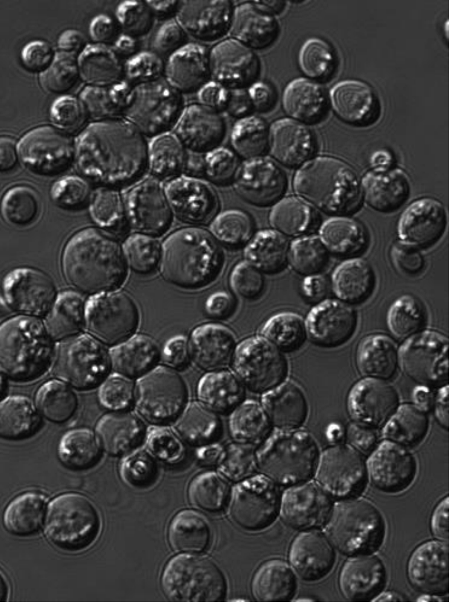

Development of superior xylose-fermenting and robust industrial yeast strains for lignocellulosic bioethanol production

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VIB (Flanders Institute of Biotechnology)

- Interuniversity non-profit research institute, with 8 departments located at 4 universities in Flanders
- Headquarters in Ghent
- ± 1200 researchers
- Financed by Ministry of Economy of Flanders
- Flemish government: strong interest in economic innovation
- Mission: excellent research, industrial valorisation, training
- Contractual agreement with Flemish government, minimum requirements for: number of papers in high impact journals, income from projects with companies, minimum number of patent applications and granted patents, one new spin-off company per year, minimum training of PhD students, education of the public, biotech community development (FlandersBio), etc.
- Own technology transfer office: patent applications, financing for translational research, limited seed capital, business development and licensing managers

Department of Molecular Microbiology

- Fundamental research on nutrient sensing and signaling in yeast and its role in control of fermentation, stress tolerance, growth, etc.
- Development of novel genomic technologies for genetic analysis of complex (polygenic) traits
- Development of superior industrial yeast strains:
  1^{st} and 2^{nd} generation bioethanol production, isobutanol production, beer brewing, bakery, wine production, etc.
- Pathogenic yeasts: *Candida albicans, Candida glabrata*
- Trehalose metabolism in yeast and plants

Yeast for 2nd-generation bioethanol

Main (best-known) challenges

- Inability of *Saccharomyces cerevisiae* to utilize **C5 sugars**: xylose and arabinose
  → Xylose constitutes up to 35% of all sugars in lignocellulosic biomass
- Inability of *Saccharomyces cerevisiae* to tolerate the high levels of **inhibitors** generated in lignocellulose hydrolysates

Other major, often overlooked, challenges

- Requirement of **robust industrial yeast strain** with optimal performance under all industrially-relevant conditions
  → **fermentation, production, drying, storage/transport**
  → major drawback of alternative microorganisms (lab strains, baker's yeast, other yeast species, bacteria, …)

Lignocellulose hydrolysates

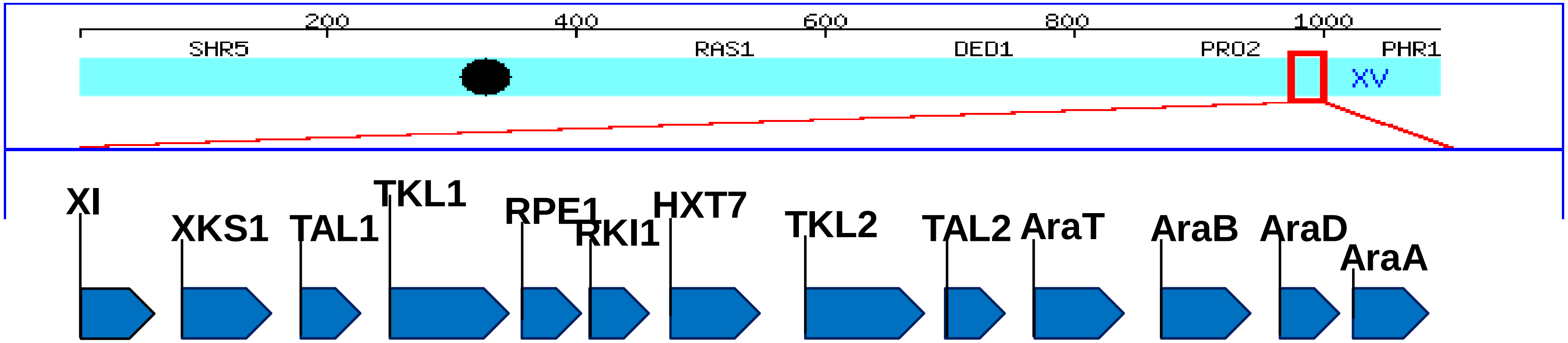
- Very dense, sticky material
- Difficult to prepare with high free sugar content (repeated enzymatic hydrolysis/addition of biomass)
- high levels of inhibitors: furan derivatives furfural and HMF; aliphatic acids: acetic acid, formic acid and levulinic acid; phenolic compounds: vanillic acid, vanillin, syringaldehyde, syringic acid and 4-hydroxybenzoic acid

Choice of the yeast strain

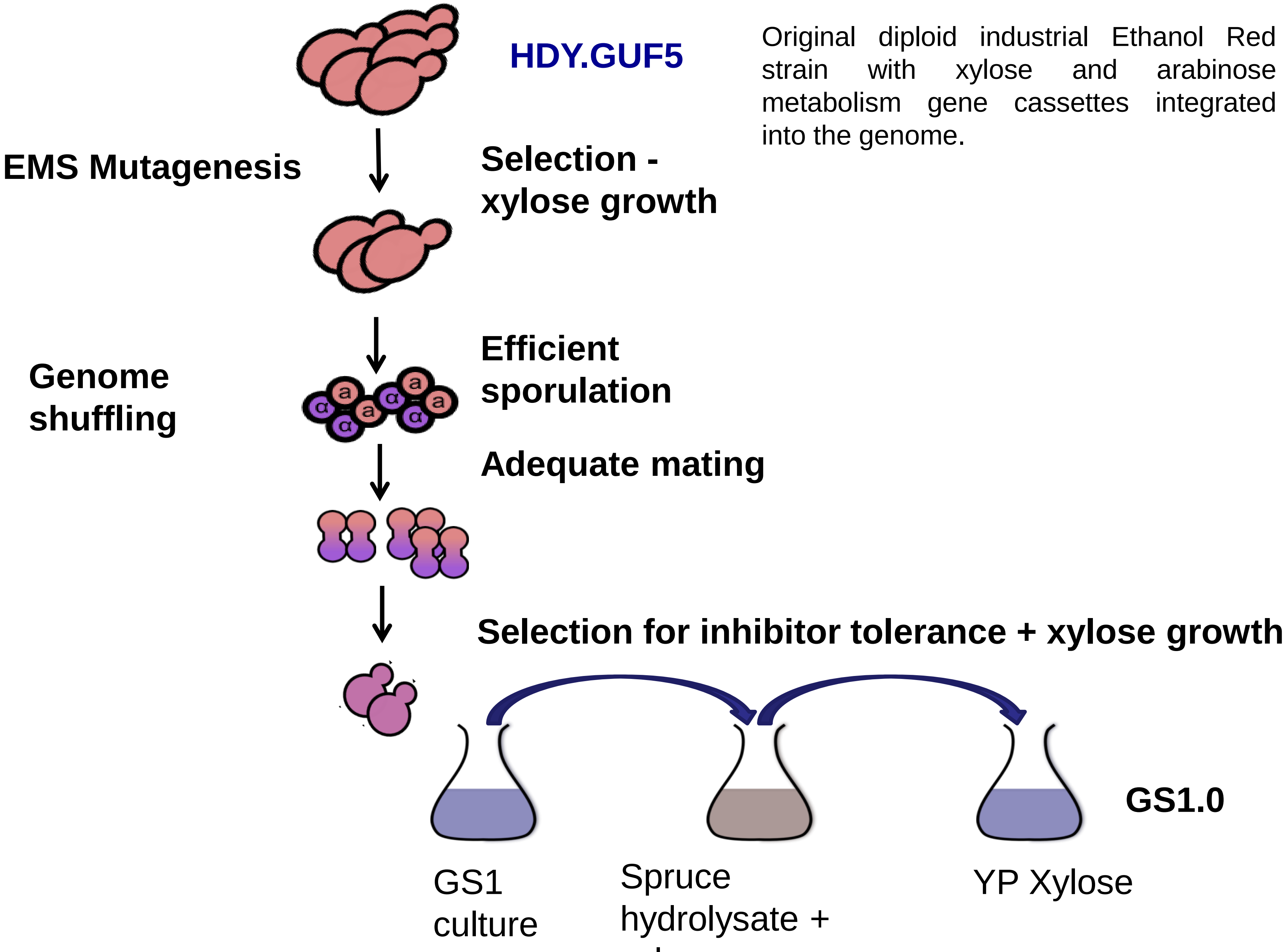
- Ethanol Red (Fermentis/Lesaffre): best bioethanol production strain available for first-generation substrates

Introduction of pentose fermentation ability

- Genes of xylose and arabinose metabolism integrated into the genome + overexpression of pentose phosphate pathway genes
  E. Boles, Frankfurt University
- → HDY.GUF5
  → no fermentation of xylose or arabinose

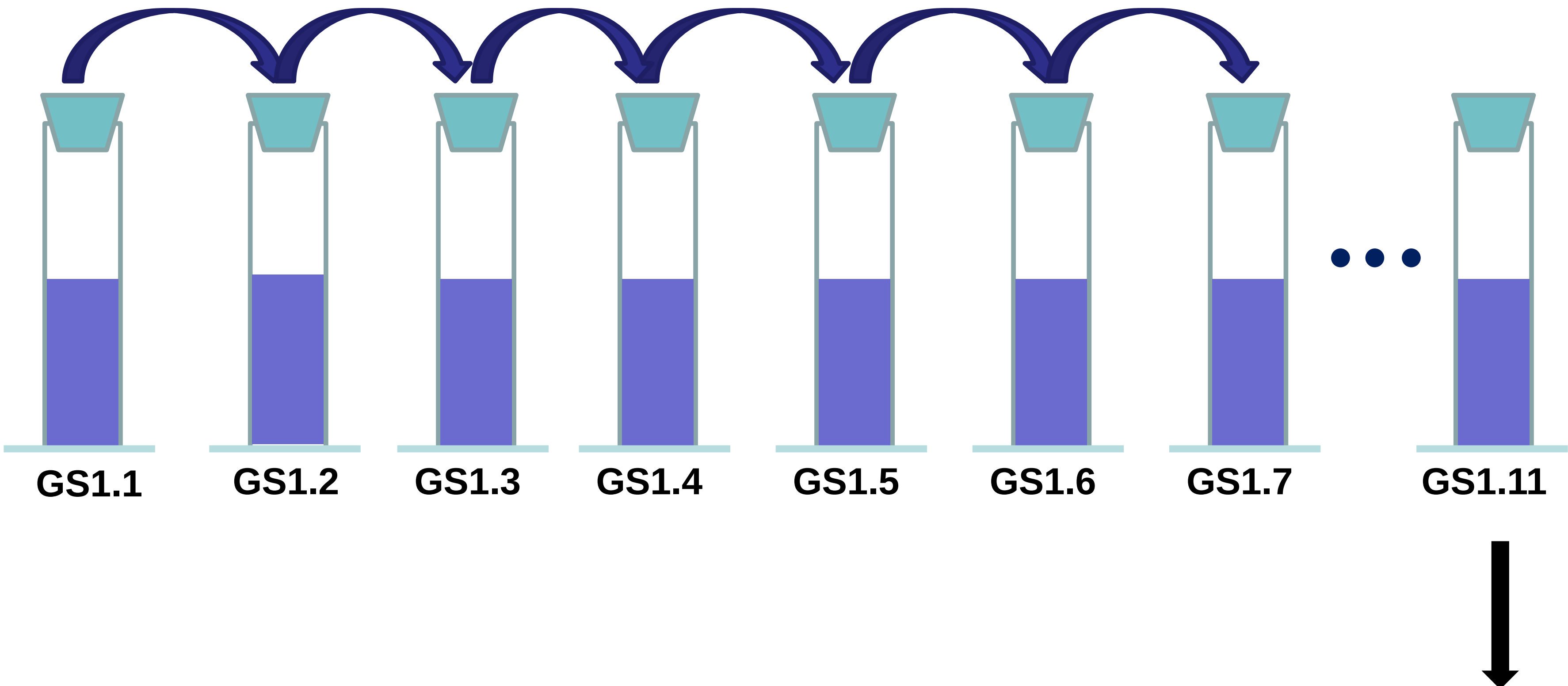


Strategy

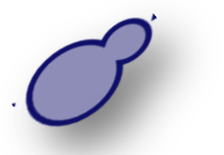

HDY.GUF5
Original diploid industrial Ethanol Red strain with xylose and arabinose metabolism gene cassettes integrated into the genome.
EMS Mutagenesis
Selection - xylose growth
Genome shuffling
Efficient sporulation
Adequate mating
a
α
Selection for inhibitor tolerance + xylose growth
GS1.0
GS1 culture
Spruce hydrolysate +
YP Xylose

Evolutionary adaptation

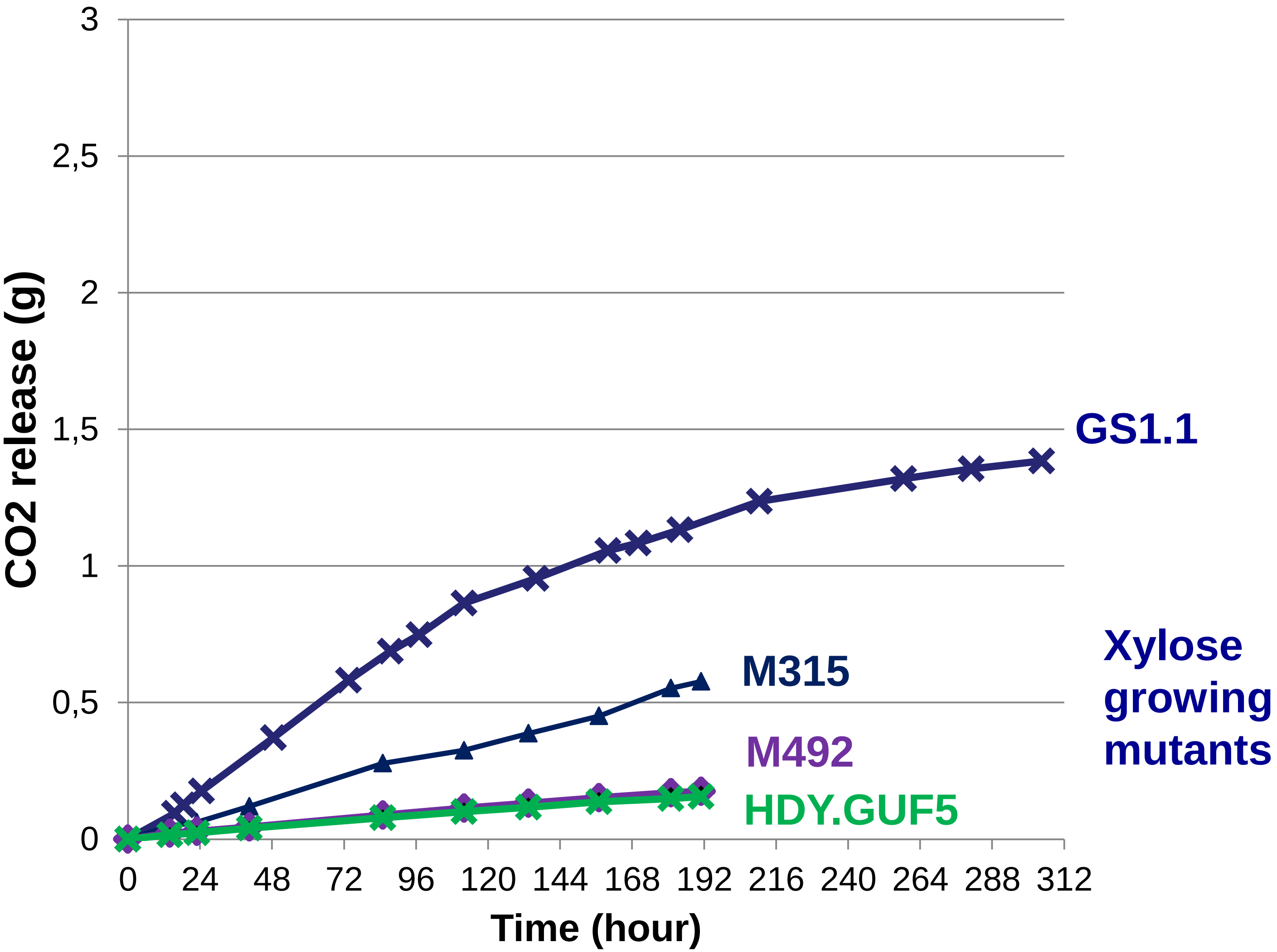
- Serial transfer in semi-anaerobic fermentation tubes (YP + 4%xylose)



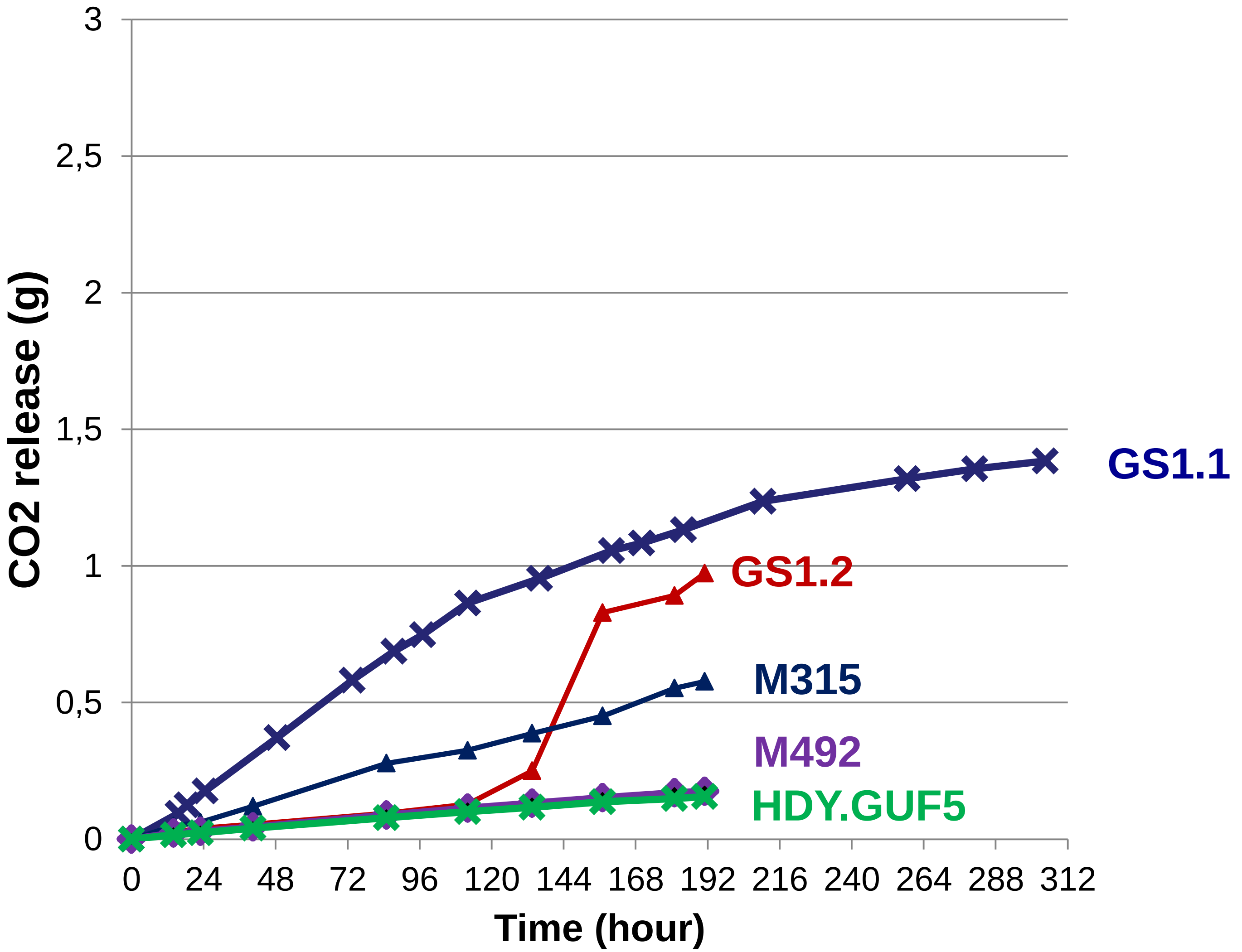
Strains with strongly improved xylose fermentation capacity



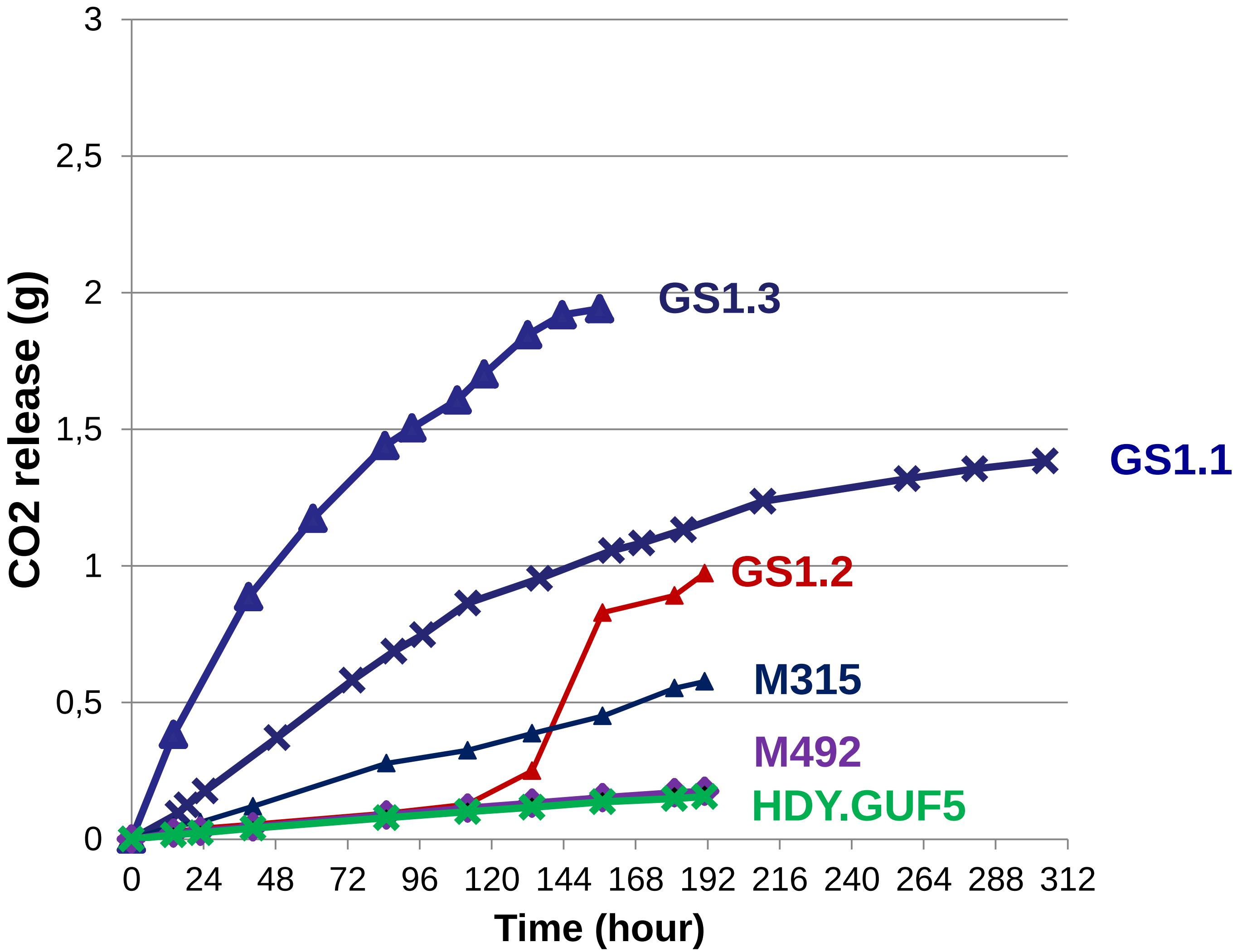
Fermentation profile during evolutionary engineering on YP + 4% xylose



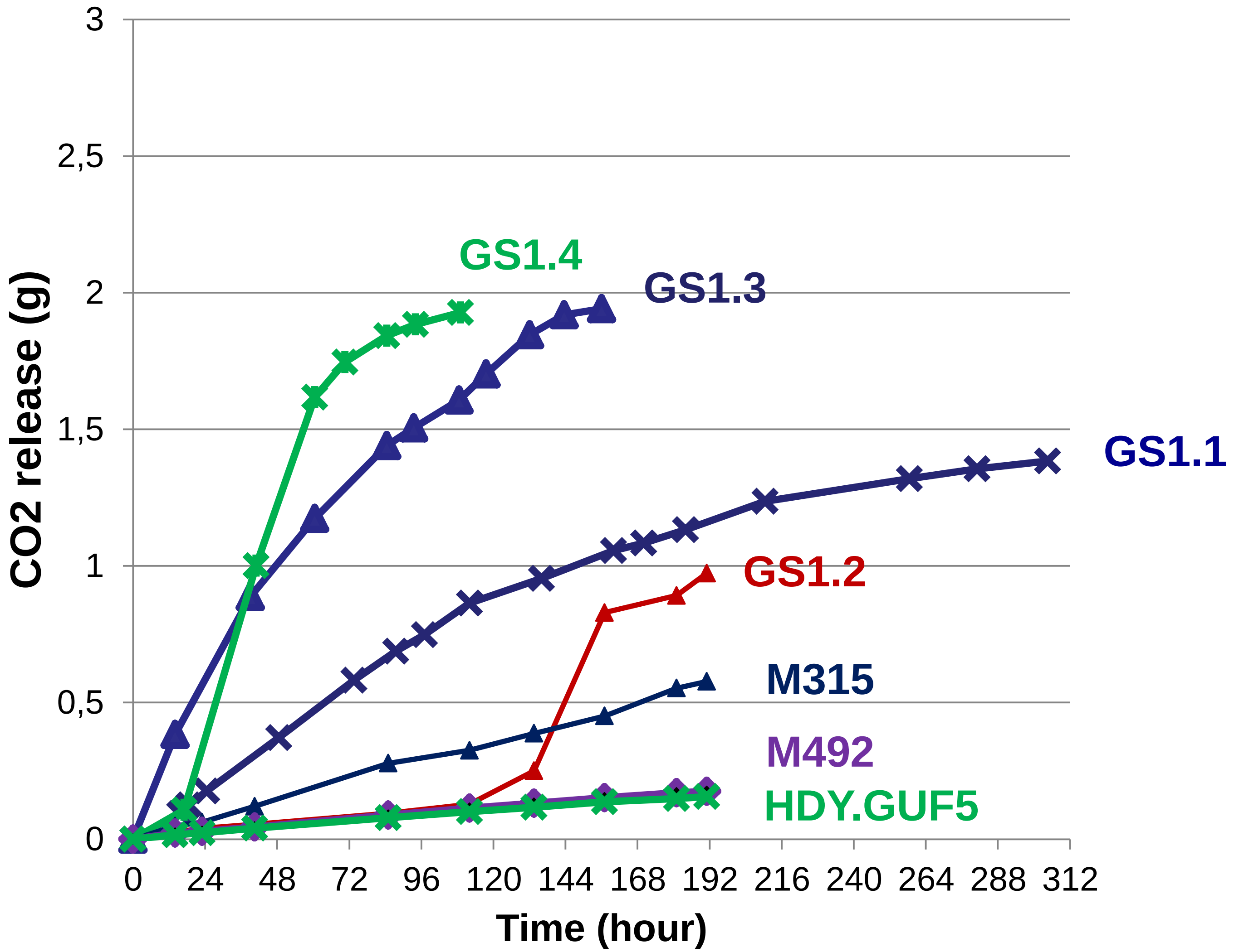
Fermentation profile during evolutionary engineering on YP + 4% xylose



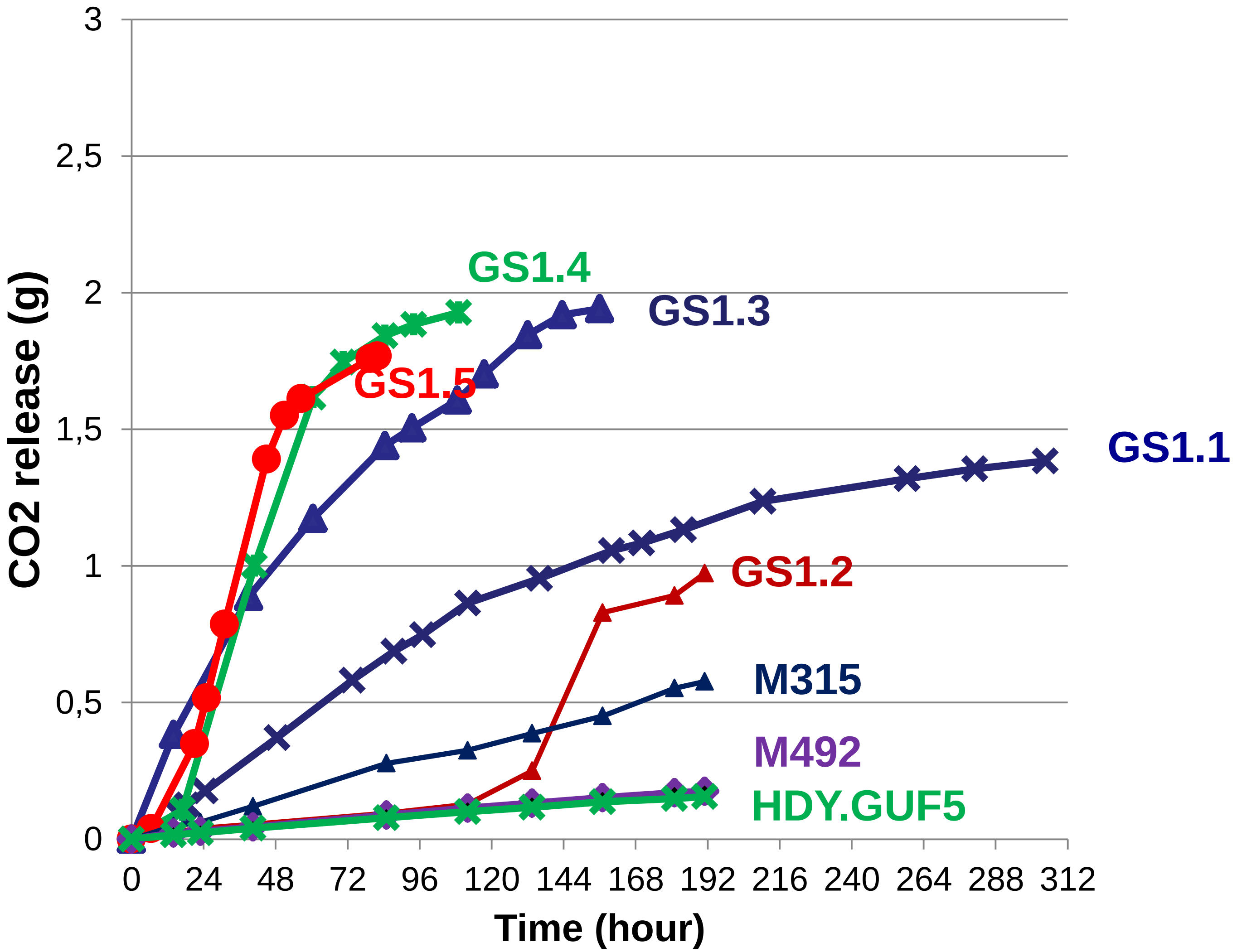
Fermentation profile during evolutionary engineering on YP + 4% xylose



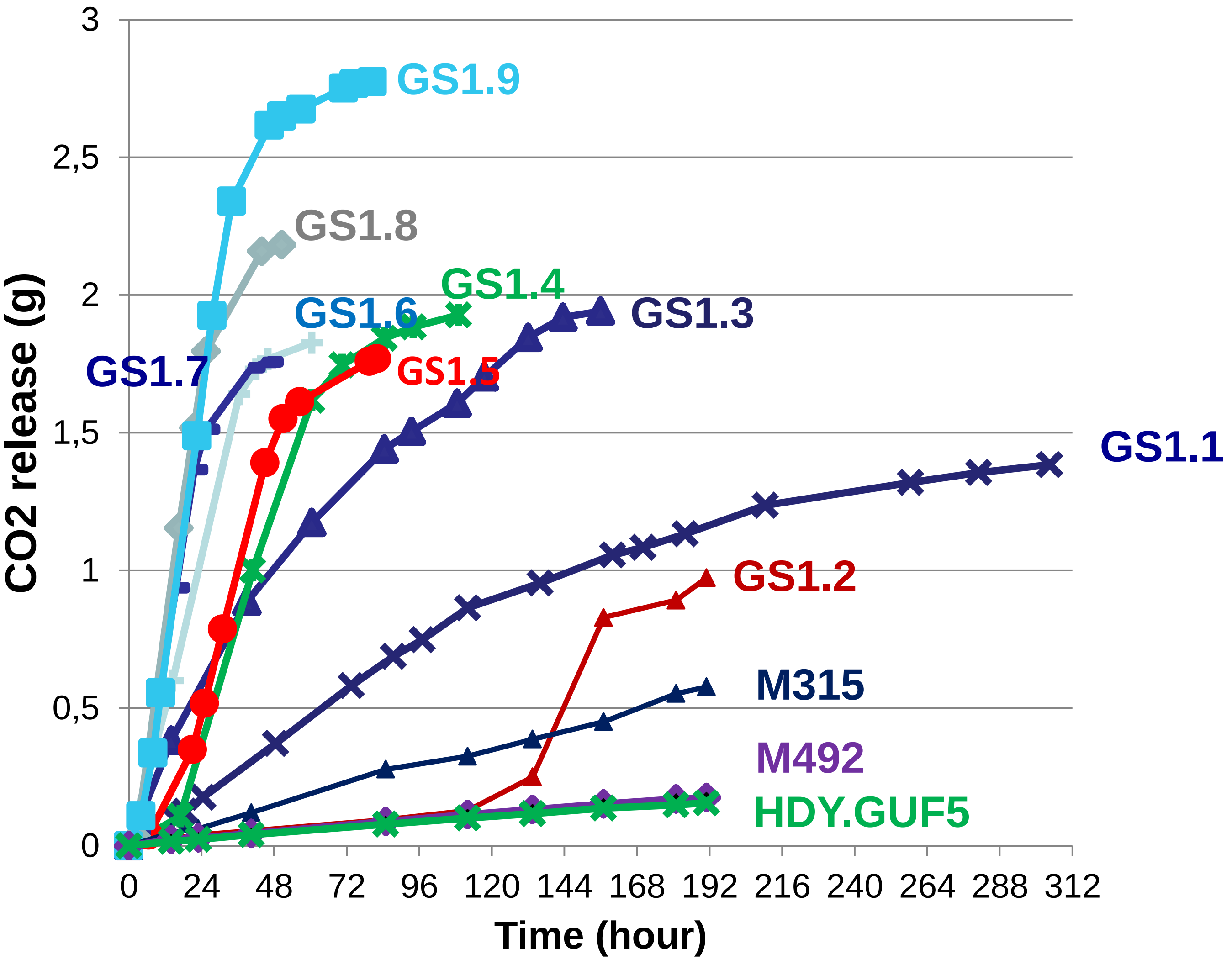
Fermentation profile during evolutionary engineering on YP + 4% xylose

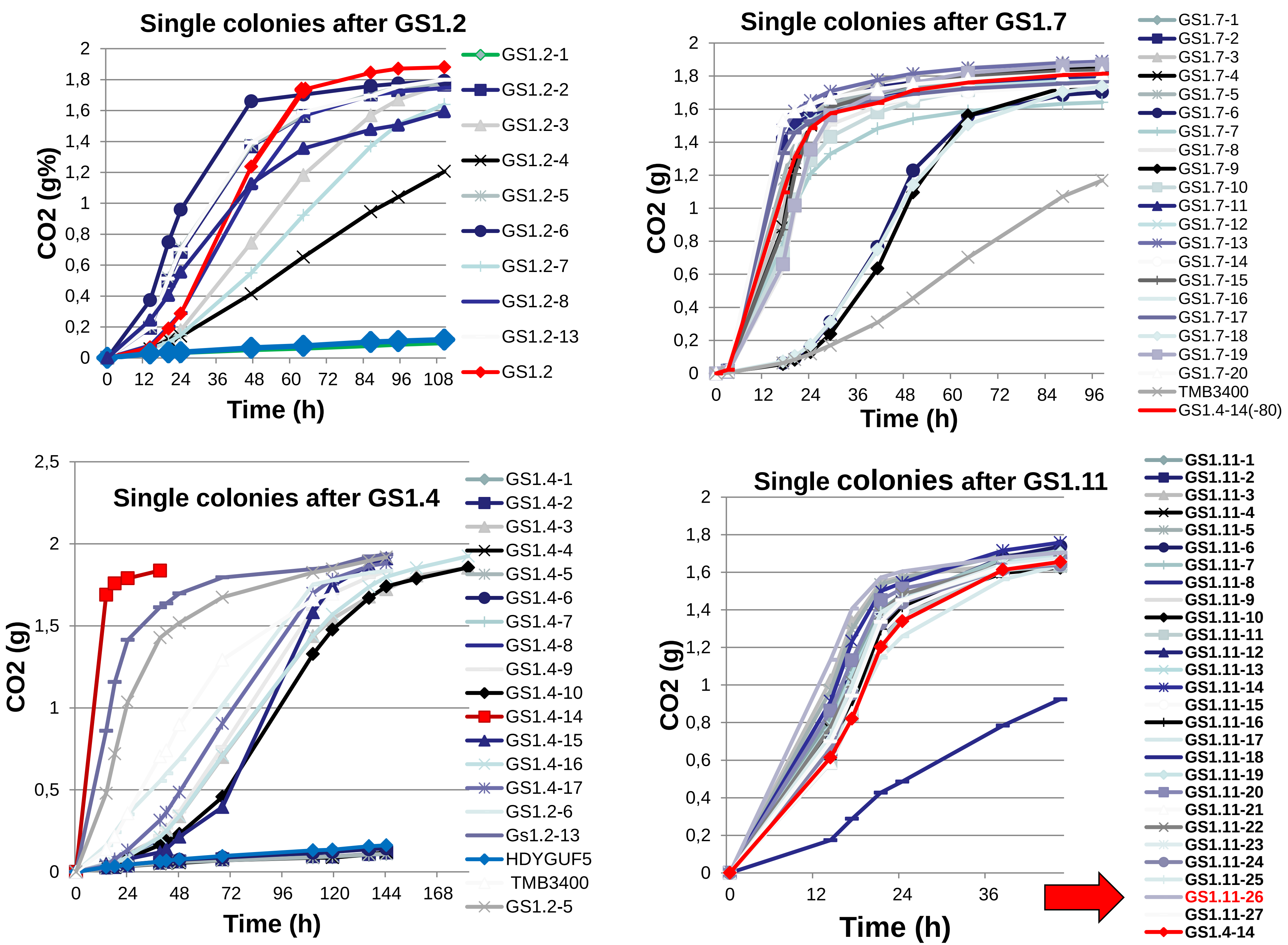


Fermentation profile during evolutionary engineering on YP + 4% xylose



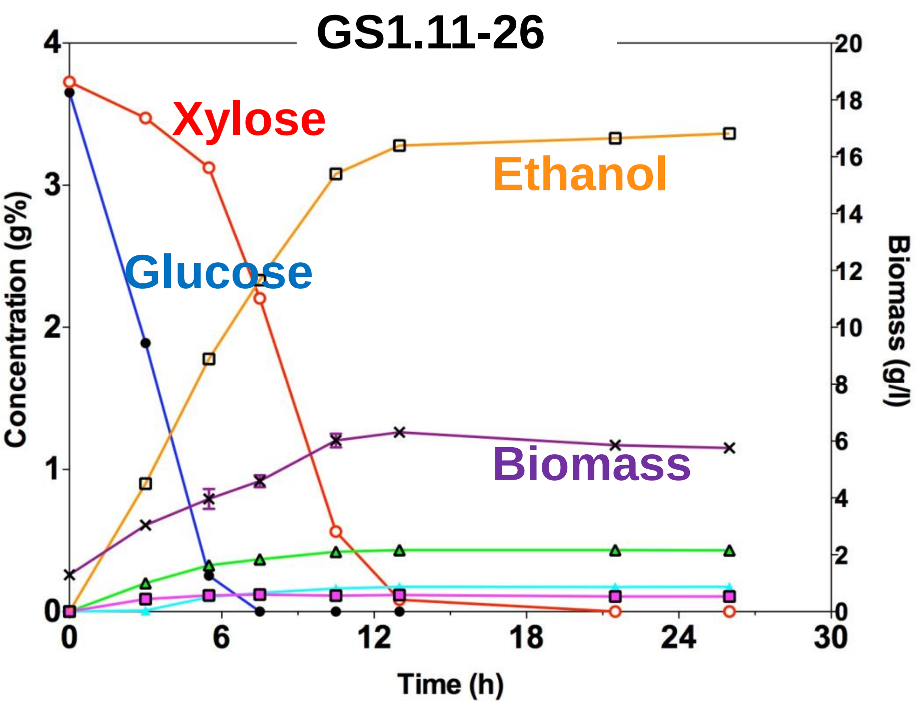
Fermentation profile during evolutionary engineering on YP + 4% xylose



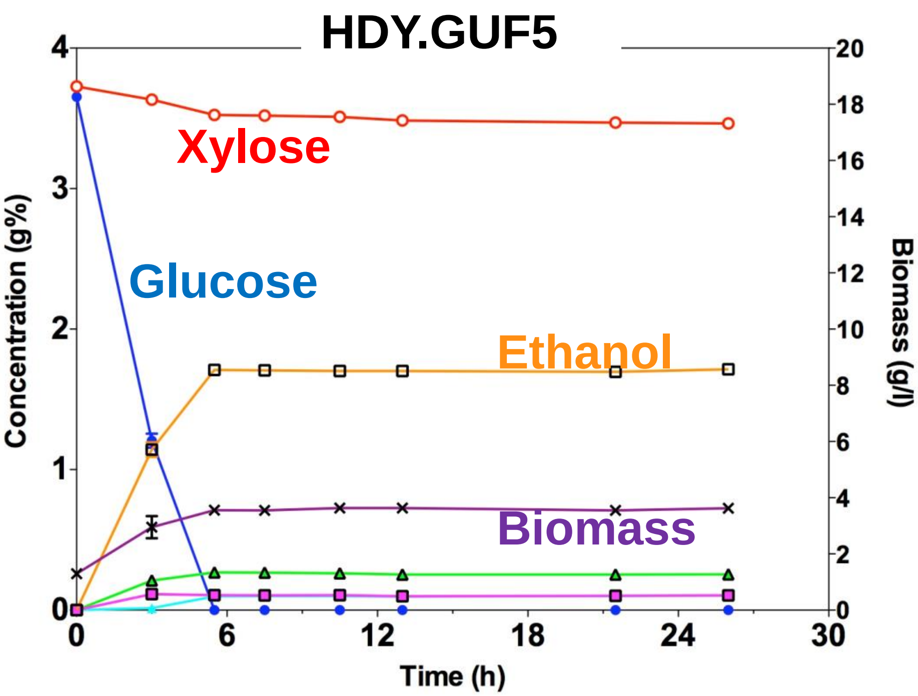

Single colonies after GS1.2
CO2 (g%)
Time (h)
GS1.2-1
GS1.2-2
GS1.2-3
GS1.2-4
GS1.2-5
GS1.2-6
GS1.2-7
GS1.2-8
GS1.2-13
GS1.2
Single colonies after GS1.7
CO2 (g)
Time (h)
GS1.7-1
GS1.7-2
GS1.7-3
GS1.7-4
GS1.7-5
GS1.7-6
GS1.7-7
GS1.7-8
GS1.7-9
GS1.7-10
GS1.7-11
GS1.7-12
GS1.7-13
GS1.7-14
GS1.7-15
GS1.7-16
GS1.7-17
GS1.7-18
GS1.7-19
GS1.7-20
TMB3400
GS1.4-14(-80)
Single colonies after GS1.4
CO2 (g)
Time (h)
GS1.4-1
GS1.4-2
GS1.4-3
GS1.4-4
GS1.4-5
GS1.4-6
GS1.4-7
GS1.4-8
GS1.4-9
GS1.4-10
GS1.4-14
GS1.4-15
GS1.4-16
GS1.4-17
GS1.2-6
Gs1.2-13
HDYGUF5
TMB3400
GS1.2-5
Single colonies after GS1.11
CO2 (g)
Time (h)
GS1.11-1
GS1.11-2
GS1.11-3
GS1.11-4
GS1.11-5
GS1.11-6
GS1.11-7
GS1.11-8
GS1.11-9
GS1.11-10
GS1.11-11
GS1.11-12
GS1.11-13
GS1.11-14
GS1.11-15
GS1.11-16
GS1.11-17
GS1.11-18
GS1.11-19
GS1.11-20
GS1.11-21
GS1.11-22
GS1.11-23
GS1.11-24
GS1.11-25
GS1.11-26
GS1.11-27
GS1.4-14

Semi-anaerobic fermentation in YP + glucose/xylose mixture (35 ° C)

Evolved strain



Original ER strain with cassette



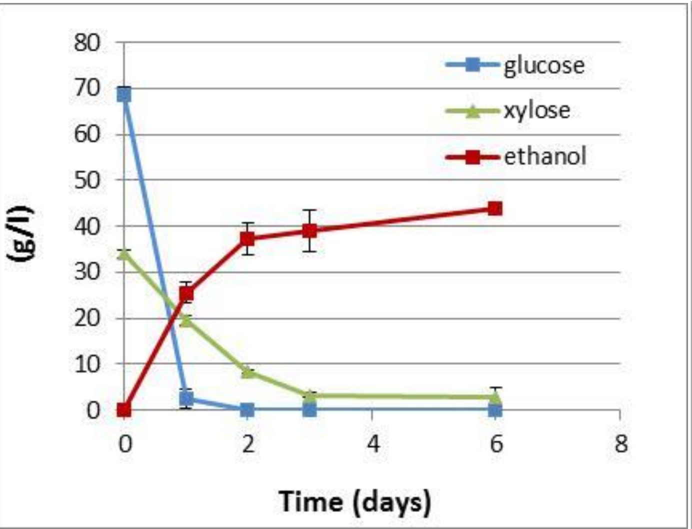
Ethanol productivity: 1.4 g/g DW/h
Max. glucose consumption rate: 2.71 g/g DW/h
Max. xylose consumption rate: 1.10 g/g DW/h
Xylose-glucose co-consumption rate: 0.4 g/g DW/h

Semi-anaerobic fermentation in lignocellulose hydrolysate: Wheat straw/hay (KAHO, Ghent, Belgium)

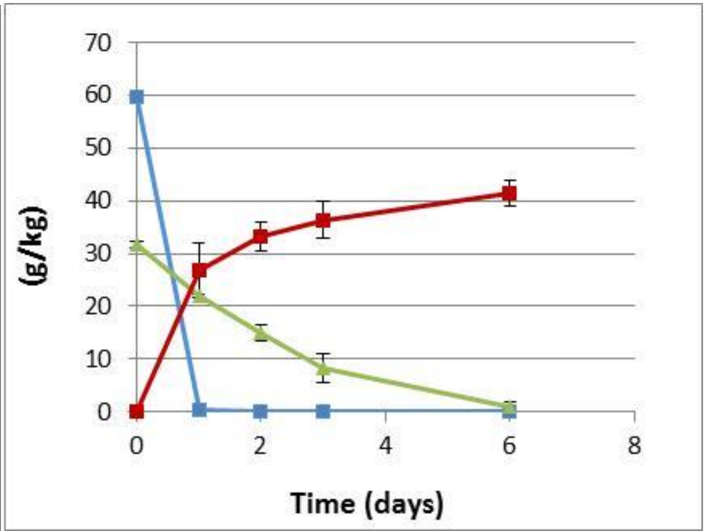
Pre-treatment
+
Enzymatic Hydrolysis

28% dry matter
60-70 g/kg glucose
30-40 g/kg xylose

SYNTHETIC MEDIUM



HYDROLYSATE

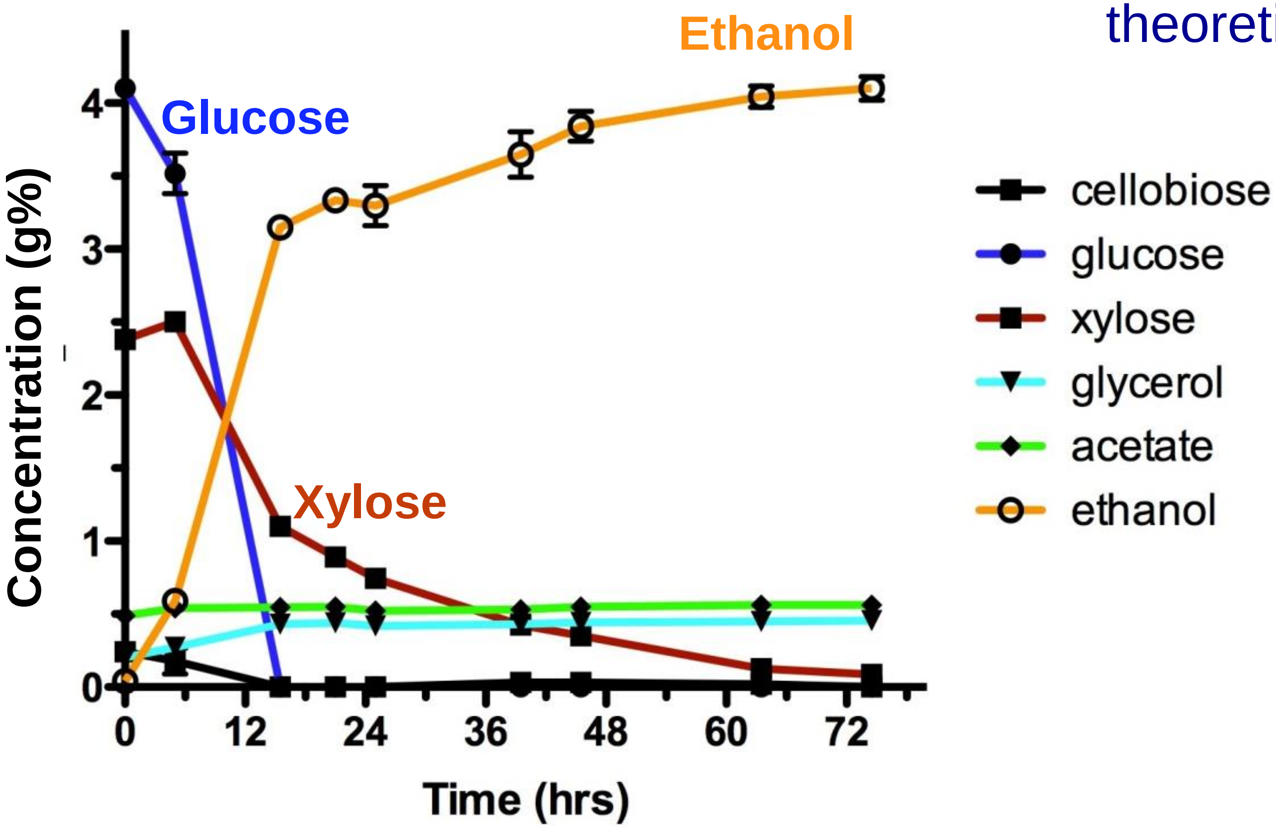


3 g yeast / L

94 % of maximum theoretical ethanol yield

Semi-anaerobic fermentation in lignocellulose hydrolysate: Giant reed (*Arundo donax*) from Chemtex (Italy) (21% dry matter)

3 g yeast / L

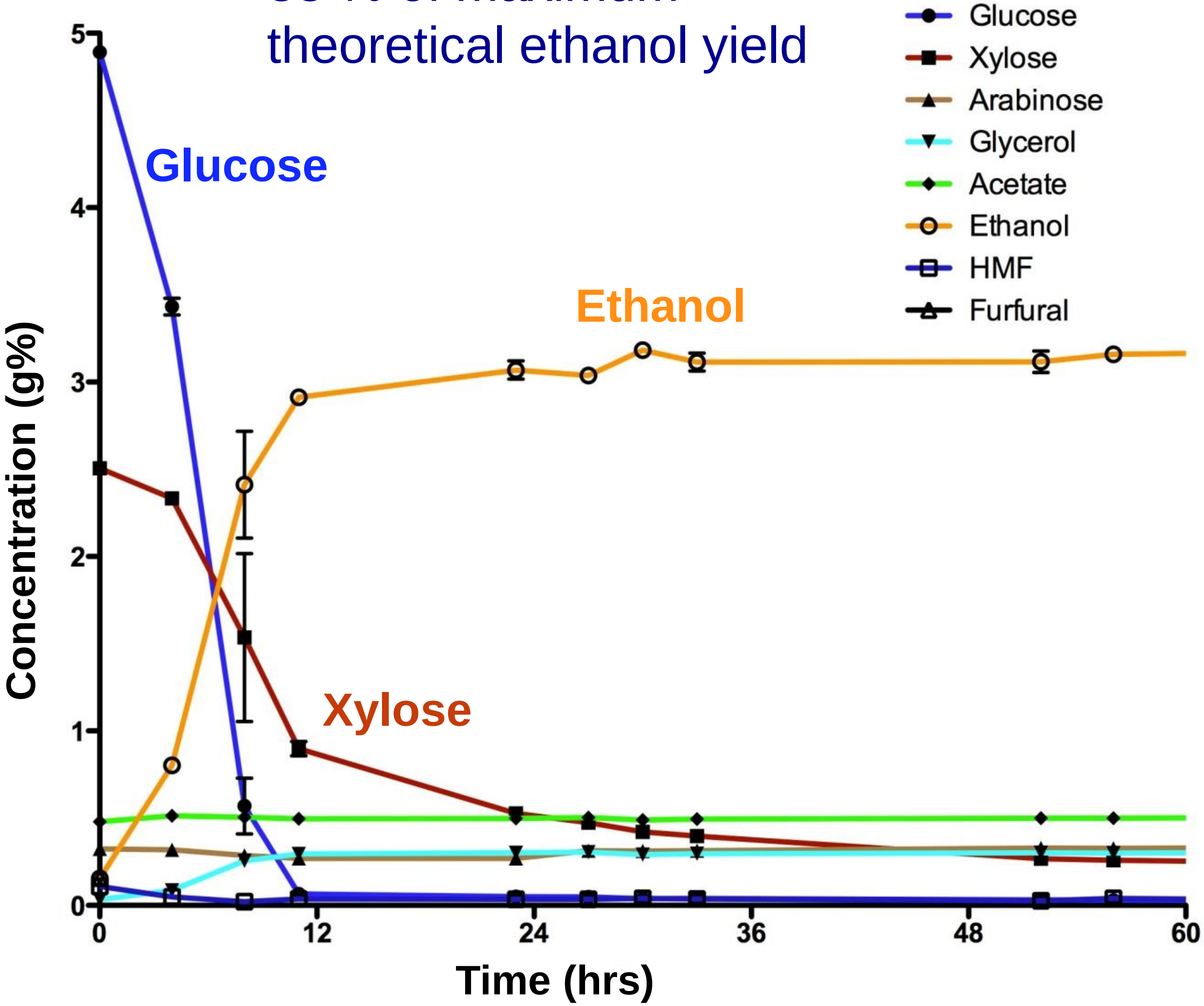


92 % of maximum theoretical ethanol yield

Semi-anaerobic fermentation in lignocellulose hydrolysate: Spruce hydrolysate from Sekab (Sweden)
(18% dry matter)

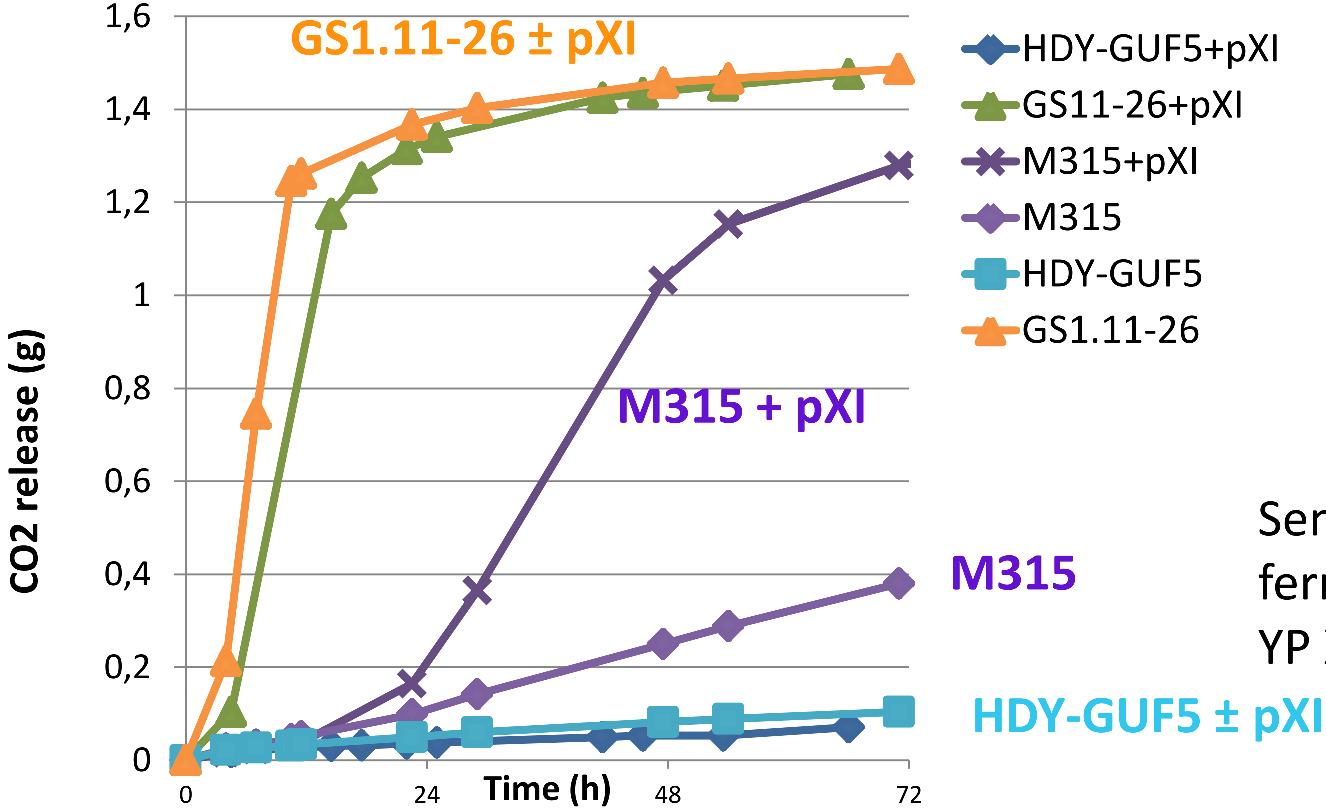
88 % of maximum
theoretical ethanol yield

3 g yeast / L



Genetic basis of GS1.11-26

Overexpression of xylose isomerase is not enough for good xylose fermentation



Semi-anaerobic fermentation in YP Xylose 4%

- Overexpression of *XylA* does not result in xylose fermentation HDY.GUF5
- Important mutation(s) generated during the mutagenesis step in M315
- Additional beneficial mutations during later steps

Further developments

- GS1.11-26: xylose fermentation capacity is stable after > 50 gen. in YP glucose
- Much information already about the genetic basis of the high xylose fermentation capacity and inhibitor tolerance
- However, negative side effects caused by mutagenesis and/or evolutionary engineering (e.g. aerobic growth defect, maximal ethanol accumulation reduced, acetic acid tolerance reduced)
- Backcrosses of GS1.11-26 with Ethanol Red and with another highly inhibitor tolerant industrial strain → selection for good aerobic growth, xylose fermentation and inhibitor tolerance in lignocellulose hydrolysates

→ Three robust xylose-fermenting industrial yeast strains with very good performance in lignocellulose hydrolysates: **GSE16, GSF335 and GSF767**

→ Under evaluation by several companies world-wide
→ Promising results → clear interest in using further improved versions of our yeast strains in commercial scale

Further improvement of the 2nd-generation bioethanol strains

- Further improvement of multiple stress tolerance traits in the most promising strains

  → Introduction of superior alleles for high tolerance to different stress conditions, identified by our technology for polygenic analysis of complex traits

- **Final goal: commercial valorisation of the best C5 strain**

  (not just publications, patent applications)

Collaboration with Praj Industries (Pune, India)

- Contact through WIP (Rainer Janssen): partner in EU-NEMO project
- Evaluation of our strains in different hydrolysates: promising results
- Currently initiating a collaboration programme for further improvement of the strains in more concentrated hydrolysates to reach even higher final ethanol levels

Other possible collaborations

- Interested in other collaborations, e.g. in new EC projects in Horizon 2020 with involvement of Indian partners

  → Biodiversity (strain screening, metagenomic libraries, etc.): source of genes for further improvement of performance and robustness

  → Evaluation of strain performance with various substrates: waste materials, bioenergy crops

  → Evaluation of strain performance under different process conditions, in large scale, etc.

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