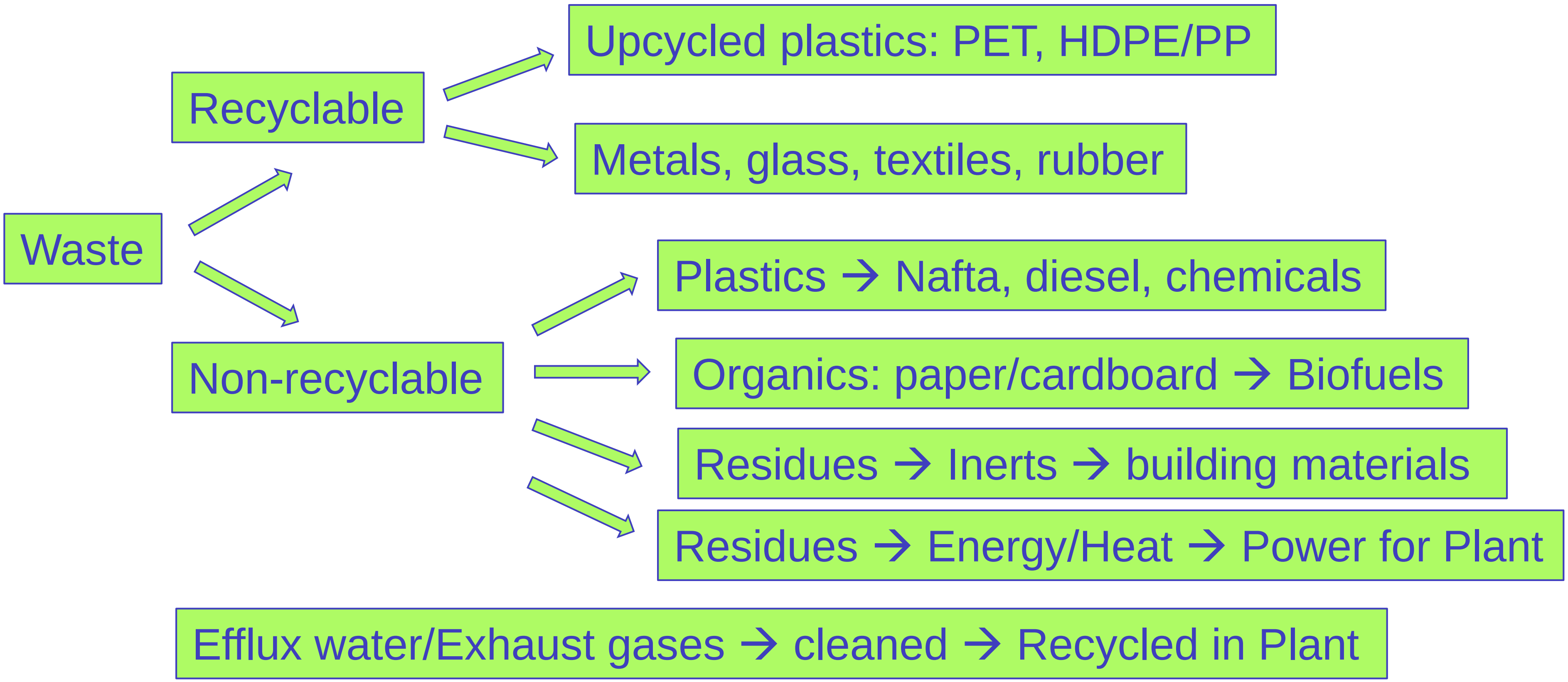
Isobutanol → Isobutene → + Glycerol (from biodiesel production) → GTBE (Glycerol Tertiary Butyl Ether: valuable fuel additive for diesel and gasoline that improves engine performance and lowers harmful exhaust emissions)
Isobutanol → Biojetfuel

EU-project *BIOREN*



Renasci

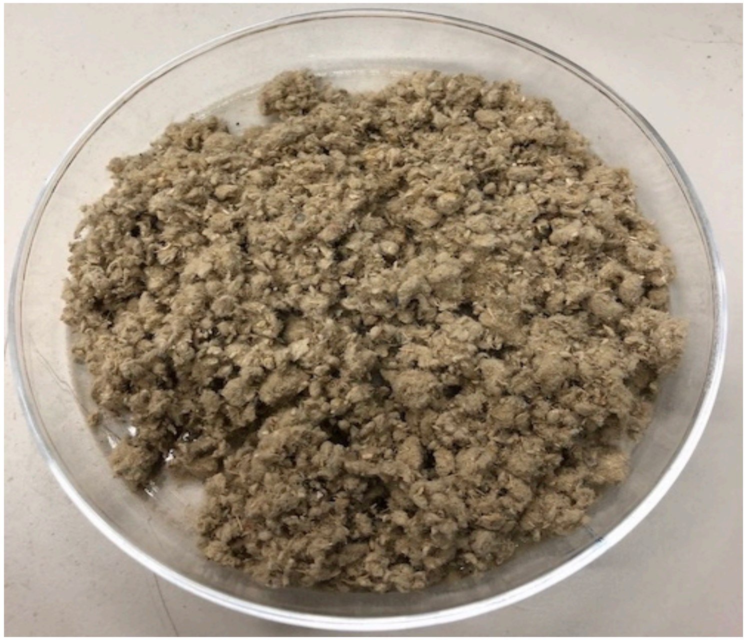
Demonstration plant



Fully integrated concept → Major synergies

Paper/cardboard fermentation unit

7 MW of energy
→ ethanol distillation
→ isobutanol distillation (vacuum at low temperature)
→ water purification by evaporation



Paper pulp

Cellulose + Hemicellulose : 55-60% (w/w)
Lignin: 8-10% (w/v)
Filler: 25% $CaCO_3$
Others: 3-5%

Sugar	Glucose	Xylose	Arabinose
Concentration (%w/v)	9.3	2.1	0.02

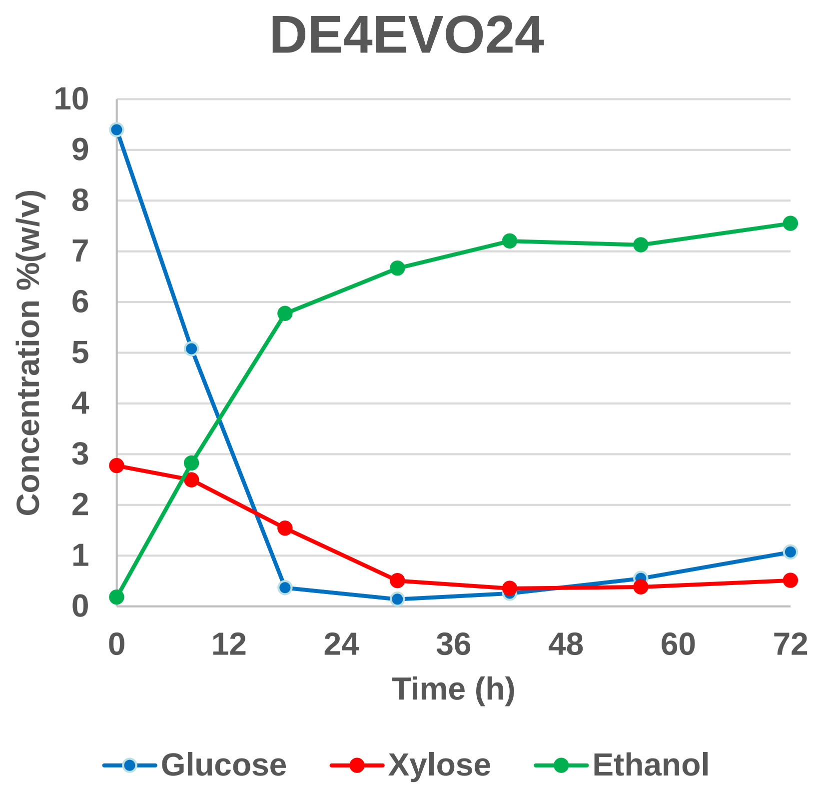
Fed-batch → high solids loads

A1 : 25 % (w/v) solids with 2.32 g Cellic CTEC 3
B1 : 27 % (w/v) solids with 2.51 g Cellic CTEC 3
C1 : 30 % (w/v) solids with 2.79 g Cellic CTEC 3
D1 : 33 % (w/v) solids with 3.07 g Cellic CTEC 3

Partial Simultaneous Saccharification and Fermentation

Enzymatic liquefaction/SSF

Total Process Time (Saccharification +Fermentation)	Total Fermentable sugar released (Glucose+ Xylose) % (w/v)	Saccharification yield (%)
Saccharification condition (55˚ C)		
18H	12.89	62.44
Fermentation condition (35˚ C)		
26H	13.217	64.00
36H	13.623	65.97
48H	16.993	82.29
60H	18.103	87.66
74H	18.536	89.76
90H	19.489	94.37



Time (h)	Ethanol produced % (v/v)	Yield (%)
42	9.1	78.0
56	9.0	75.4
72	9.6	76.0

Major challenge in 2G bioethanol production
High cost of commercial enzyme cocktails

- Enzymatic hydrolysis of paper pulp
  → ±25 % of the cost of the ethanol

→

- **Enzymatic liquefaction at low solids load**
- **Slow fed-batch to increase solids load**
- **Switch to Simultaneous Saccharification and Fermentation**

→

E2G yeast with secreted enzymes

- reduce enzyme requirement

- Holy grail: 'Consolidated bioprocessing'

yeast: enzymatic hydrolysis + fermentation

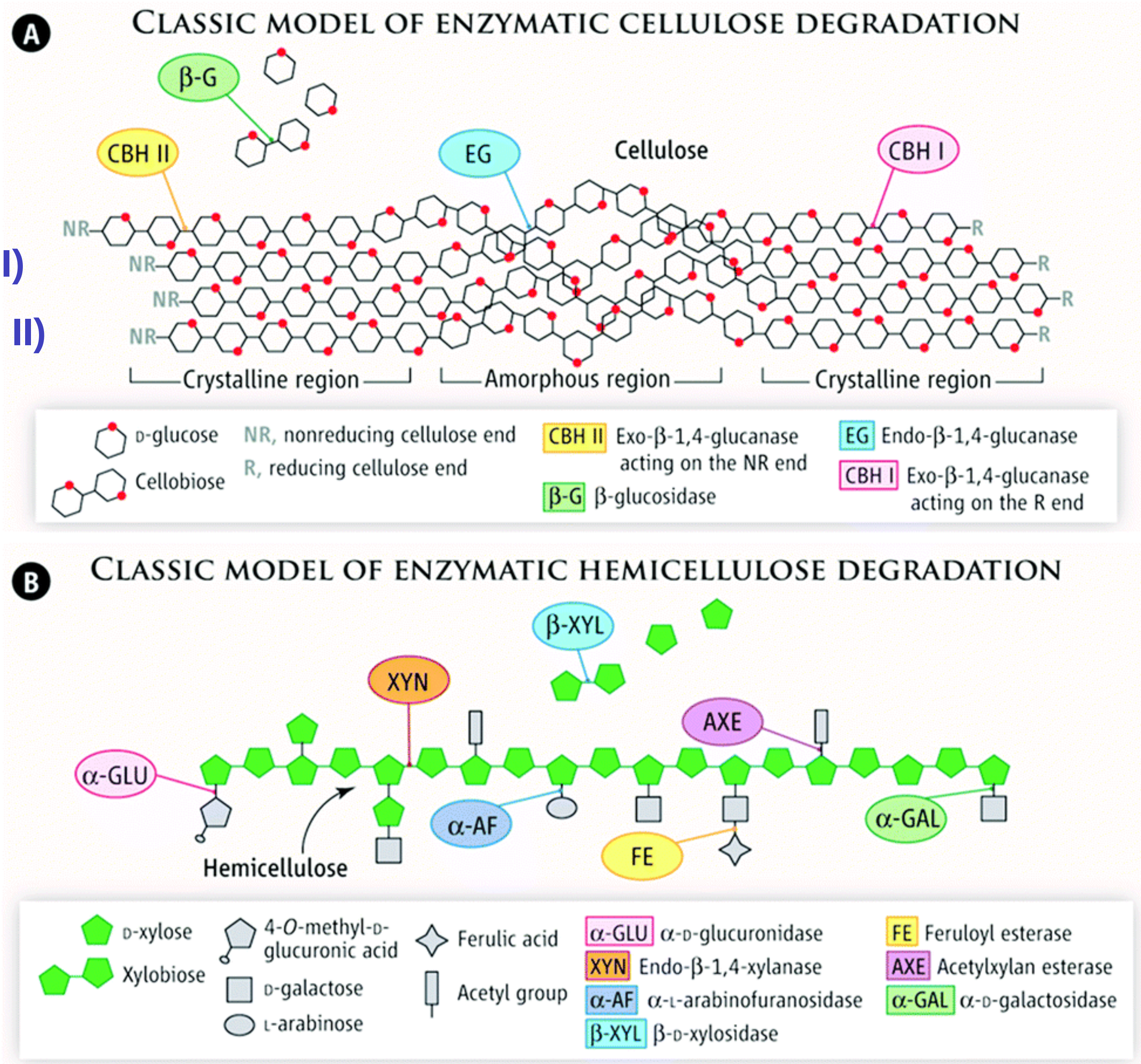
Types of enzymes required

Cellulolytic enzymes:

- β-glucosidase (BGL)
- Endoglucanase (EG)
- Cellobiohydrolase I (CBH I)
- Cellobiohydrolase II (CBH II)

Hemicellulolytic enzymes:

- β-xylosidase (β-XYL)
- Xylanase (XYN)



Consecutive integration of 4-5 copies of each gene

MD4
- Industrial xylose-fermenting inhibitor-tolerant *S. cerevisiae* strain
- 4n-5n; aneuploid for some chromosomes

AC1
- Genomic integration of ß-glucosidase (BGL) of *Trichoderma reesei* at IS2.1 in ChrII
- Capacity for cellobiose hydrolysis

AC6
- Genomic integration of ß-xylosidase (BXL) of *Aspergillus niger* at IS7.1 in ChrVII
- Capacity for xylobiose hydrolysis

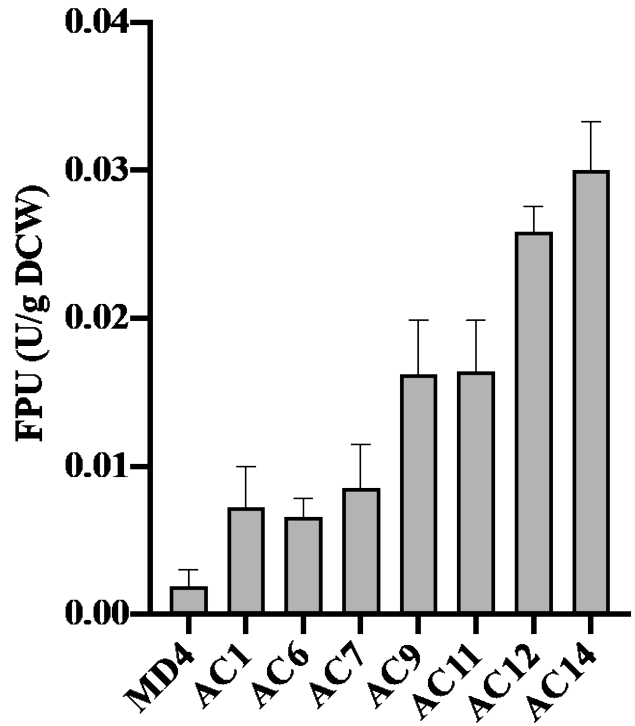
AC7
- Genomic integration of xylanase (XYL) of *Aspergillus niger* at IS16.1 in ChrXVI
- Capacity for breakdown of corncob and beechwood xylan

AC9
- Genomic integration of endoglucanase (EG) of *Aspergillus oryzae* at IS16.2 in ChrXVI
- Capacity for hydrolysis of amorphous regions of cellulose

AC11
- Genomic integration of cellobiohydrolase II (CBHII) of *Chrysosporium lucknowense* at IS4.2 in ChrIV
- Capacity for hydrolysis of non-reducing ends of cellulose

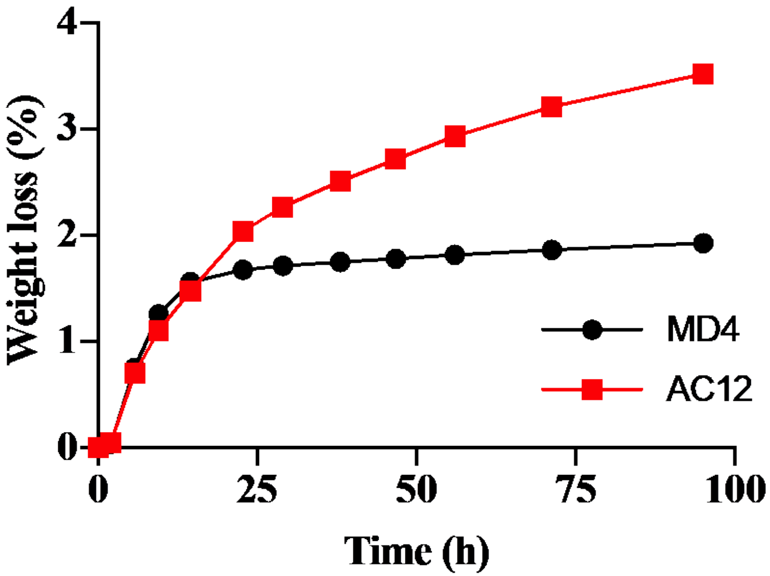
AC12
- Genomic integration of cellobiohydrolase I (CBHI) of *Talaromyces emersonii* at IS4.1 in ChrIV
- Capacity for hydrolysis of reducing ends of cellulose; complete breakdown of cellulose

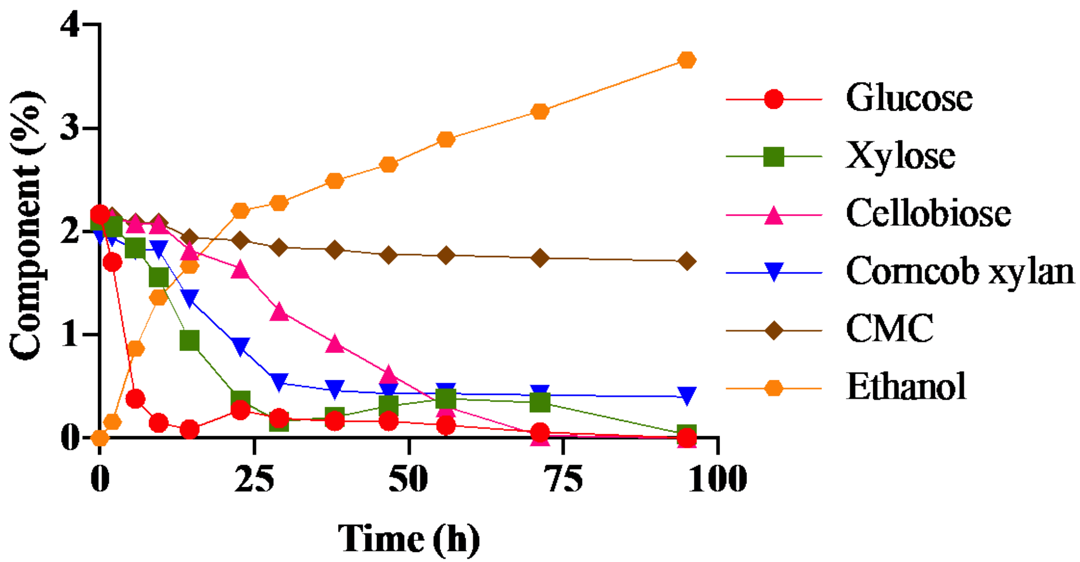
AC14
- Genomic integration of acetylxylan esterase/xylanase (AXE) of *Clostridium cellulovorans* at IS16.3 in ChrXVI
- Improved breakdown of corncob xylan



Fermentation performance of AC12 (6 enzymes)

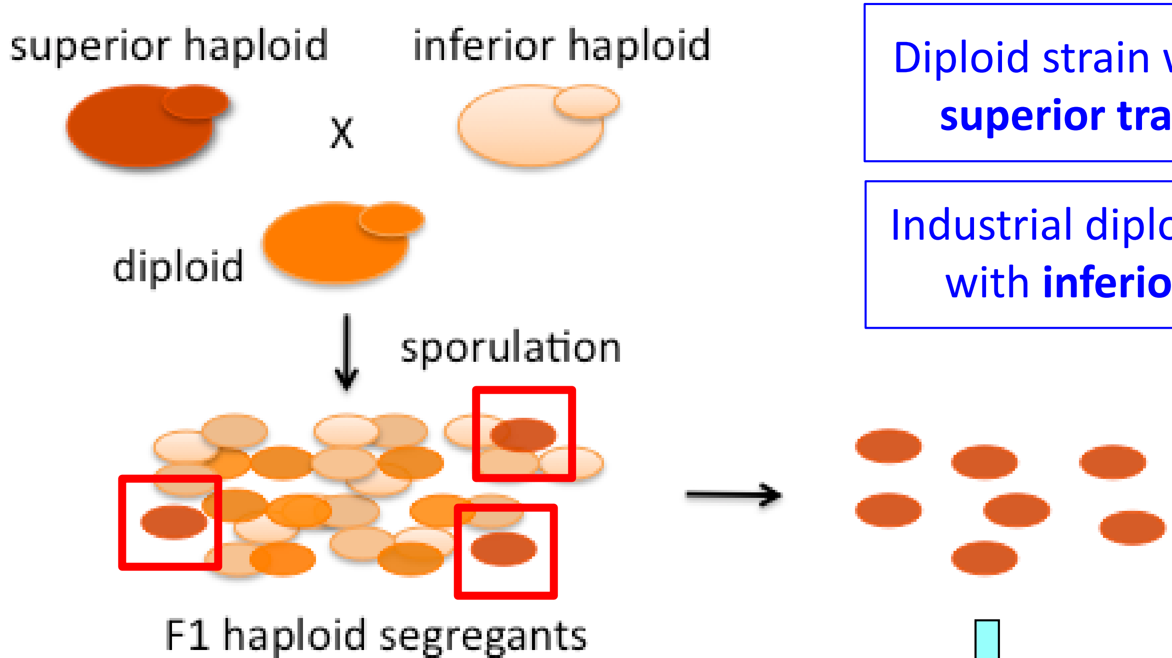
- Fermentation of YP with 2% glucose, 2% xylose, 2% cellobiose, 2% corncob xylan and 2% CMC at 35° C, initial pitching of 1 g DCW/L





- Monomeric substrates are utilized and converted to ethanol at the same rate with AC12 as with MD4
- Polymeric substrates are consumed by AC12
- CMC breakdown is very slow

Polygenic analysis platform for complex traits: pooled-segregant whole-genome sequence analysis



F1 superior haploids
min. ± 30 segr.

Diploid strain with **superior trait** ⇦ Screen of *S. cerevisiae* strain collection

Industrial diploid strain with **inferior trait**

Pooled

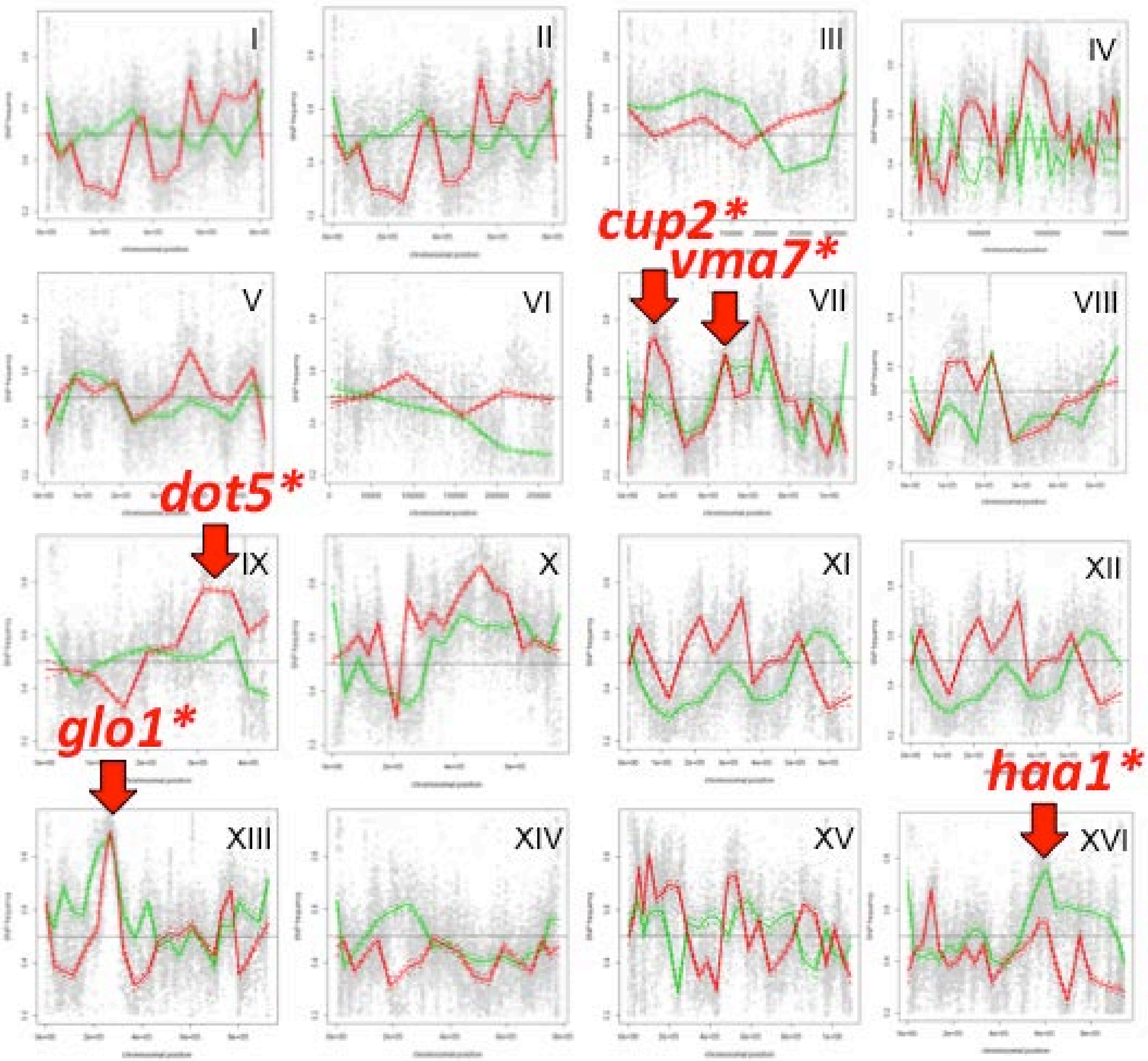
Extraction of genomic DNA

2 parent strains ⇨ Whole-genome sequence analysis (Illumina) ⇨ QTL mapping (Quantitative Trait Locus)

Acetic acid tolerance of fermentation

F1 segregants

F7 segregants



Known:
Haa1: transcription factor involved in acetic acid tolerance

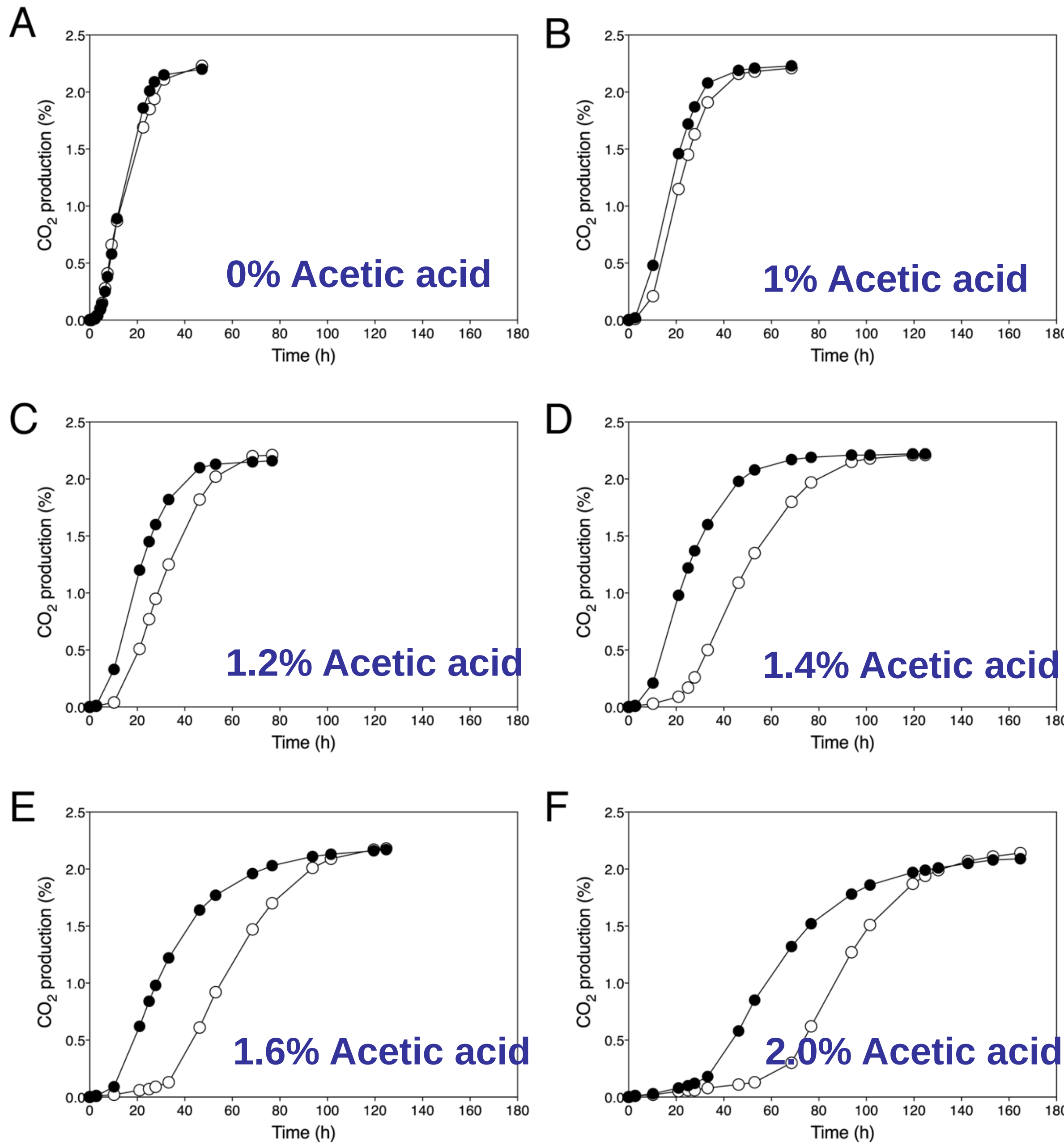
New:
Cup2: homolog of Haa1
Dot5
Glo1
Vma7

HAA1* **: unique mutation in acetic acid tolerant strain**

Insertion of G → A mutation in *HAA1* (2 alleles) of T18

○ **T18**

● **T18 HAA1***



Predictable improvement of stress tolerance

Polygenic analysis of industrially important traits for 2G bioethanol production

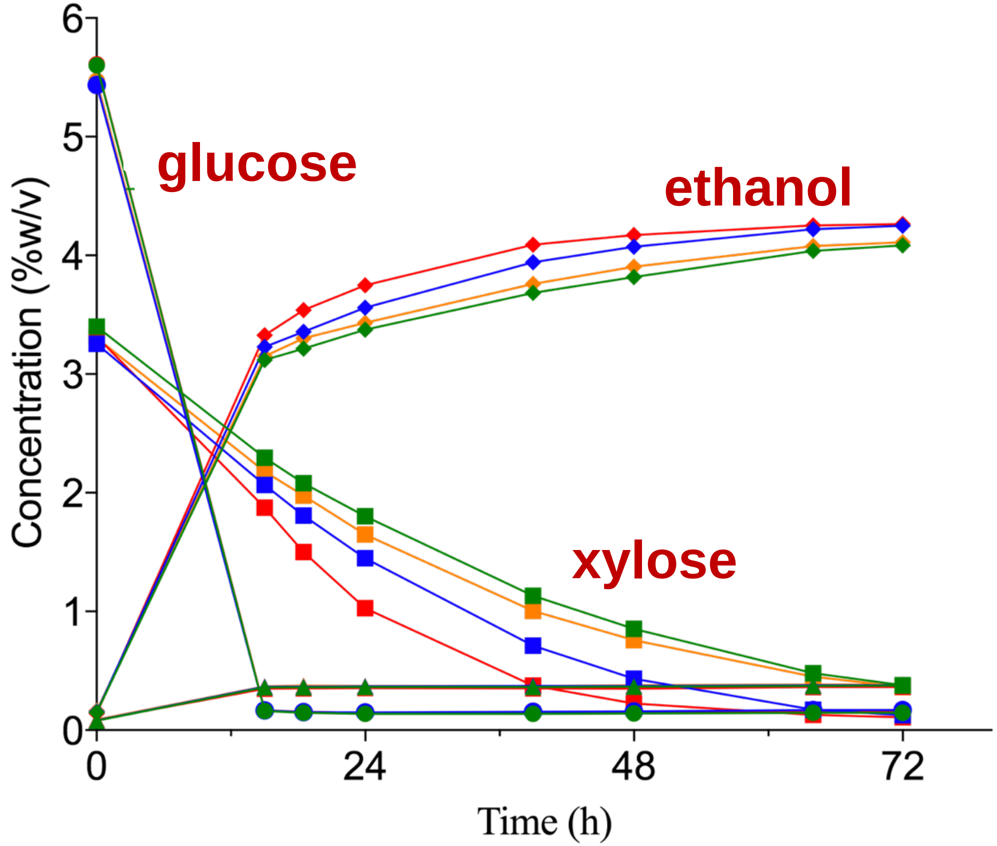
- Xylose fermentation rate
- Acetic acid tolerance
- Furfural tolerance
- HMF tolerance
- Thermotolerance
- Low glycerol/high ethanol production

➡ **Portfolio of superior alleles for targeted industrial strain improvement**

Industrial strains with high xylose fermentation capacity

Further improvement by evolutionary adaptation, genome shuffling, targeted genetic engineering with superior alleles, whole-genome transformation

→ steady improvement of performance



Semi-anaerobic static fermentation in bagasse hydrolysate

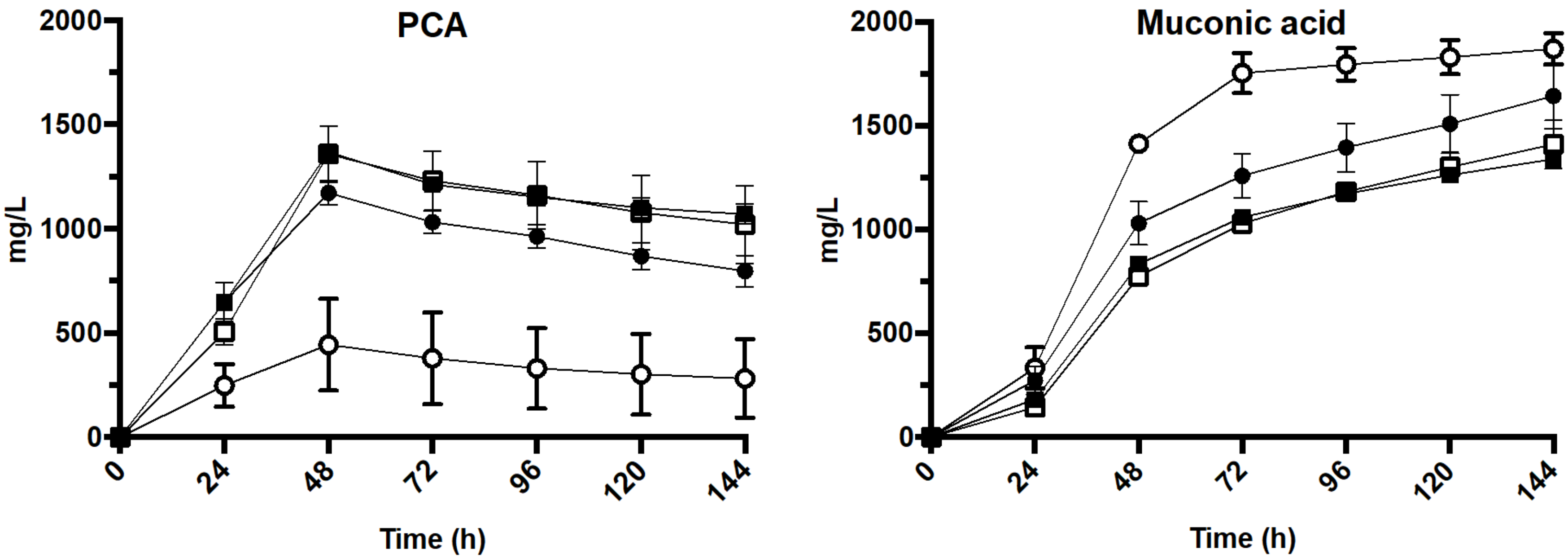
Goal for commercial E2G production

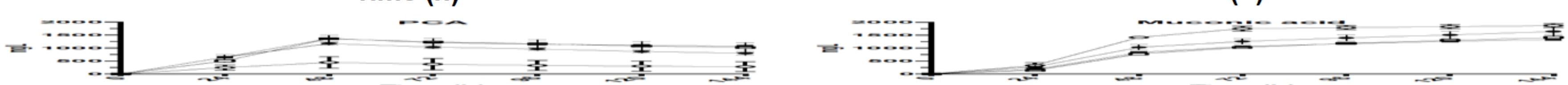
> 80% of sugar in 48 h with 1 g DW yeast/L and > 5% (v/v) ethanol titer

Production of muconic acid with glucose as substrate

Muconic Acid pathway

Glucose ----→ PCA → Catechol → Muconic acid





- Muconic acid (2 – 2.5 g/L) toxicity limits muconic acid production
- In situ removal of muconic acid during fermentation essential
- Appropriate solvent recently identified

Conclusions

2G bioethanol and bio-chemicals production

- Efficient industrial yeast strains for second-generation bioethanol production available: xylose utilization + high inhibitor tolerance

- Further improvement for better performance in undetoxified lignocellulose hydrolysates → cheaper pretreatment technologies

- Demonstration plant for paper pulp → ethanol/isobutanol/GTBE (Q3-4 2020/Q1-2 2021)

- Strong 2G platform strain for secreted enzyme expression
  Reduction of enzyme load/cost → Partial Consolidated BioProcessing

- Strong platform for cell factory strains to produce bio-based chemicals with lignocellulosic biomass

Thank you for your attention