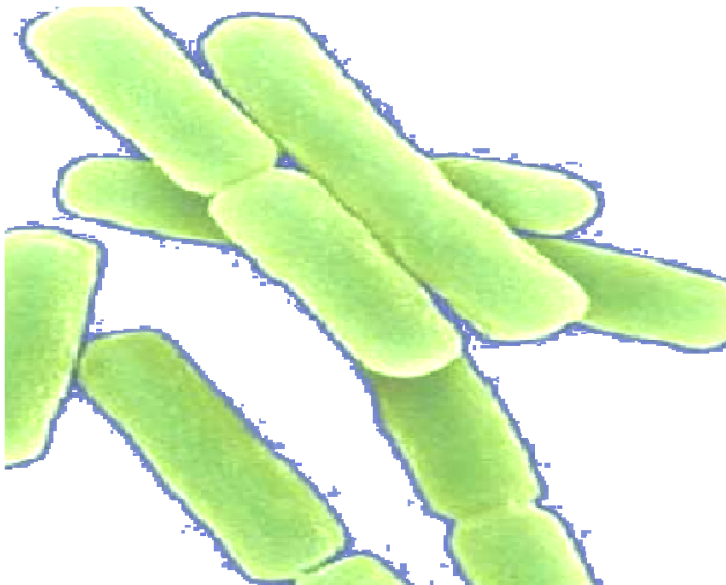
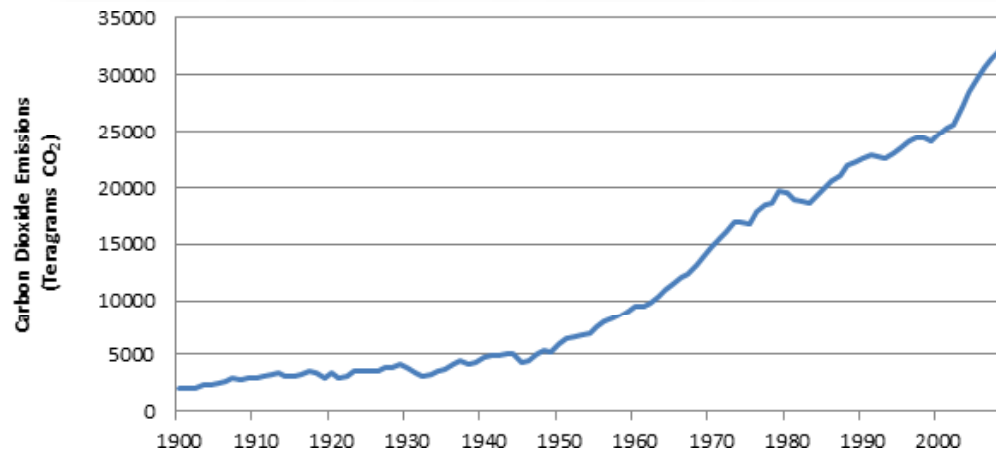


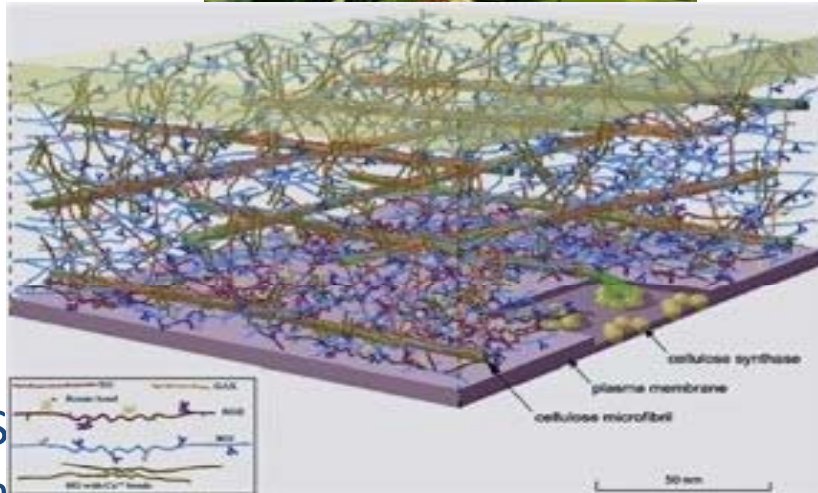
Geobacillus thermoglucosidasius – a catabolically versatile host for metabolic engineering



Global warming & energy security



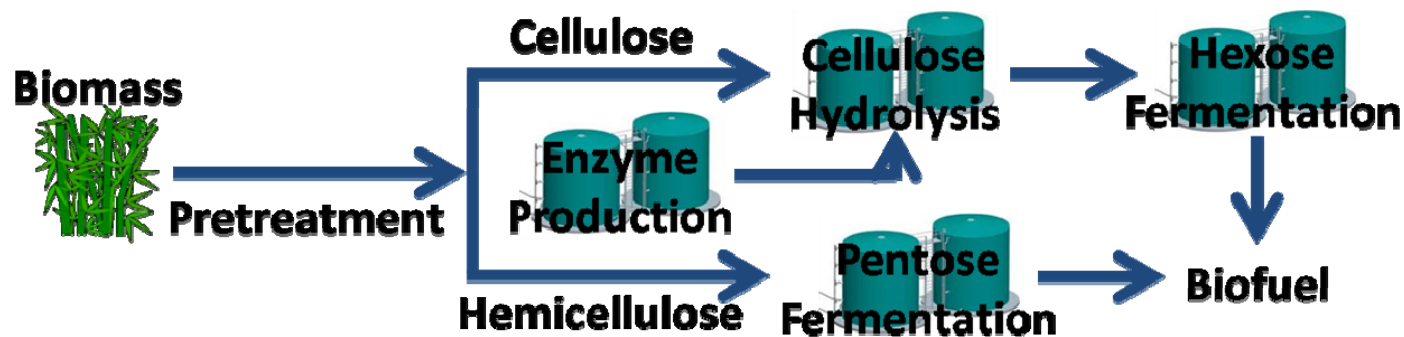
Biofuel Feedstock (Food vs Fuel)



- Sugars are easily digested to monomers.



- Non edible lignocellulosics are composed of lignin and difficult to breakdown sugars in the form of (hemi)cellulose.



Biofuels from lignocellulose - Options

Strategies

- Create pentose and oligosaccharide utilising ethanologens (*S. cerevisiae*, *Z. mobilis*)
- Transfer known ethanol fermentation pathways into catabolically versatile strains (*Zymomonas pdc* and *adh* in *E. coli*, *K. oxytoca* etc)
- Create novel ethanol fermentation pathways in catabolically versatile strains

Geobacillus spp for ethanol production

Geobacillus spp Gm +ve, 40-70°C , spore forming - reclassified 2001 (Nazina et al)

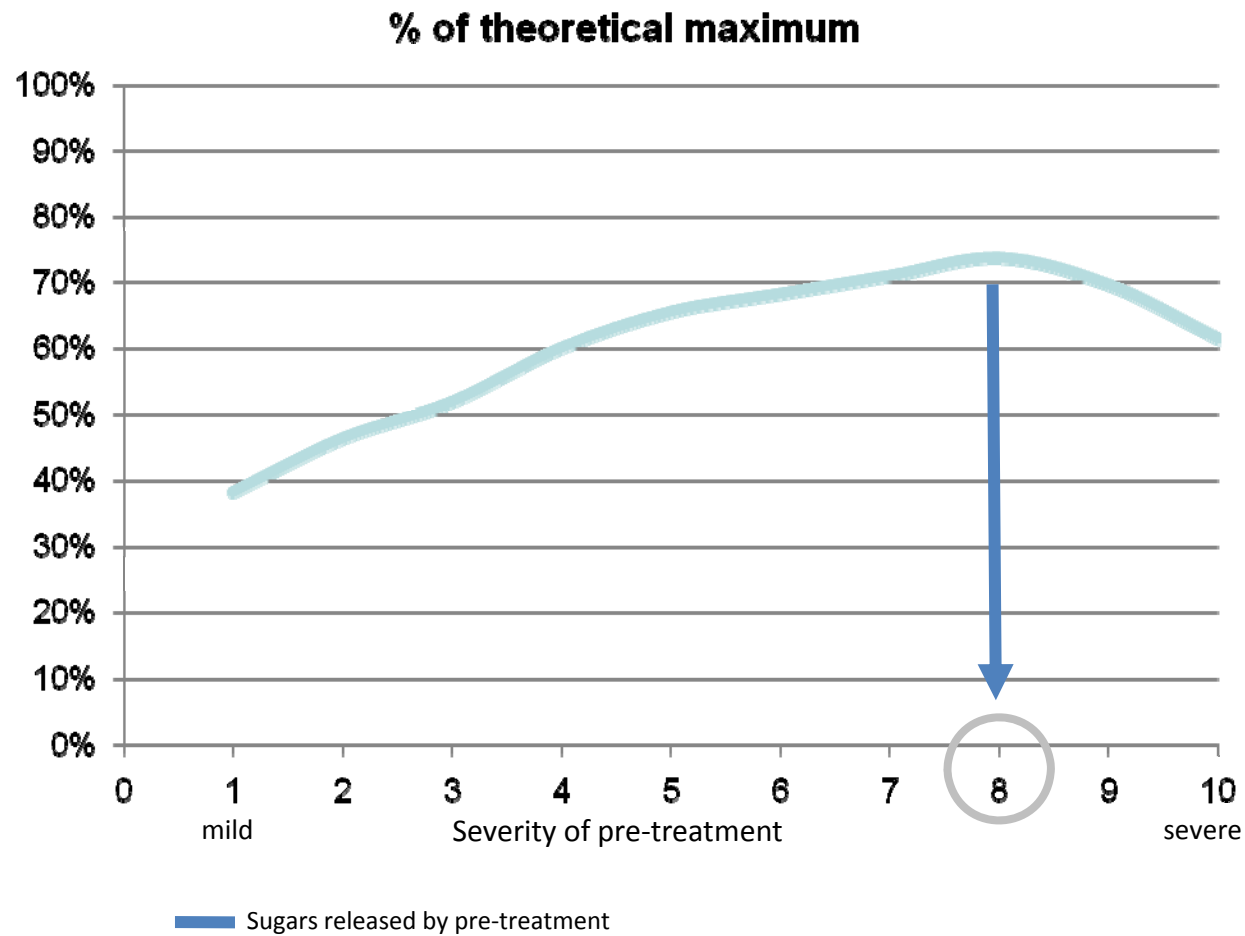
Advantages

- Metabolically versatile (hexose, pentose, cellobiose, xylans)
- Rapid growth (eg $\mu_{\text{glc}} = 0.85 \text{ h}^{-1}$, $\mu_{\text{xyI}} = 0.60 \text{ h}^{-1}$ aerobic 66°C)
- Growth at ~70°C allows ethanol removal by gas stripping
- Growth temperature compatible with typical process operations
- Facultative anaerobes allow
 - 1) aerobic biomass
 - 2) anaerobic fermentation

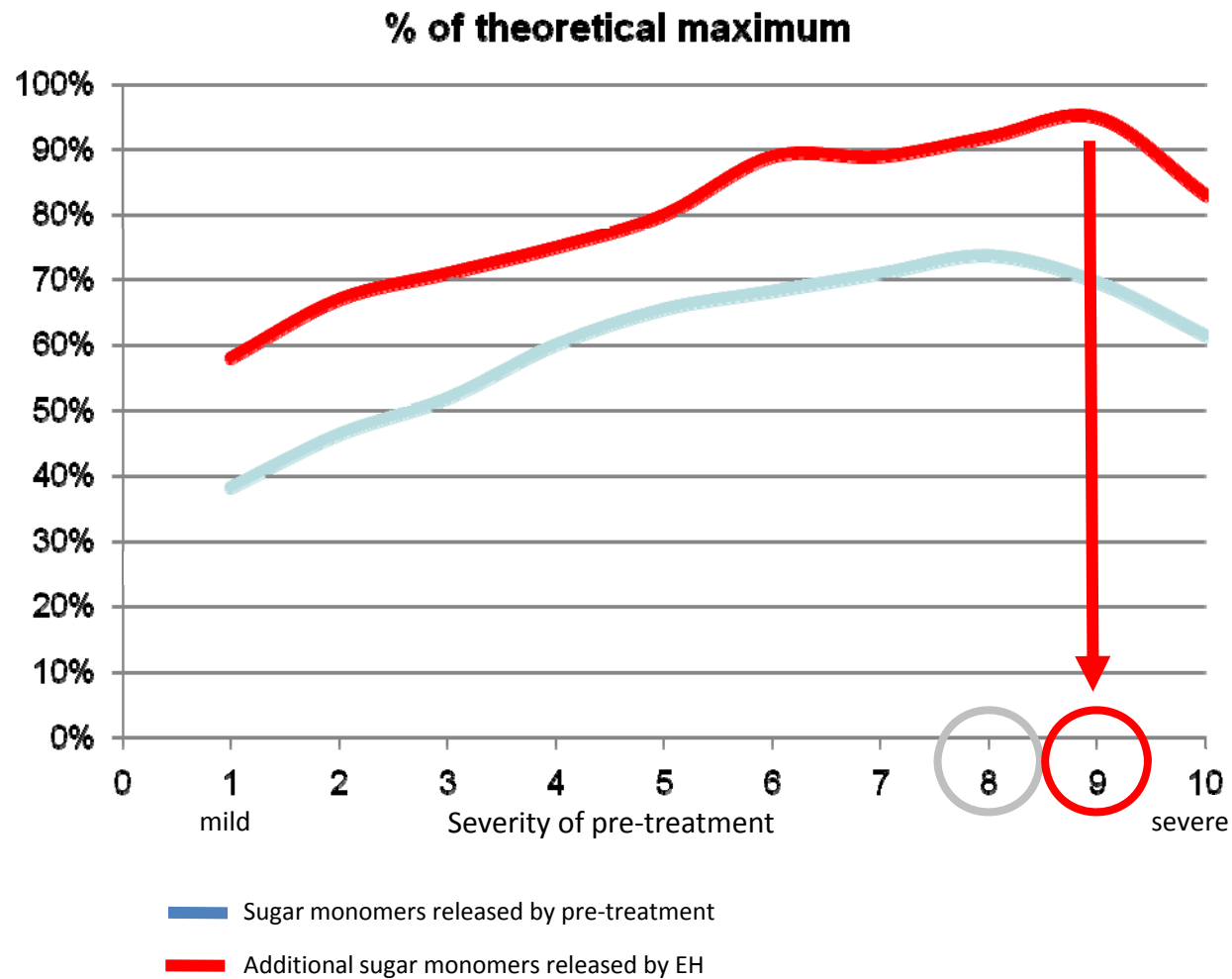
Disadvantages

- Mixed acid fermentation pathway
- Low ethanol tolerance (compared to *S. cerevisiae*)
- Limited genetic tools

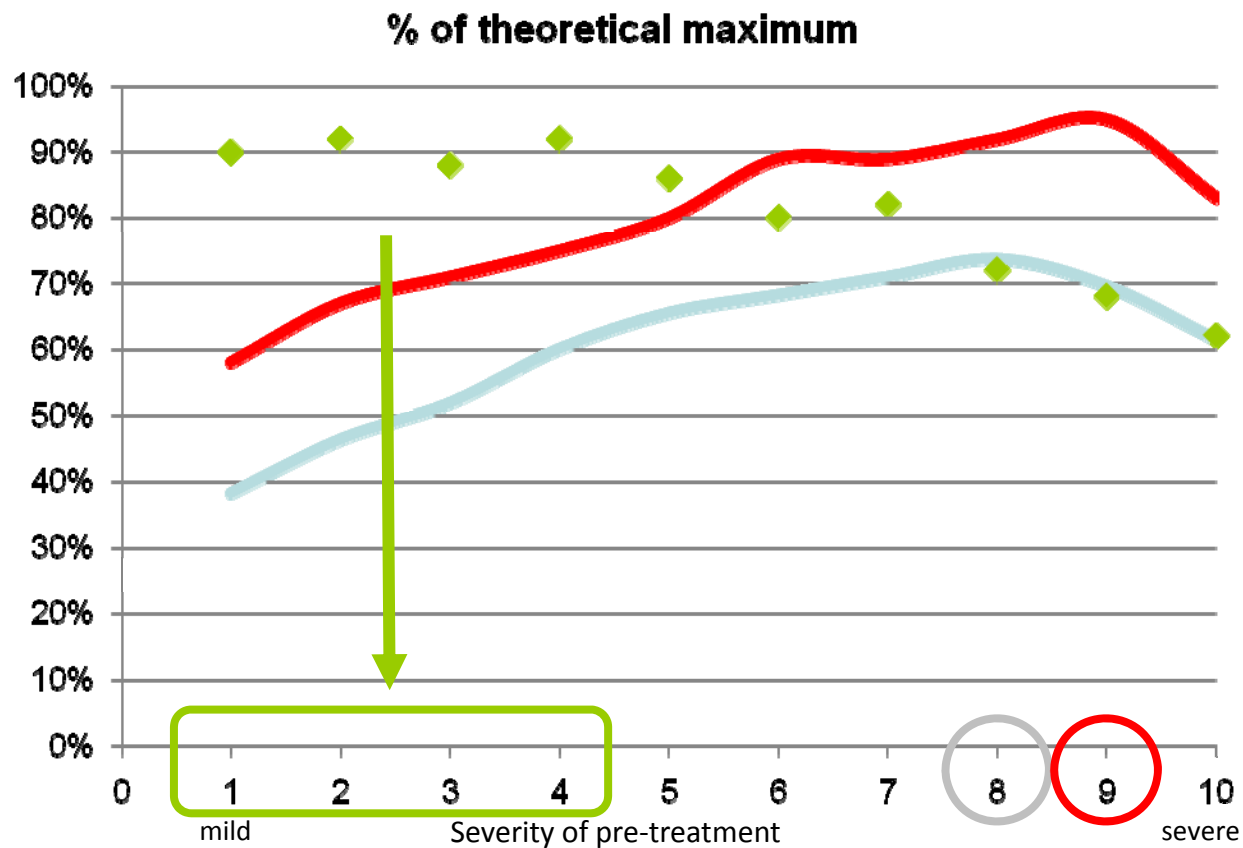
Initial pre-treatment studies



Following enzyme hydrolysis

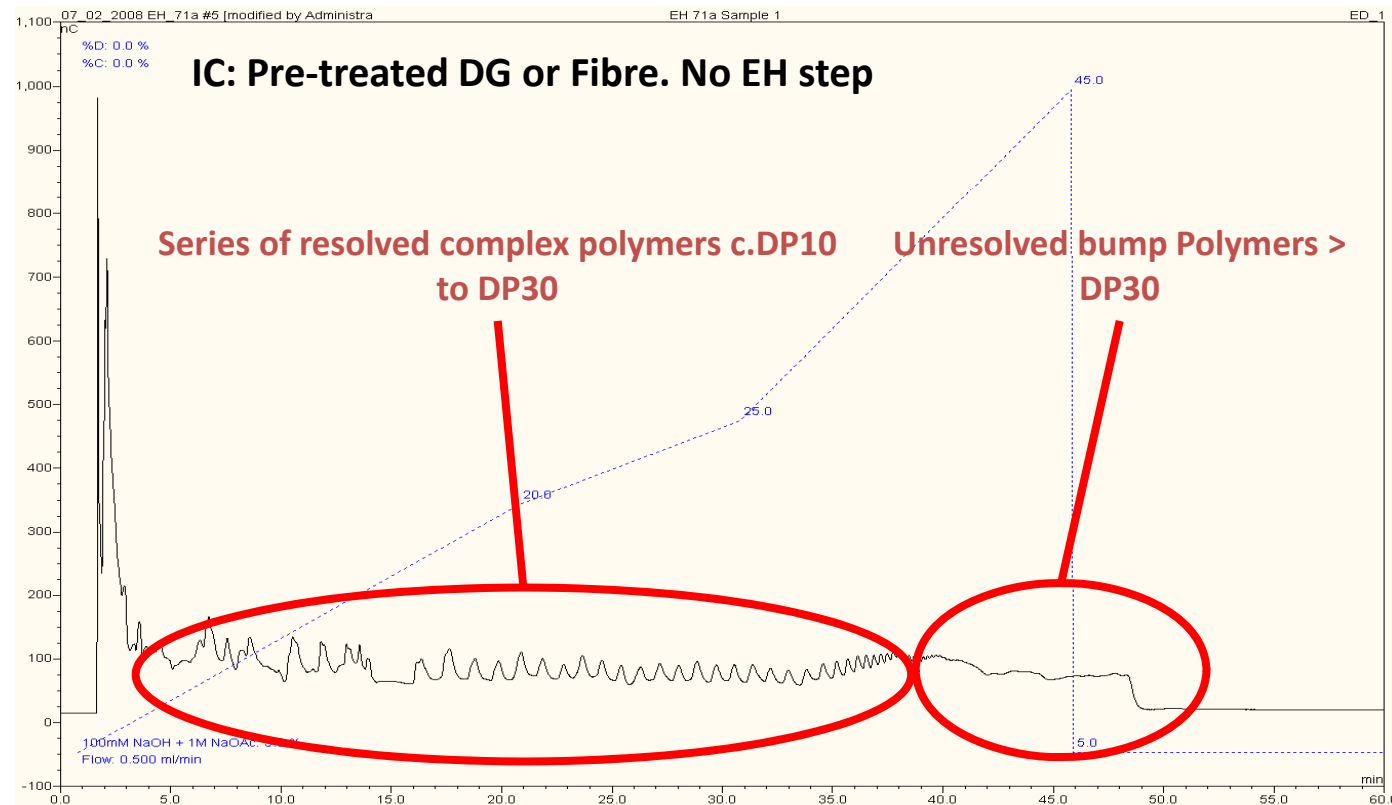


After Fermentation



- Sugar monomers released by pre-treatment
- Additional sugar monomers released by EH
- ◆ Ethanol yield

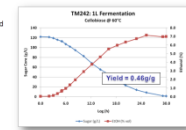
Utilisation of oligomers/polymers



Utilisation of Cellobiose



- **Cellobiose**
Breakdown product of cellulose
Possibility to reduce enzyme loading and residence time
Need to manage end-product inhibition (SSP)
- **Fermentation Productivity**
Yields are good (0.46g/g)
Uptake rates similar to glu.
(No optimisation)
Minimal organic acids



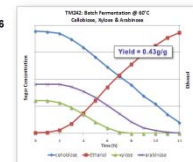
There is significant know-how in understanding how to exploit TM242

7% Ethanol

Mixed sugars simultaneously

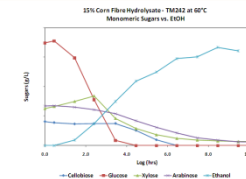


- **TM242 co-ferments C5 & C6**
 - Monomers and polymers
 - High yields
 - Rapid conversion
- **No separation of sugar streams**
- **Single fermentation step**



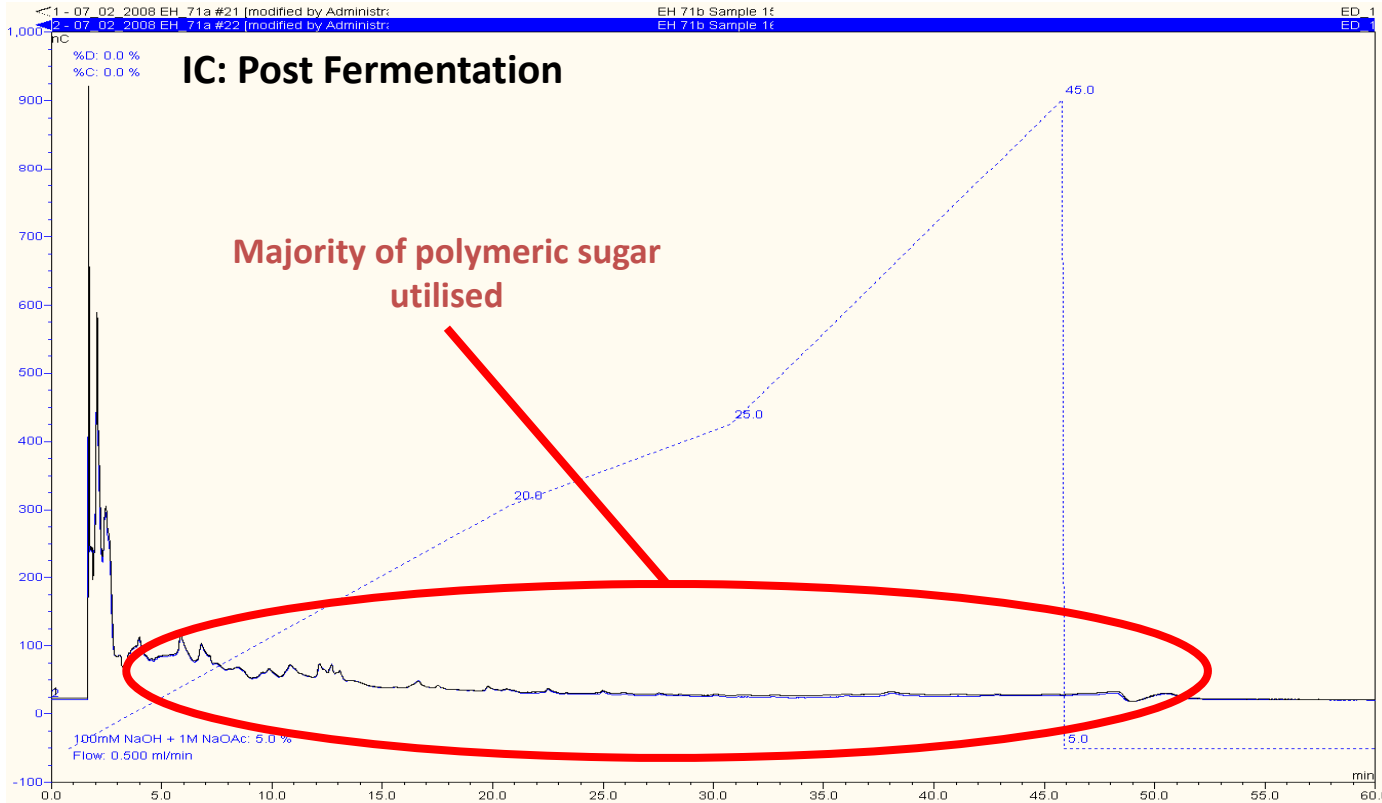
Mixed sugars

Real Feedstocks



- Mild pretreatment - low levels of inhibitors
- Low enzyme loadings - solubilisation > 90%
- 96% of monomers utilised - yields > 0.4 g/g

Real feedstocks

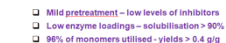


-
- TM242: 11 Fermentation**
Cellulose @ 37°C
- Yield = 0.66g/g
- | Time (h) | Sugar (g/L) | Yield (g/g) |
|----------|-------------|-------------|
| 0.0 | 120 | 0.00 |
| 2.0 | 118 | 0.02 |
| 4.0 | 115 | 0.05 |
| 6.0 | 110 | 0.08 |
| 8.0 | 105 | 0.12 |
| 10.0 | 95 | 0.18 |
| 12.0 | 80 | 0.25 |
| 14.0 | 65 | 0.32 |
| 16.0 | 50 | 0.38 |
| 18.0 | 35 | 0.45 |
| 20.0 | 20 | 0.52 |
| 22.0 | 10 | 0.58 |
| 24.0 | 0 | 0.66 |

7% Ethanol

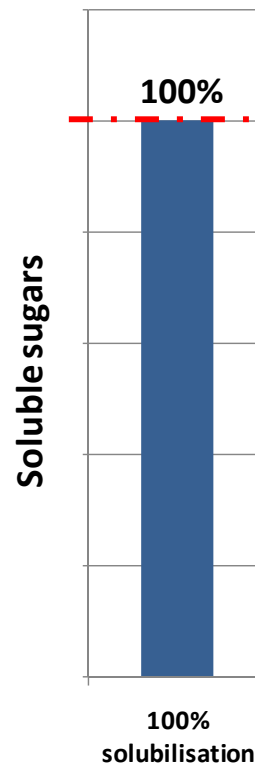
-
- Figure 1 is a line graph titled "TM342 Batch Fermentation @ 80°C" with the subtitle "Cellulose, Sucrose & Galactose". The x-axis is labeled "Time (h)" and ranges from 0 to 10. The y-axis is labeled "Sugar Concentration" and ranges from 0 to 1.0. There are four data series: Glucose (blue circles), Sucrose (purple squares), Fructose (green triangles), and Galactose (red diamonds). Glucose starts at 1.0 g/g and decreases to 0.0 g/g by 10 hours. Sucrose starts at 0.5 g/g and decreases to 0.0 g/g by 10 hours. Fructose starts at 0.3 g/g and decreases to 0.0 g/g by 10 hours. Galactose starts at 0.0 g/g and increases to 0.642 g/g by 10 hours. A callout box indicates "Yield = 0.642g/g" for Galactose.
- | Time (h) | Glucose (g/g) | Sucrose (g/g) | Fructose (g/g) | Galactose (g/g) |
|----------|---------------|---------------|----------------|-----------------|
| 0 | 1.0 | 0.5 | 0.3 | 0.0 |
| 2 | 0.9 | 0.45 | 0.25 | 0.05 |
| 4 | 0.8 | 0.4 | 0.2 | 0.1 |
| 6 | 0.7 | 0.35 | 0.15 | 0.2 |
| 8 | 0.6 | 0.3 | 0.1 | 0.35 |
| 10 | 0.0 | 0.0 | 0.0 | 0.642 |

Mixed sugars



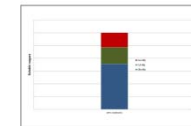
Real feedstocks

Saccharification strategy



Utilisation of Oligomeric Sugars on Fibre

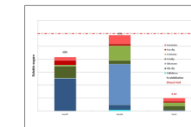
100% Solubilisation



- The total available sugars available in the feedstock for hydrolysis and fermentation
- "Perfect Hydrolysis"

Utilisation of Oligomeric Sugars on Fibre

Severe Pretreatment, High Enzyme Loading

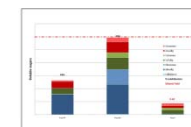


- High Level of monomerisation
- 90% Monomerisation
- 97% of all sugars solubilised
- 0.43 g of ethanol produced/gram of sugar used
- Not a commercially viable process

Yield is ~70 US gal/ton with unaffordable enzyme cost and high energy input

Utilisation of Oligomeric Sugars on Fibre

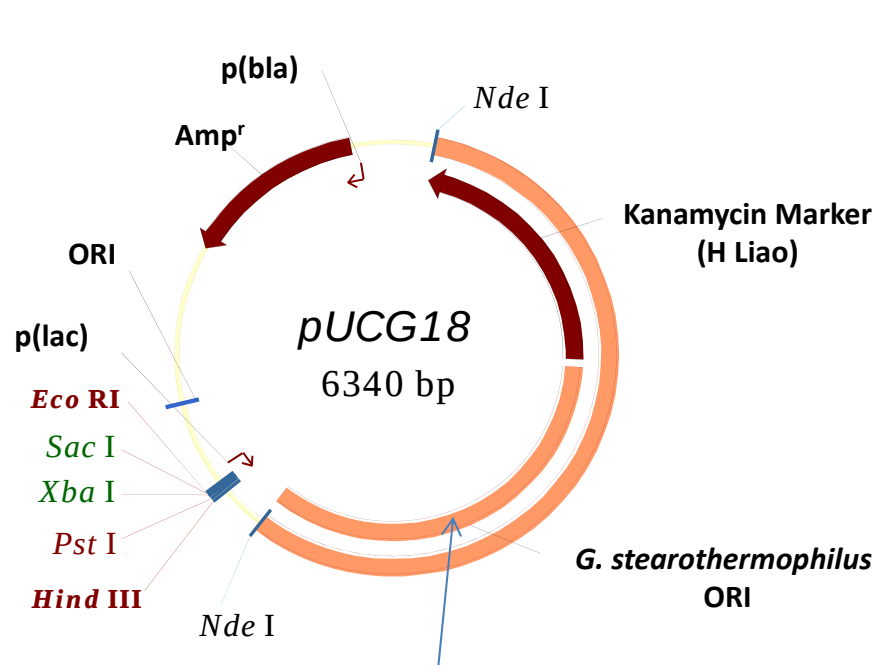
Mild Pretreatment, Low Enzyme Loading



- High Level of monomerisation
- 30% Monomerisation
- 99% of all sugars solubilised
- 0.42 g of ethanol produced/gram of sugar used
- A commercially viable process

Yield is ~70 US gal/ton with \$0.30/gal relative enzyme cost and lower energy costs

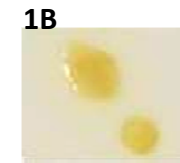
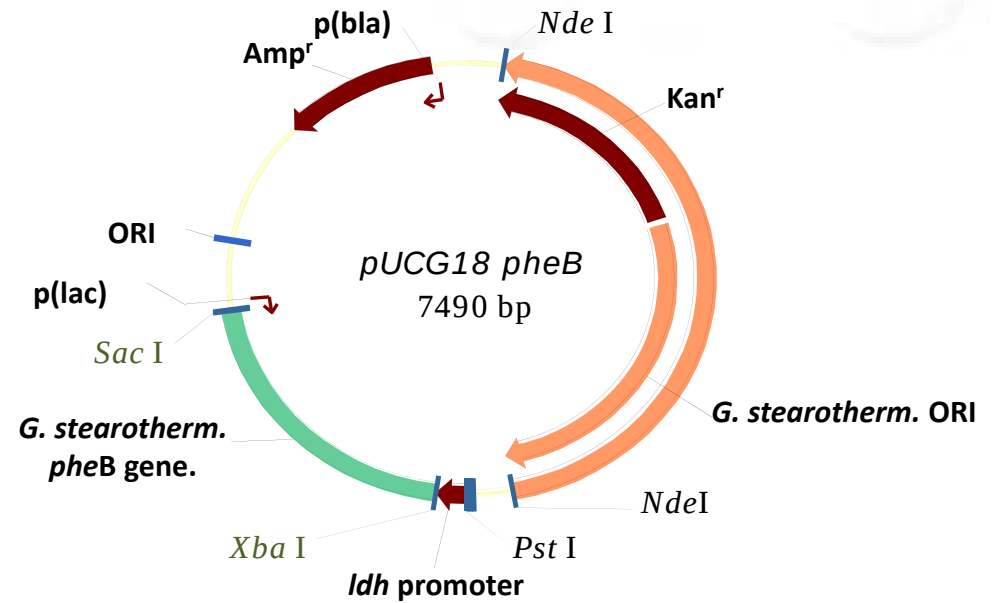
Shuttle vectors and reporter genes



Exchange for the ORI from pUB110 for T^s (55°C max) suicide vector for knockouts



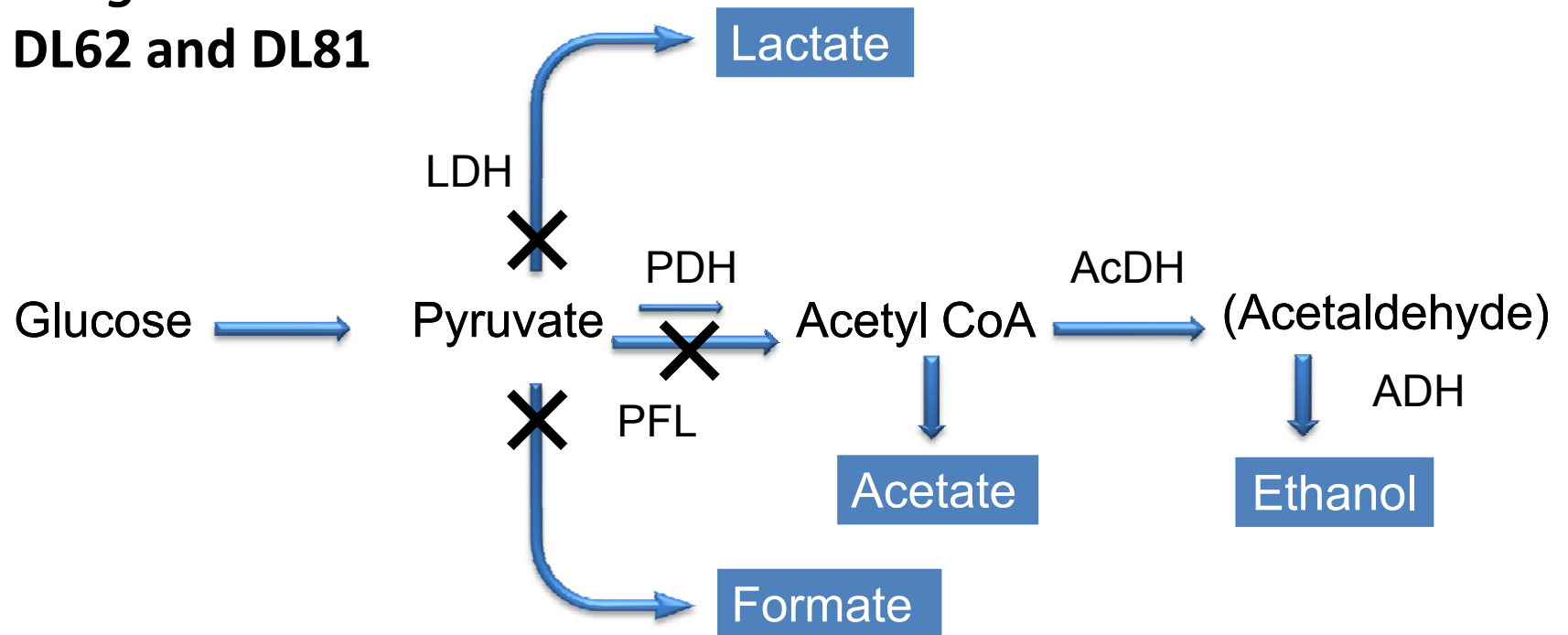
Expressing superfolder GFP



C230
Catechol → 2-hydroxymuconic semialdehyde

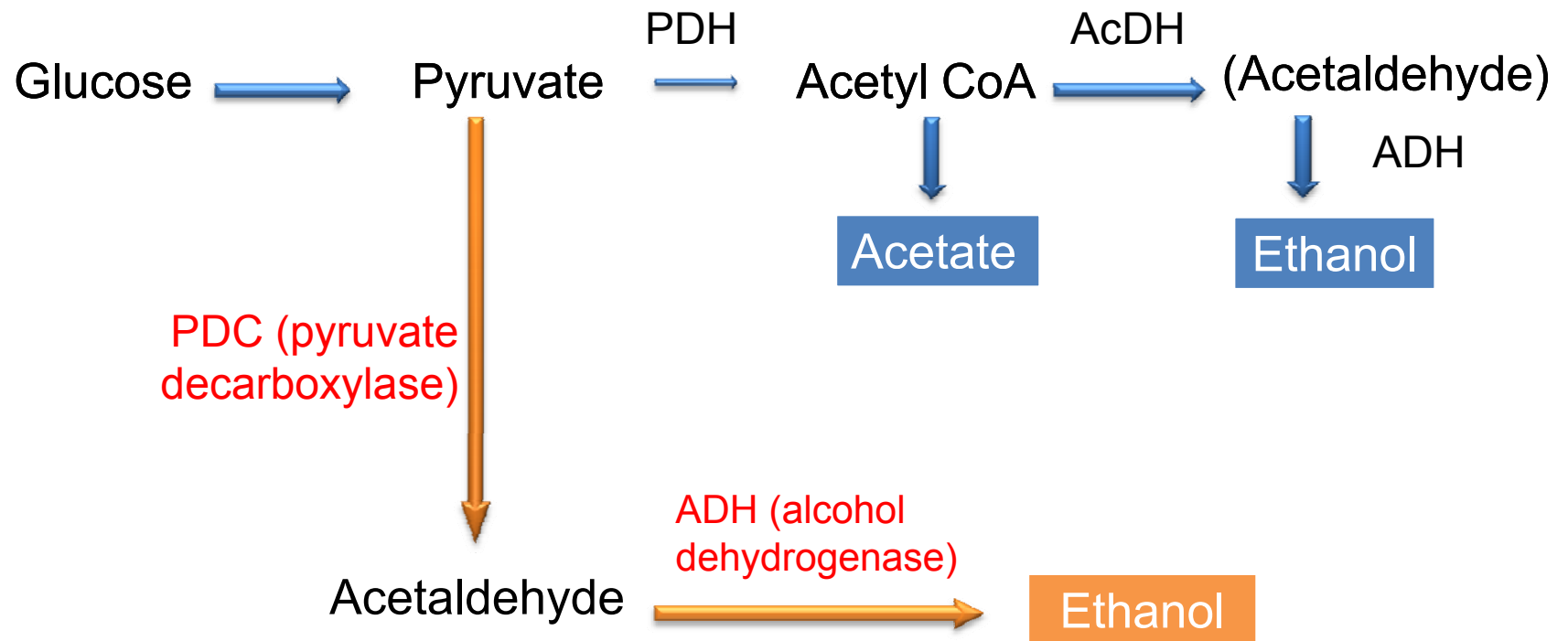
Ethanologenic Engineering Strategies

Fermentation pathways in
G. thermoglucosidasius
DL33, DL62 and DL81



Ethanologenic Engineering Strategies

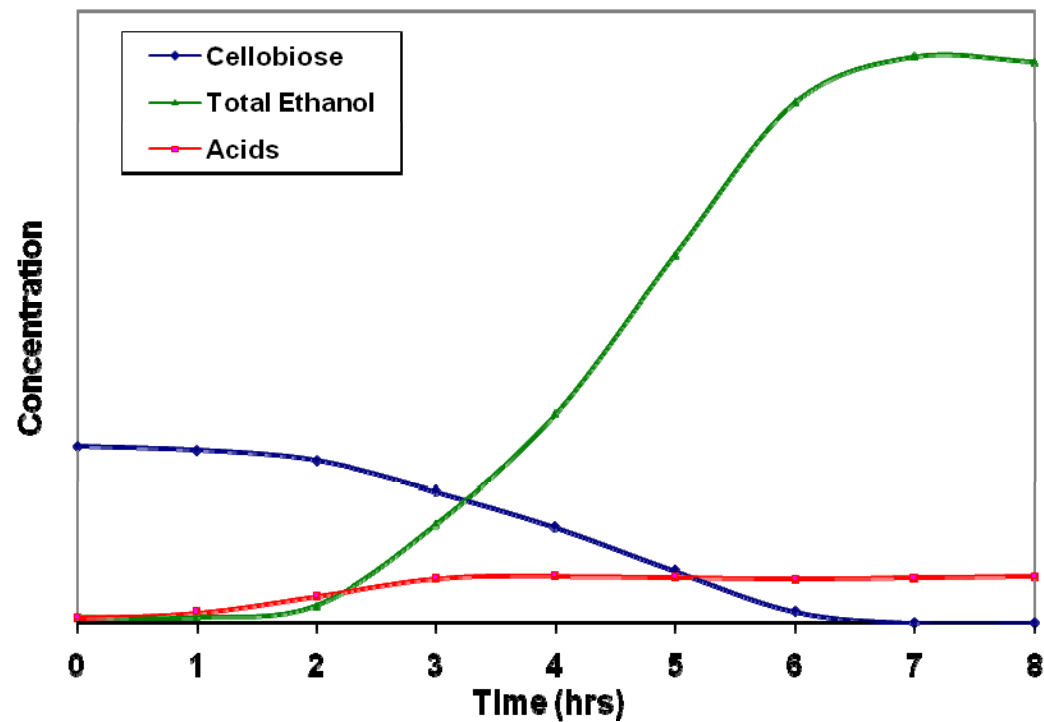
Fermentation pathways in
G. thermoglucosidasius
DL62



Growth of TM242 on cellobiose

Cellobiose - breakdown product from biomass hydrolysis

TM242 with Cellobiose at 60°C



Typical Ethanol yield = 0.45 g/g sugar

(90% of theoretical)

TMO Cellulosic Ethanol Demonstration Facility



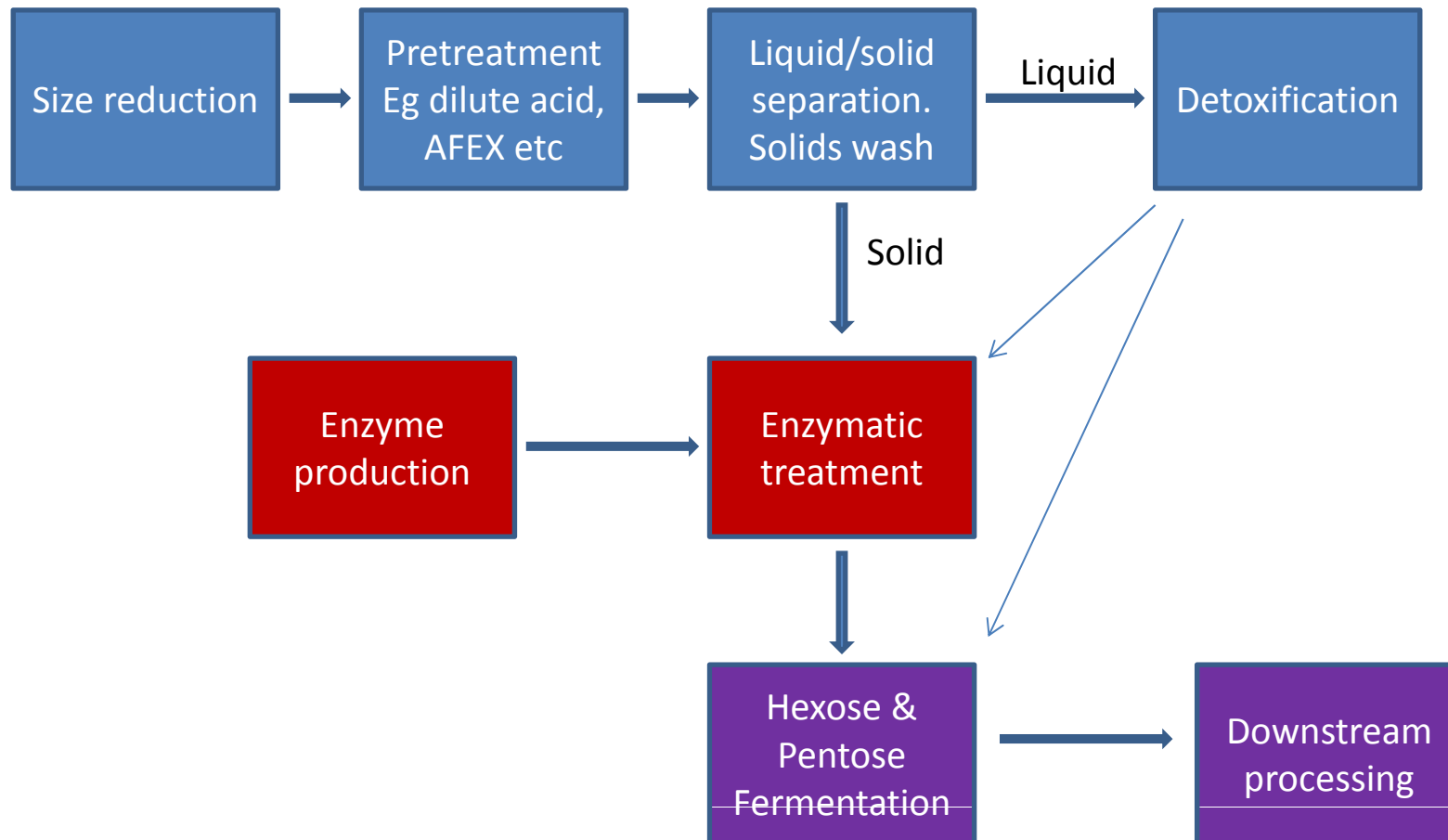
Cost: £7.8M
Pipework: 5km
Cabling: 50km
I/O: >2000

Flexible Capability
Multiple Feedstocks
High Solids Handling
24/7 Operation



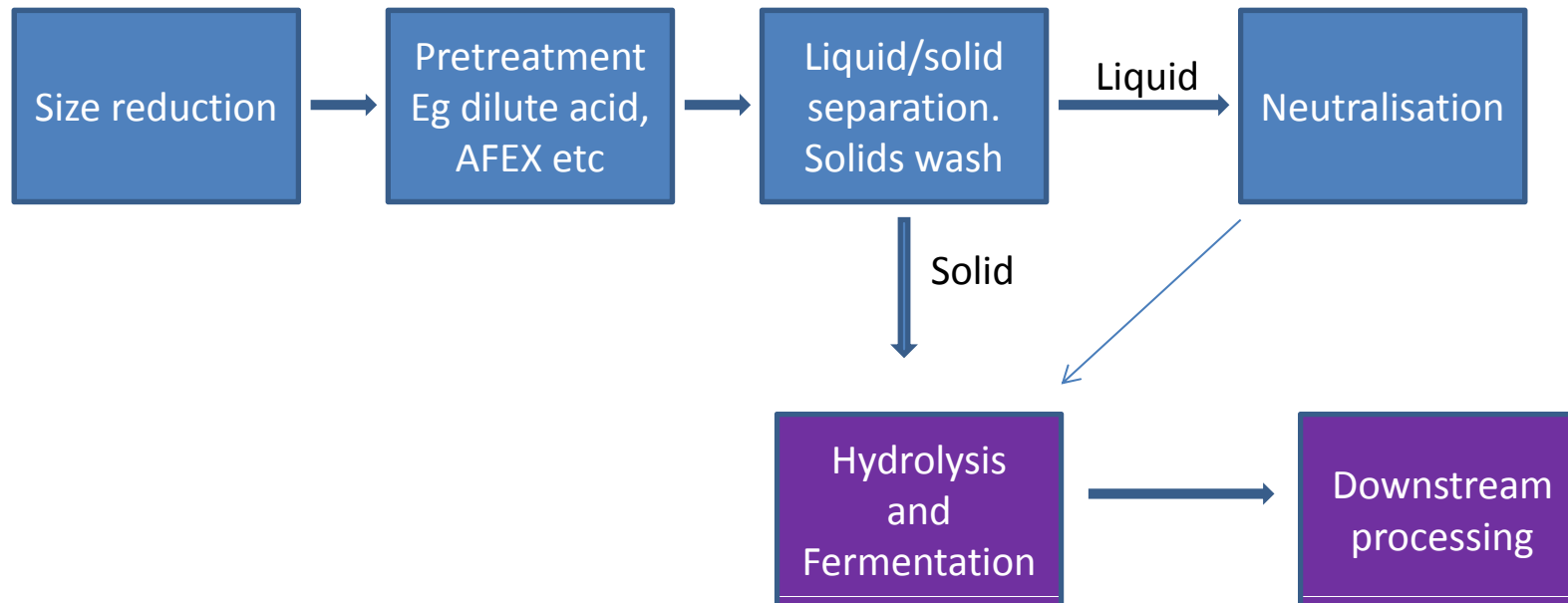
Lignocellulosics - process diagram

Typical Process Stream

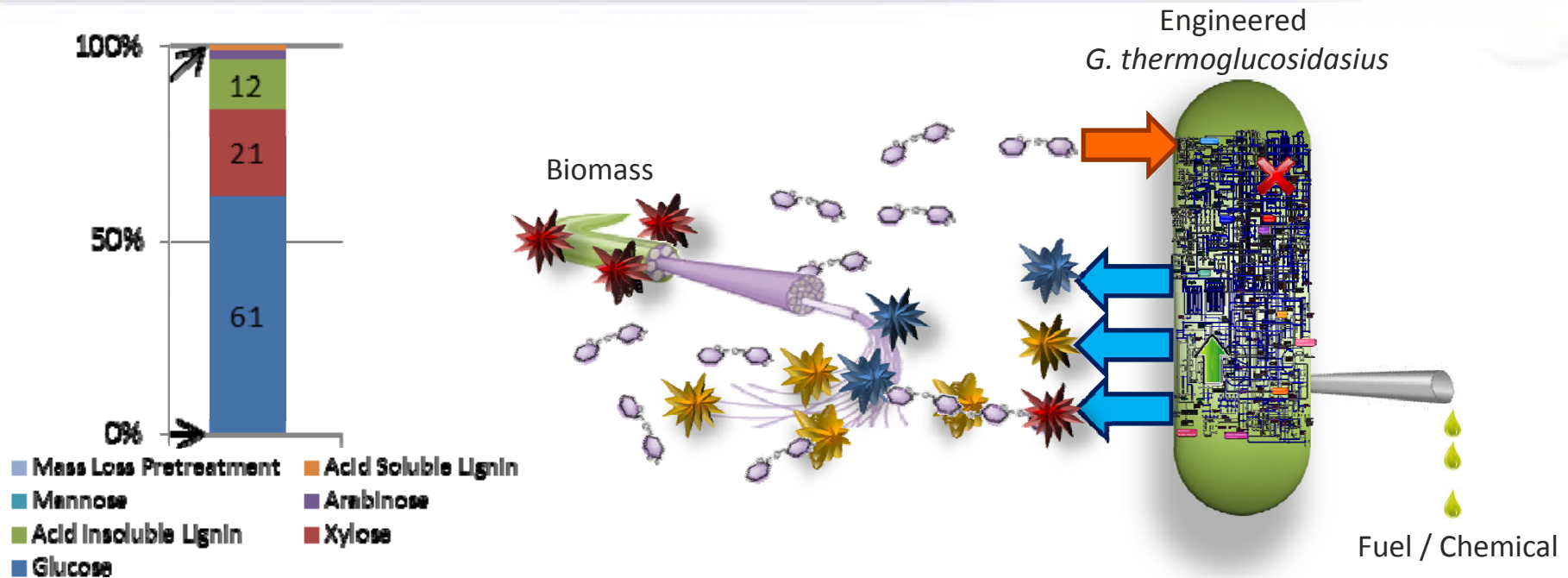


Lignocellulosics – consolidated bioprocess

Consolidated Process Stream

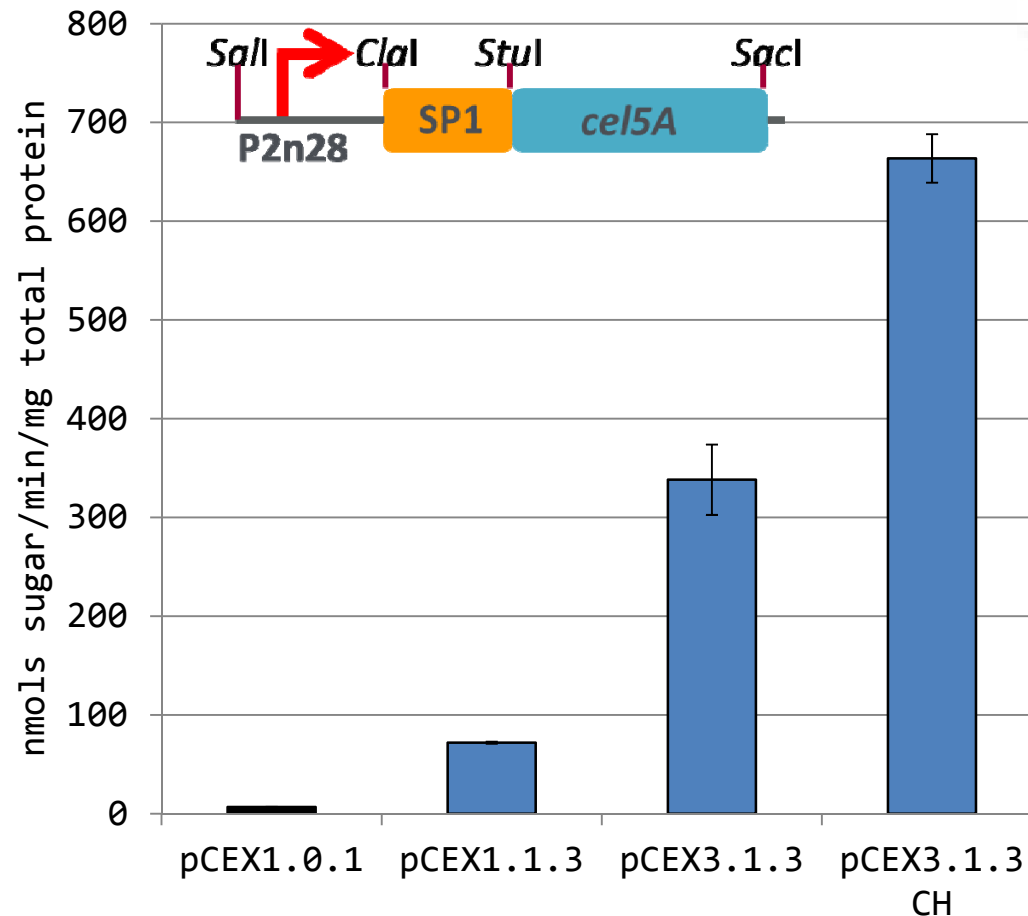


Consolidated Bioprocessing with *G. thermoglucosidasius*



Enzyme	CAZY GH Family	Organism
α -L-arabinofuranosidase1	GH51	<i>Cellvibrio japonicus</i> Ueda107
α -L-arabinofuranosidase	GH43	<i>Thermoanaerobacterium thermosaccharolyticum</i> M0795
Xylanase	GH10	<i>Caldicellulosiruptor saccharolyticus</i> DSM 8903
Xylanase	GH11	<i>Thermoanaerobacterium saccharolyticum</i> JW/SL-YS485
Acetylxyylan esterase	GH11	<i>Clostridium thermocellum</i> ATCC 27405
Exoglucanase	GH48	<i>Thermobifida fusca</i> YX
Exo&Endoglucanase	GH48 &GH9	<i>Caldicellulosiruptor saccharolyticus</i>
Endoglucanase	GH5	<i>Thermotoga maritima</i>
Endoglucanase	GH5	<i>Caldicellulosiruptor saccharolyticus</i>
Endoglucanase	GH9	<i>Clostridium thermocellum</i>

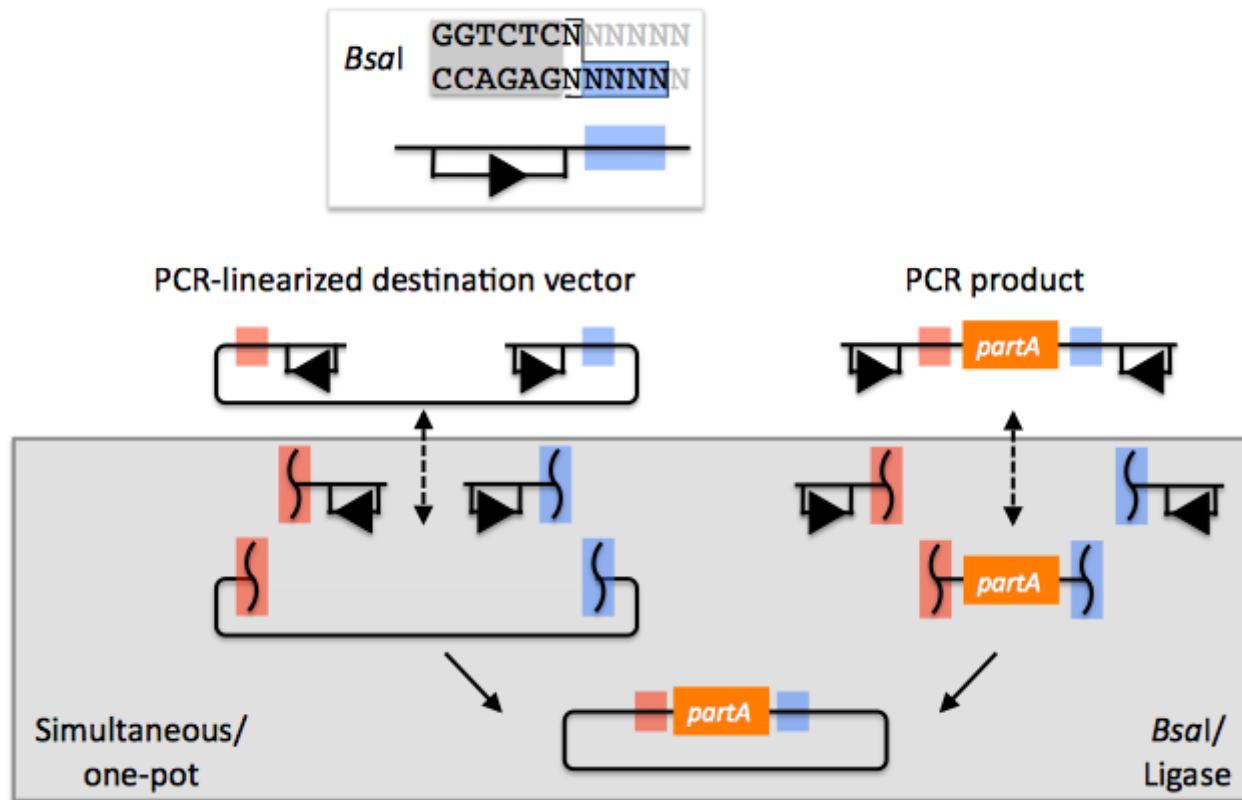
Improvements in GH activity



- 10 fold increase in CMCase activity in culture supernatant.
- Published activity of 616 U/mg on CMC.

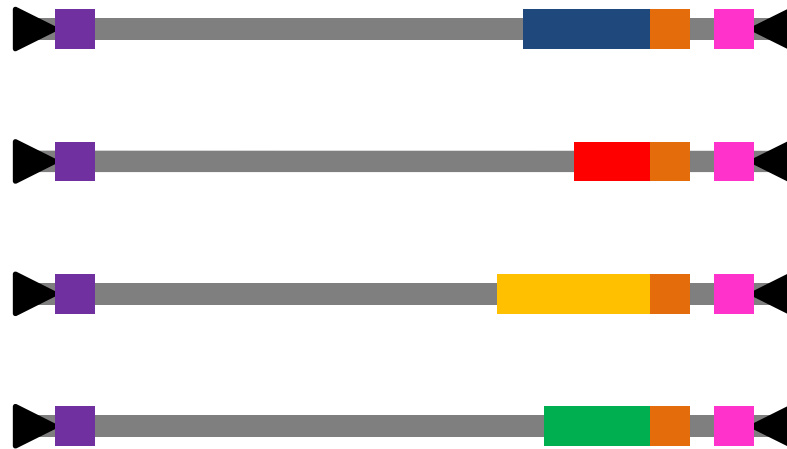
Assembling pCEX variants

- *Golden Gate assembly – using BsaI (Type IIs restriction enzyme)*



Destination vectors

- Generate linearized product which *Bsal* will cut in the *StuI* and *SacI* sites.



■ *StuI* - AGGCCT

■ *SacI* - GAGCTC

▶ *Bsal* - GGTCTC

GGTCTC**GAGCTC**
CCAGAG**CTCGAG**



AGGCCTGAGACC
TCCGGACTCTGG

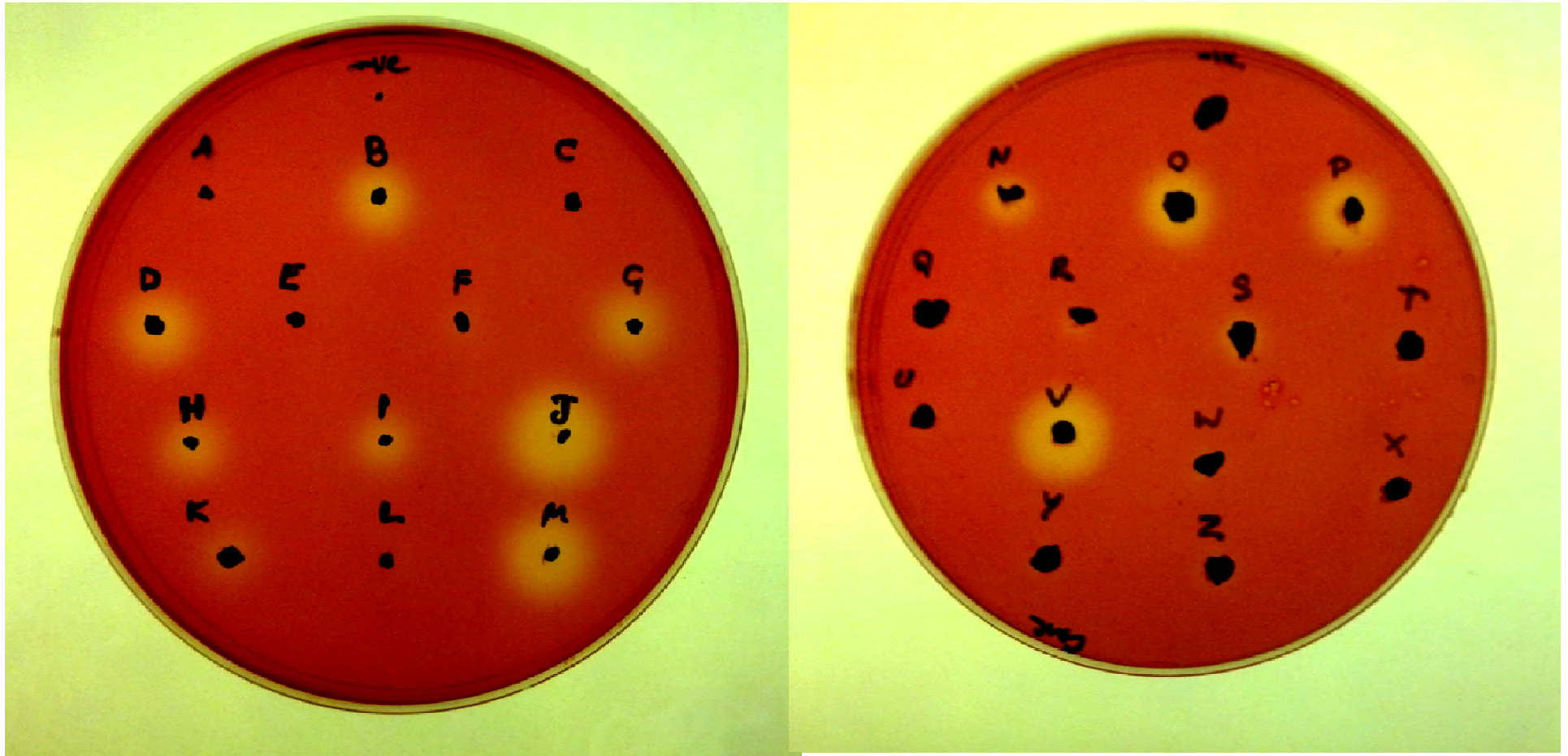


GGTCTC**G**...**AGCTC**
CCAGAG**CTCGA**...**G**



A...**GGCCT**GAGACC
TCCGG...**ACTCTGG**

Screening of signal sequences

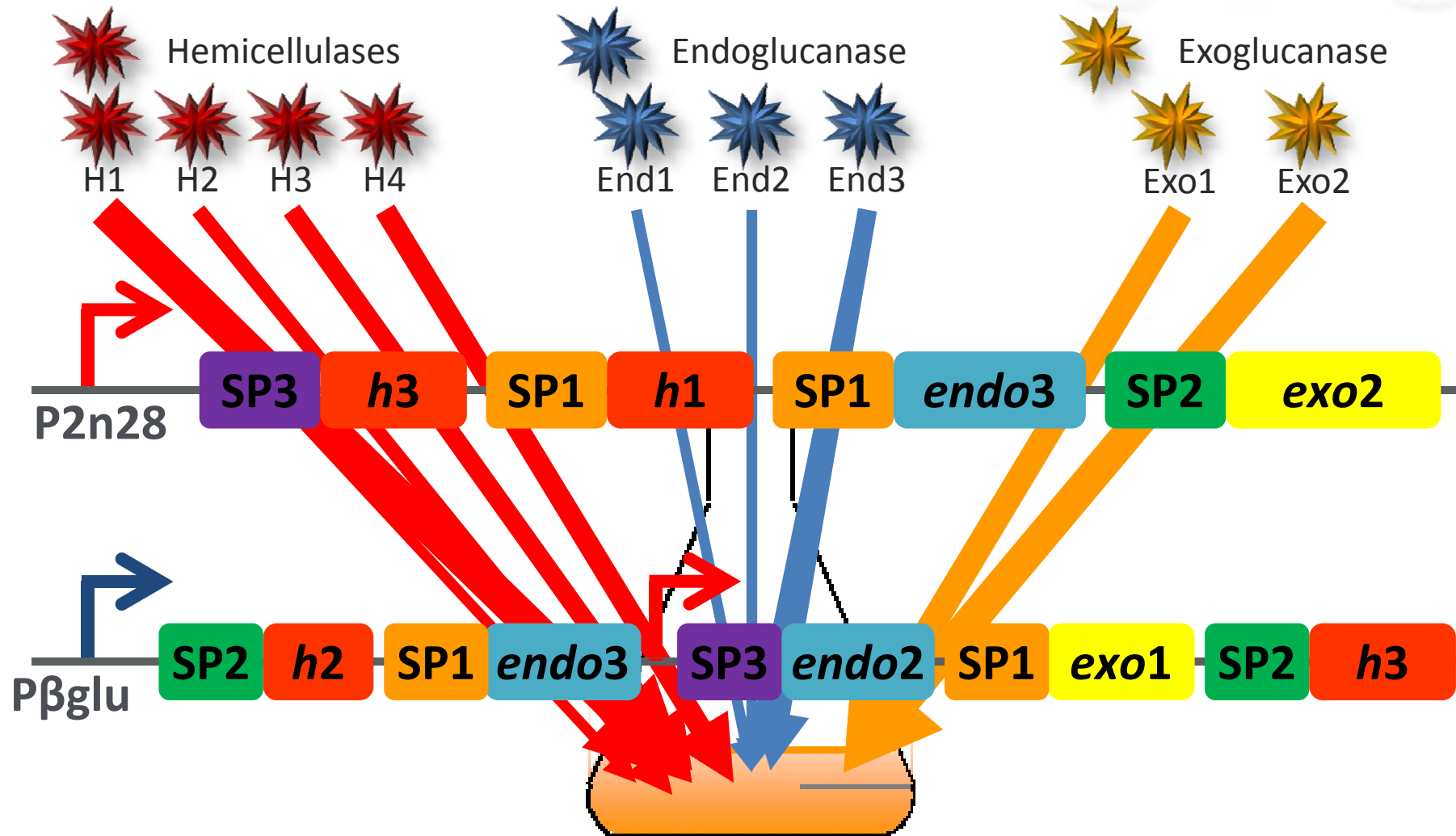


Preliminary GH activities

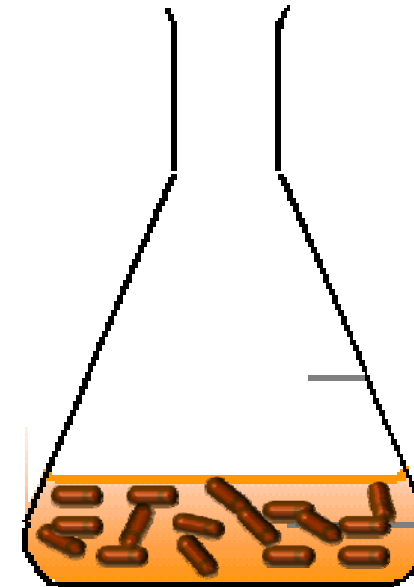
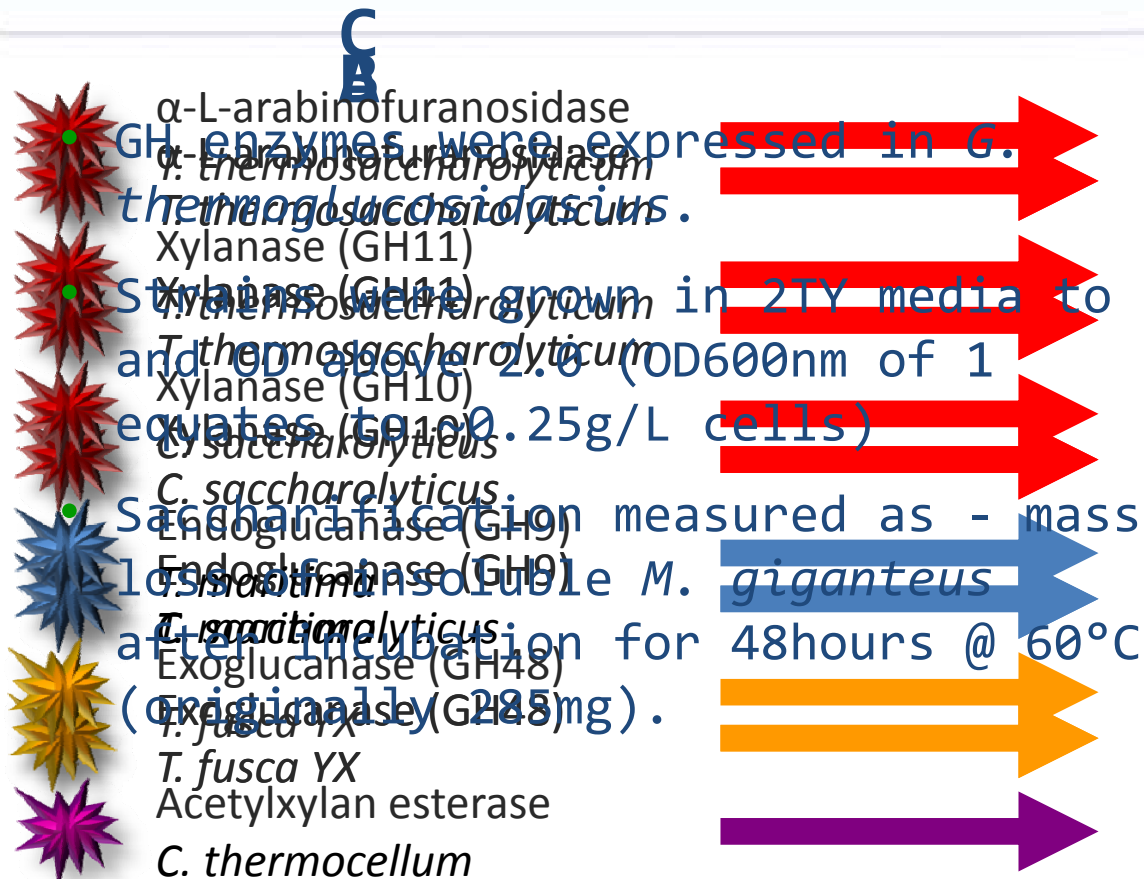
- Optimised a cassette for GH expression
- Expressed and measured activities in culture supernatants

Enzyme	Organism	Substrate	Activity @ 60°C pH7.0
α-L-arabinofuranosidase1	<i>T. thermosaccharolyticum</i> M0795	Oat Spelt Xylan, o/p-nitrophenyl-α-l-arabinofuranoside	20 μmol min ⁻¹ mg ⁻¹
α-L-arabinofuranosidase	<i>C. japonicus</i> Ueda107T	As above	3 μmol min ⁻¹ mg ⁻¹
Xylanase	<i>C. saccharolyticus</i> DSM 8903	Beech/Oat spelt xylan o-nitrophenyl-β-d-xylobioside	100 μmol min ⁻¹ mg ⁻¹
	<i>T. saccharolyticum</i> JW/SL-YS485	As above	
Acetylxytan esterase	<i>C. thermocellum</i> ATCC 27405	Accelerant of xylanase activity	N/D
Exoglucanase	<i>T. fusca</i> YX	Filter paper, PASC, Avicel	0.3 μmol min ⁻¹ mg ⁻¹
Exo&Endoglucanase	<i>C. saccharolyticus</i>	As above	N/D
Endoglucanase	<i>T. maritima</i>	CMC, HEC	0.6 μmol min ⁻¹ mg ⁻¹
Endoglucanase	<i>C. saccharolyticus</i>	As above	0.1 μmol min ⁻¹ mg ⁻¹
Endoglucanase	<i>C. thermocellum</i>	As above	0.2 μmol min ⁻¹ mg ⁻¹

Assessing recombinant GH activity



Evaluating CBP – *M. giganteus*



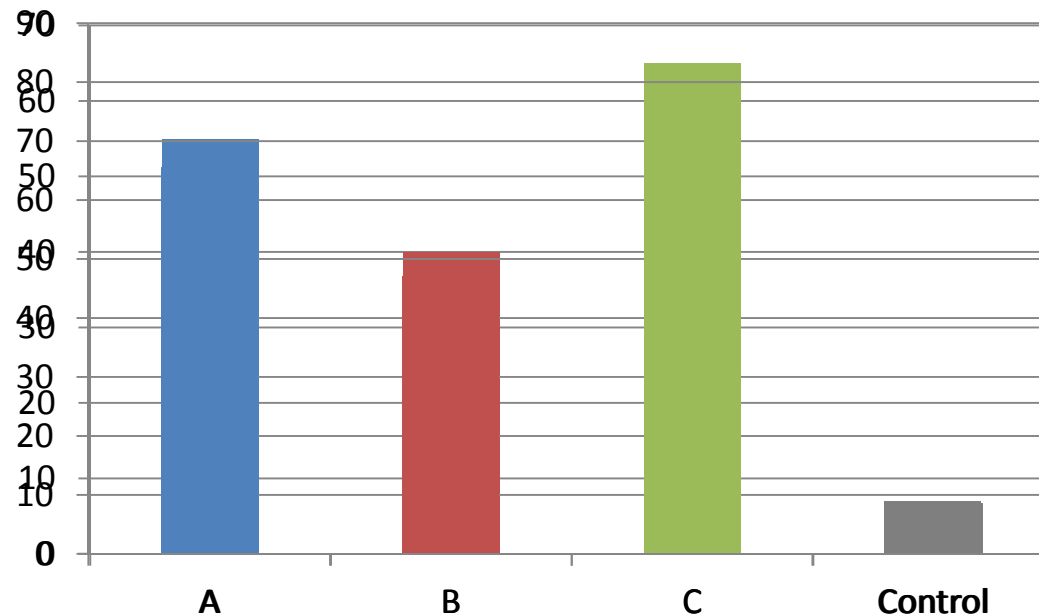
- A**
- α -L-arabinofuranosidase
T. thermosaccharolyticum
 - Xylanase (GH11)
T. thermosaccharolyticum
 - Xylanase (GH10)
C. saccharolyticus
 - Endoglucanase (GH9)
T. maritima
 - Exoglucanase (GH48)
T. fusca YX

- B**
- α -L-arabinofuranosidase
T. thermosaccharolyticum
 - Xylanase (GH11)
T. thermosaccharolyticum
 - Xylanase (GH10)
C. saccharolyticus
 - Endoglucanase (GH9)
C. saccharolyticus
 - Exoglucanase (GH48)
T. fusca YX

- C**
- α -L-arabinofuranosidase
T. thermosaccharolyticum
 - Xylanase (GH11)
T. thermosaccharolyticum
 - Xylanase (GH10)
C. saccharolyticus
 - Endoglucanase (GH9)
T. maritima
 - Exoglucanase (GH48)
T. fusca YX
 - Acetylxylen esterase
C. thermocellum

CBP – Mass loss

- % of available carbohydrates released as after CBP. Assumes lignin is not degraded.



A

- α-L-arabinofuranosidase
T. thermosaccharolyticum
- Xylanase (GH11)
T. thermosaccharolyticum
- Xylanase (GH10)
C. saccharolyticus
- Endoglucanase (GH9)
T. maritima
- Exoglucanase (GH48)
T. fusca YX

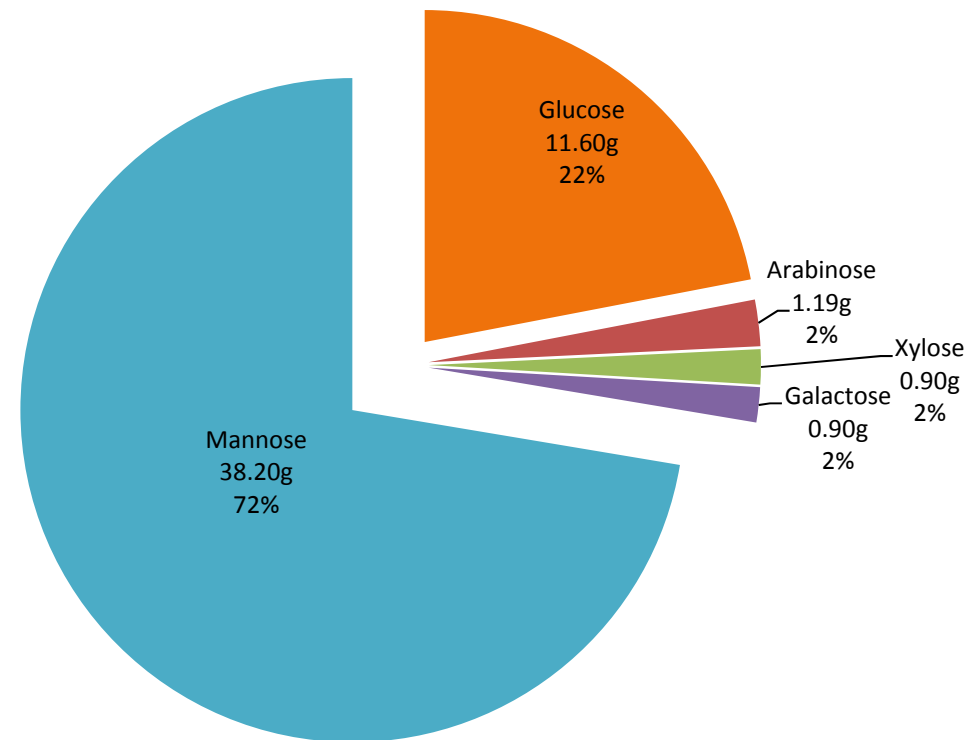
B

- α-L-arabinofuranosidase
T. thermosaccharolyticum
- Xylanase (GH11)
T. thermosaccharolyticum
- Xylanase (GH10)
C. saccharolyticus
- Endoglucanase (GH9)
C. saccharolyticus
- Exoglucanase (GH48)
T. fusca YX

C

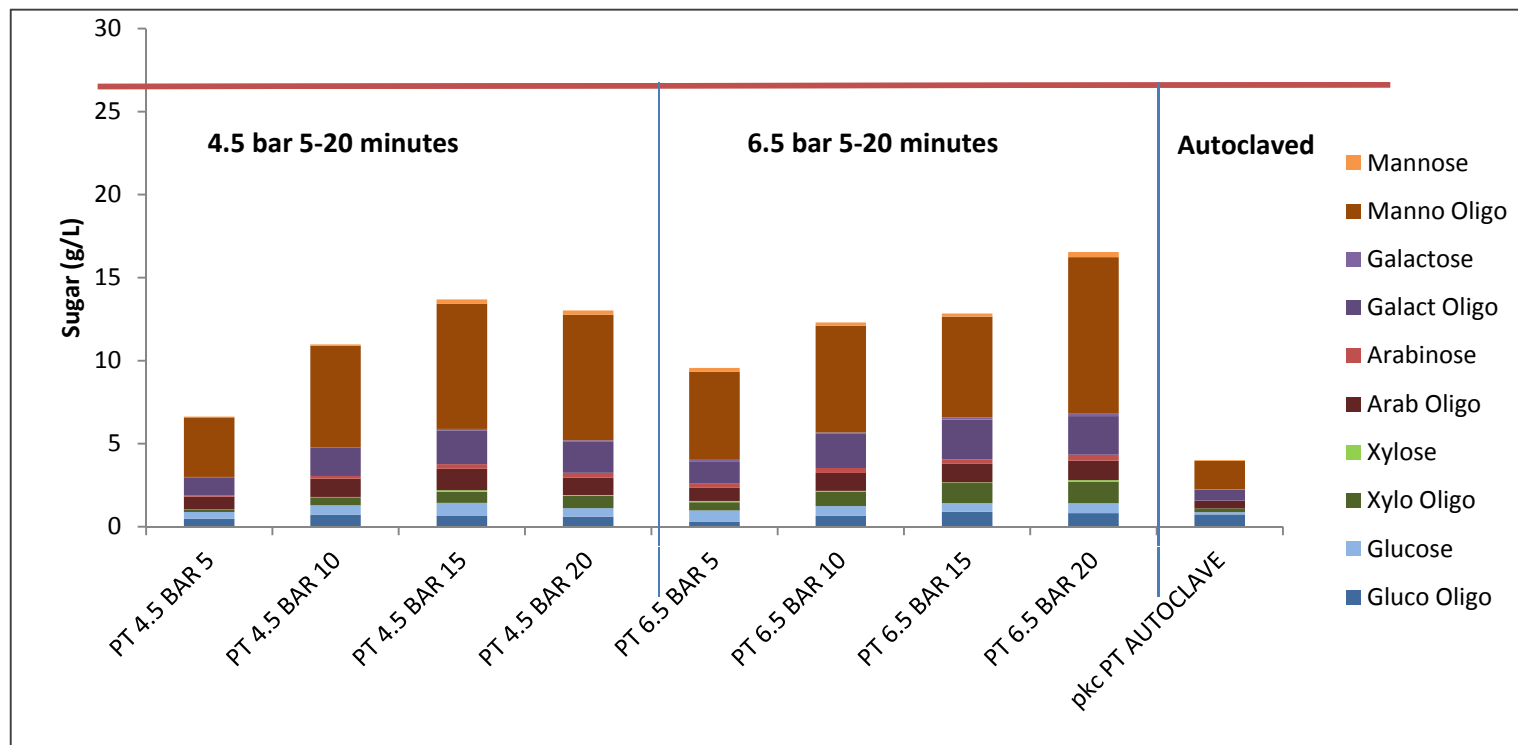
- α-L-arabinofuranosidase
T. thermosaccharolyticum
- Xylanase (GH11)
T. thermosaccharolyticum
- Xylanase (GH10)
C. saccharolyticus
- Endoglucanase (GH9)
T. maritima
- Exoglucanase (GH48)
T. fusca YX
- Acetylxylan esterase
C. thermocellum

Carbohydrate composition of PKC



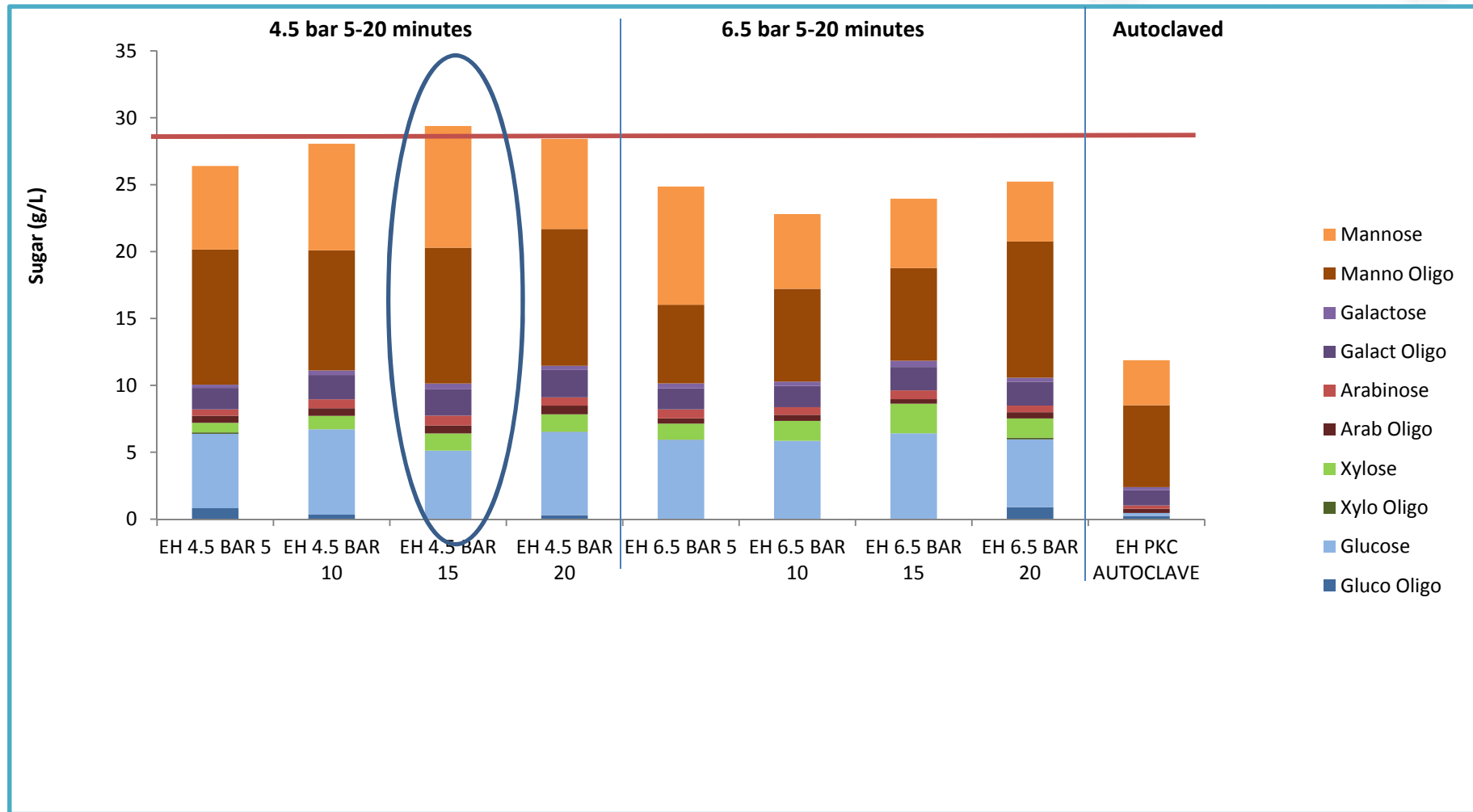
- Mainly Mannose and Cellulose (mannan and glucan) in PKC
- Possibility of producing biofuels from this biomass
- Enzyme required for hydrolysis are cellulase, mannanase + mannosidase

Steam Pre-treatment of PKC (5% DS)



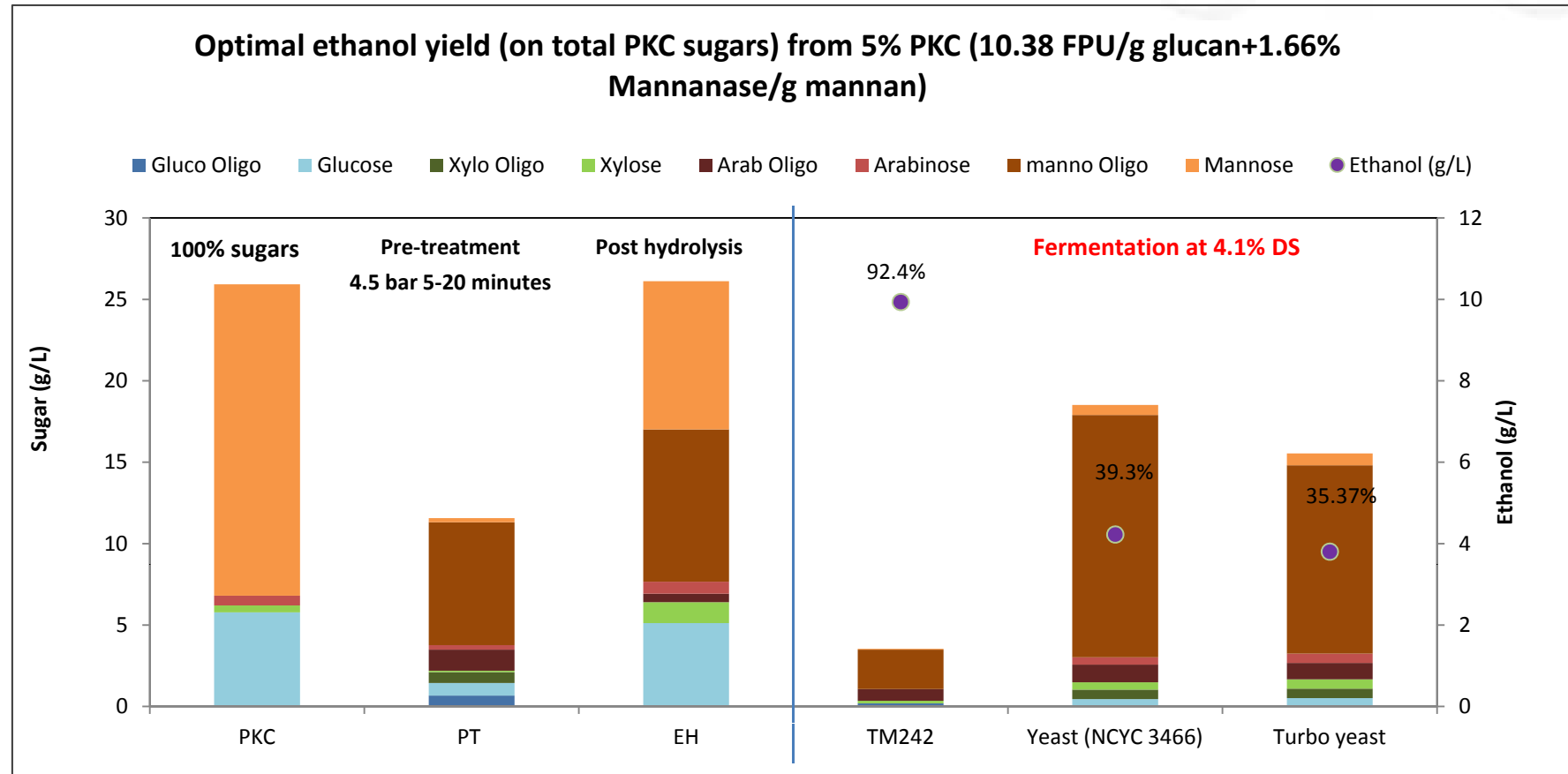
- Various pre-treatments (PT) were carried out for 5-20 minutes released mainly oligomeric sugars (soluble sugars shown).
- Increasing sugar release with increasing time of PT.

Enzyme hydrolysis of 5% DS PKC



- This graph suggests 4.5 bar 15 as optimum (pH5, 50°C, 72hr), while sugar degradation at higher temperature, pressure and time.

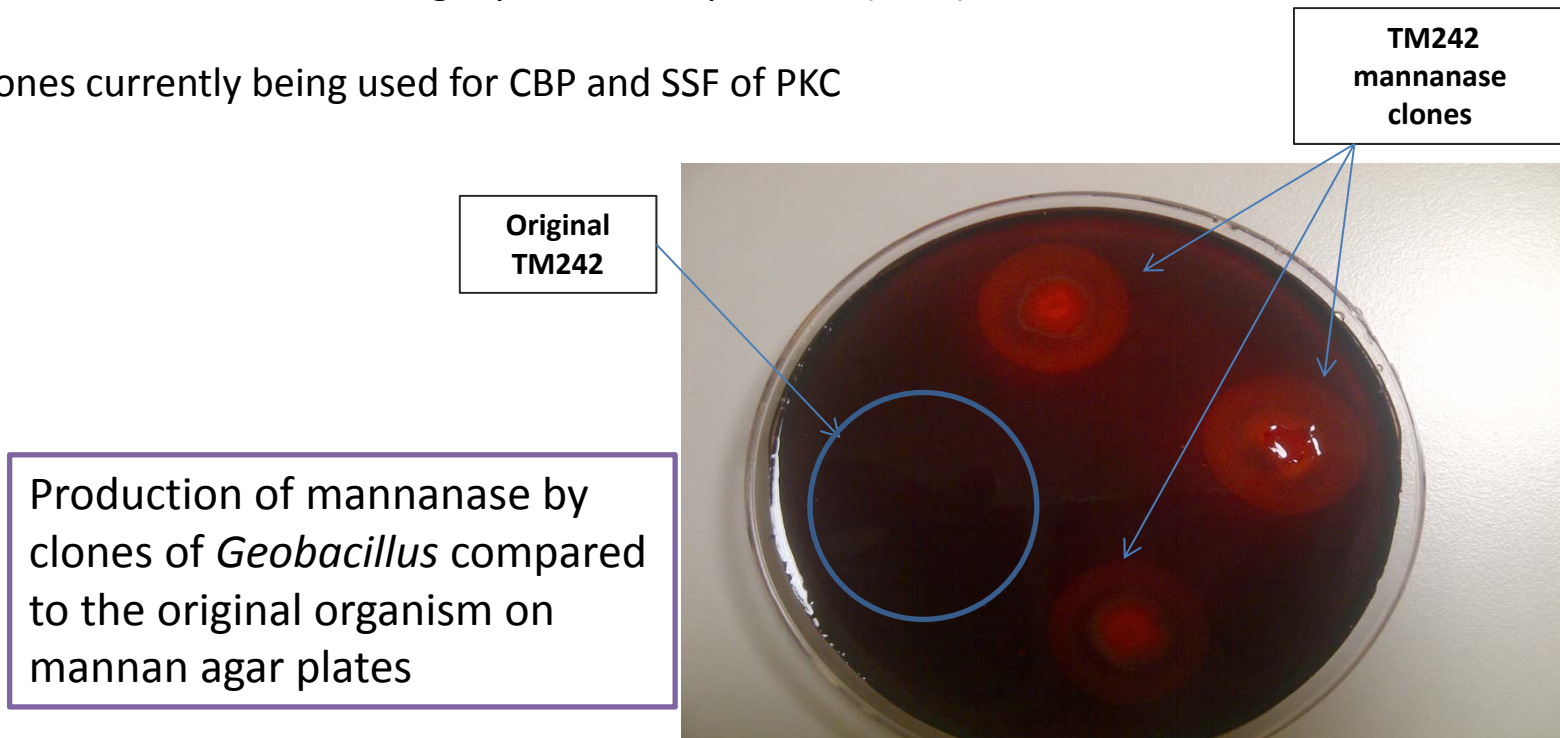
Production of ethanol from 5% PKC



- Post enzyme hydrolysis, 100% solubilisation of sugars (glucan and mannan) after 72hrs, but only 50% monomerisation of mannan (100% for glucan).
- *Geobacillus* ferments most of the sugars including the oligomers, while yeast only ferments the monomeric sugars

Mannanase production by TM242

- Mannanase gene PCR extracted from *Bacillus subtilis*
- Engineered into *E.coli* with a holding vector (and colony PCR checked)
- Cloned into *Geobacillus* TM242 (constitutive expression vector) and confirmed by PCR
- Clones tested on mannan agar plates for expression (24hr).
- Clones currently being used for CBP and SSF of PKC



Thank you



- Jeremy Bartosiak-Jentys & Ali Hussein¹ - CBP and SigP
- Chris Ibenegbu, Charlie Bennett¹, Marisa Raita ^{1,2} - PKC
- Steve Martin & Kirstin Eley³

¹Dept of Biology & Biochemistry, University of Bath

²Joint Graduate School of Energy and Environment, King Mongkut's University of Technology Thonburi

³TMO Renewables

Any Questions?



IBTI • INTEGRATED BIOREFINING RESEARCH AND TECHNOLOGY CLUB

Palm Kernel Cake (PKC) Production



Palm Tree



Fresh Fruit Bunches (FFB)

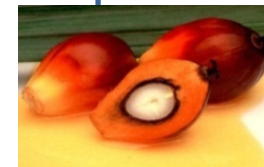


Empty Palm Fruit Bunch (EPFB) 22%

Sterilization and stripping



Palm Oil Milled Effluent (POME)



Palm Fresh Fruit

Digestion, clarification, pressing



Crude Palm Oil



Palm Nut



Palm Fiber

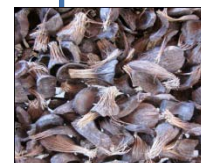
Oil palm tree (25 years)

- To start **bearing bunches** 2½–3 years after field planting
- After **harvesting round** 10–15 days per month

Cracking



Palm Kernel Cake (PKC) 6%



Palm Kernel Shell (PKS)



Crude Palm Kernel Oil

High Solids Loading CBP

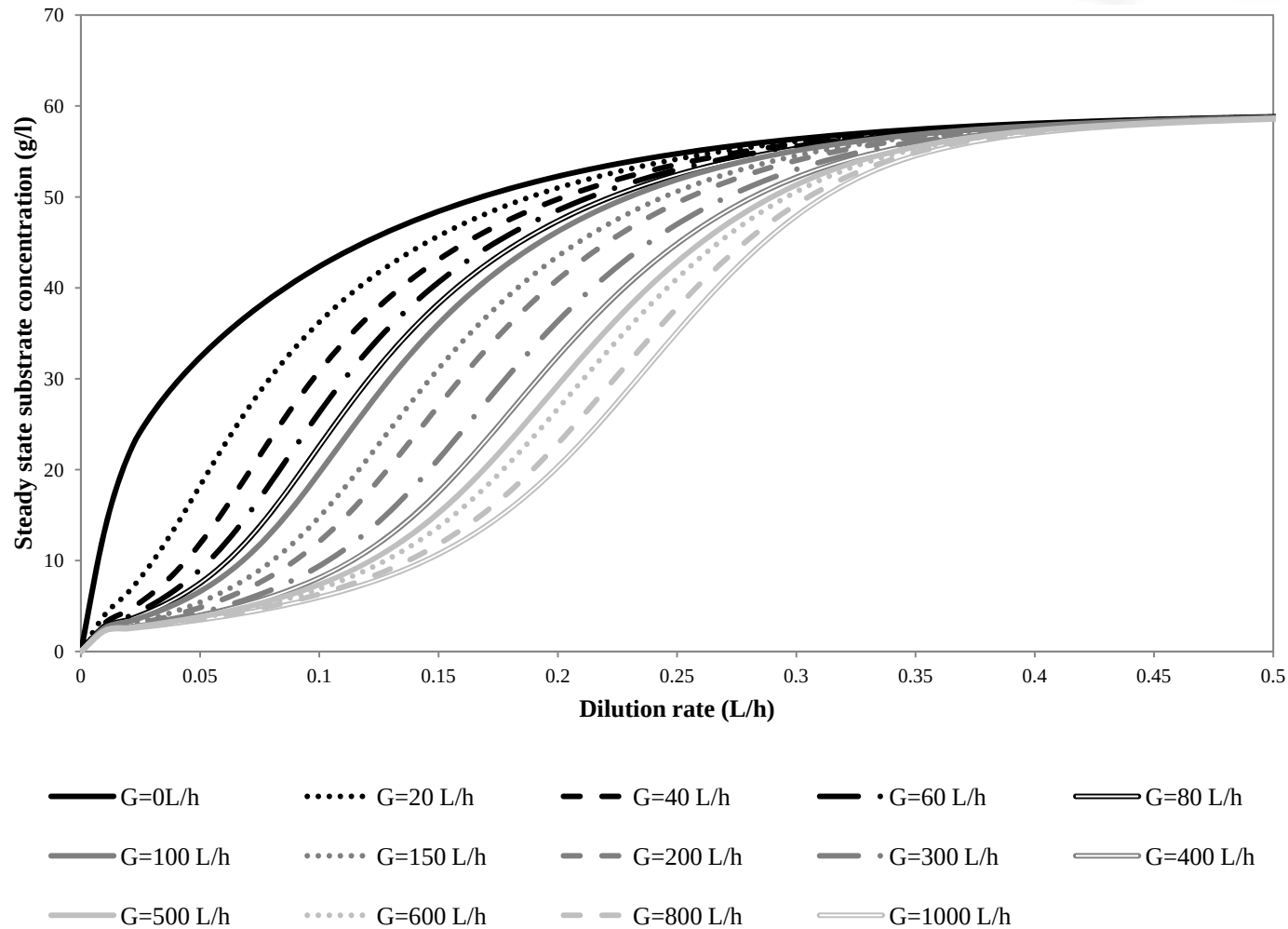
- Effective CBP requires solids loadings of ~30% (w/w)



Pretreated (Lig. Cor.) Post Sacch. (Lig. Cor.)

- Particle size distribution changes during process.
- Monosaccharide release was rapid over first 18 hours and then linear over remaining 5 days.
- Changing rheological properties of the slurry during CBP are likely to have a significant effect on its operation.
- Compositional analysis indicated extent of degradation.
- Recommendation is to run a fed batch process.

Modelling gas-stripping requirement for continuous culture



Blank

