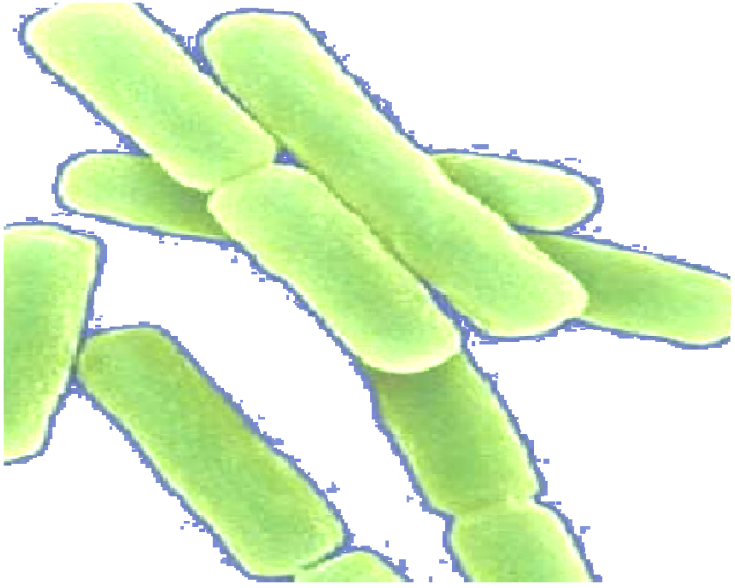
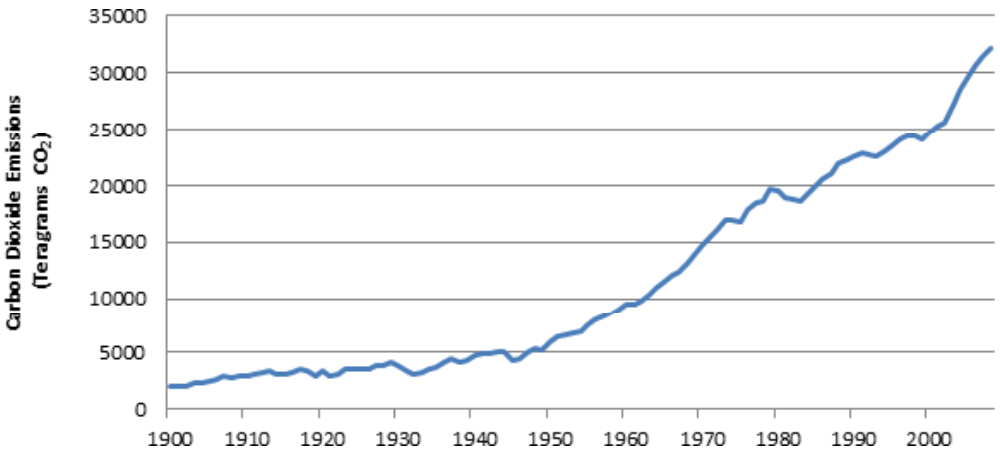
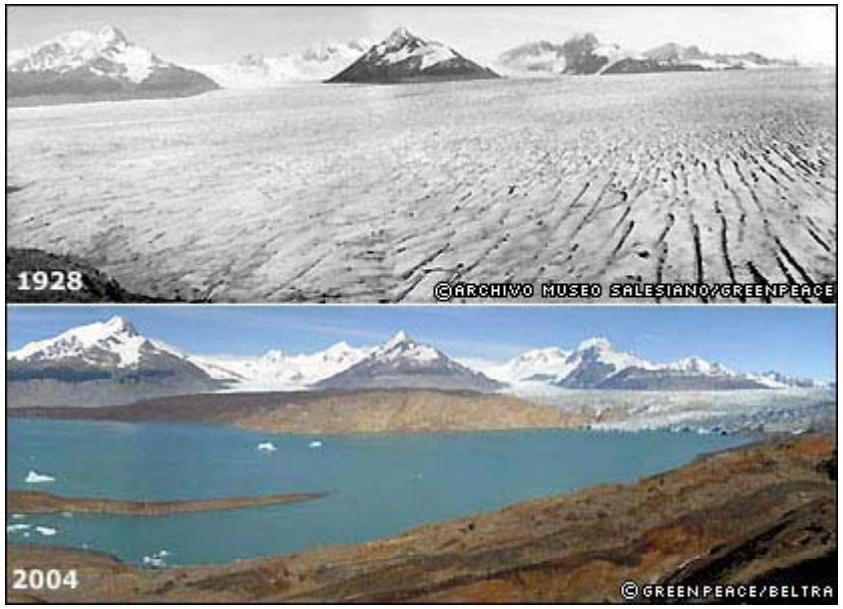
Geobacillus thermoglucosidasius – a catabolically versatile host for metabolic engineering

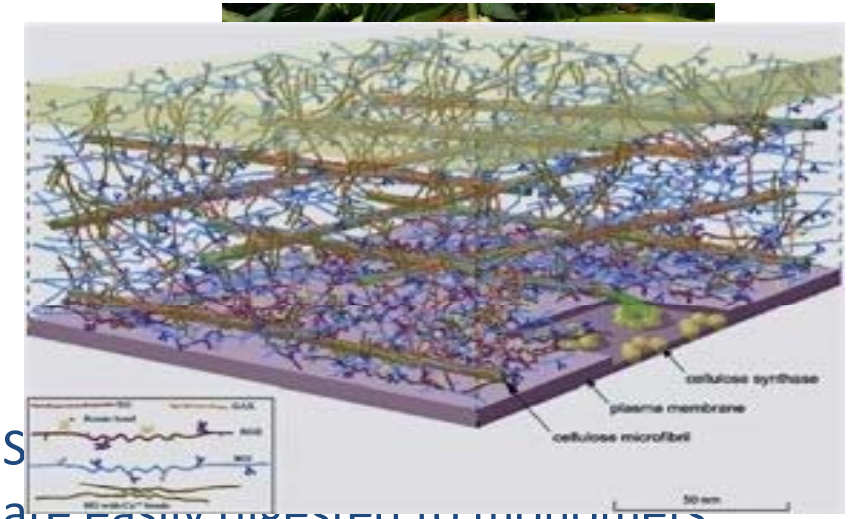


Global warming & energy security



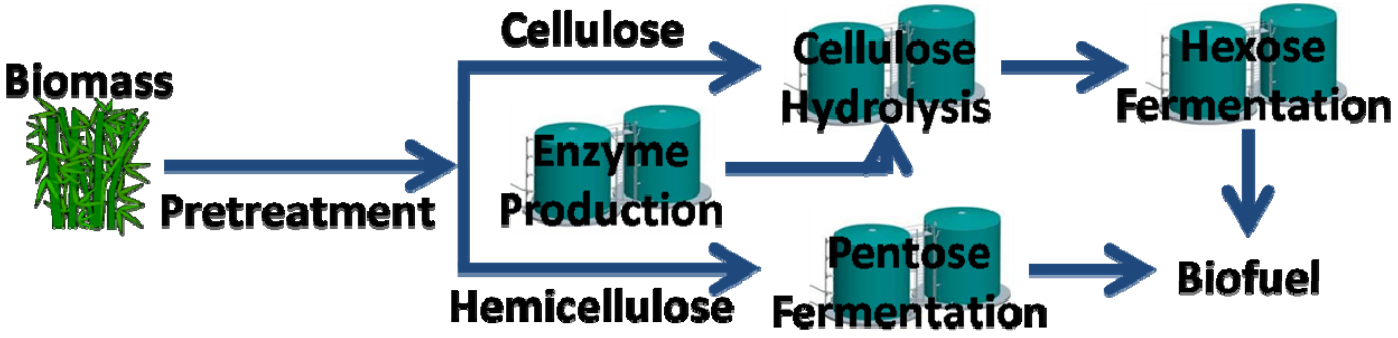


Biofuel Feedstock (Food vs Fuel)



- S
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^{o}C , spore forming - reclassified 2001 (Nazina et al)

Advantages

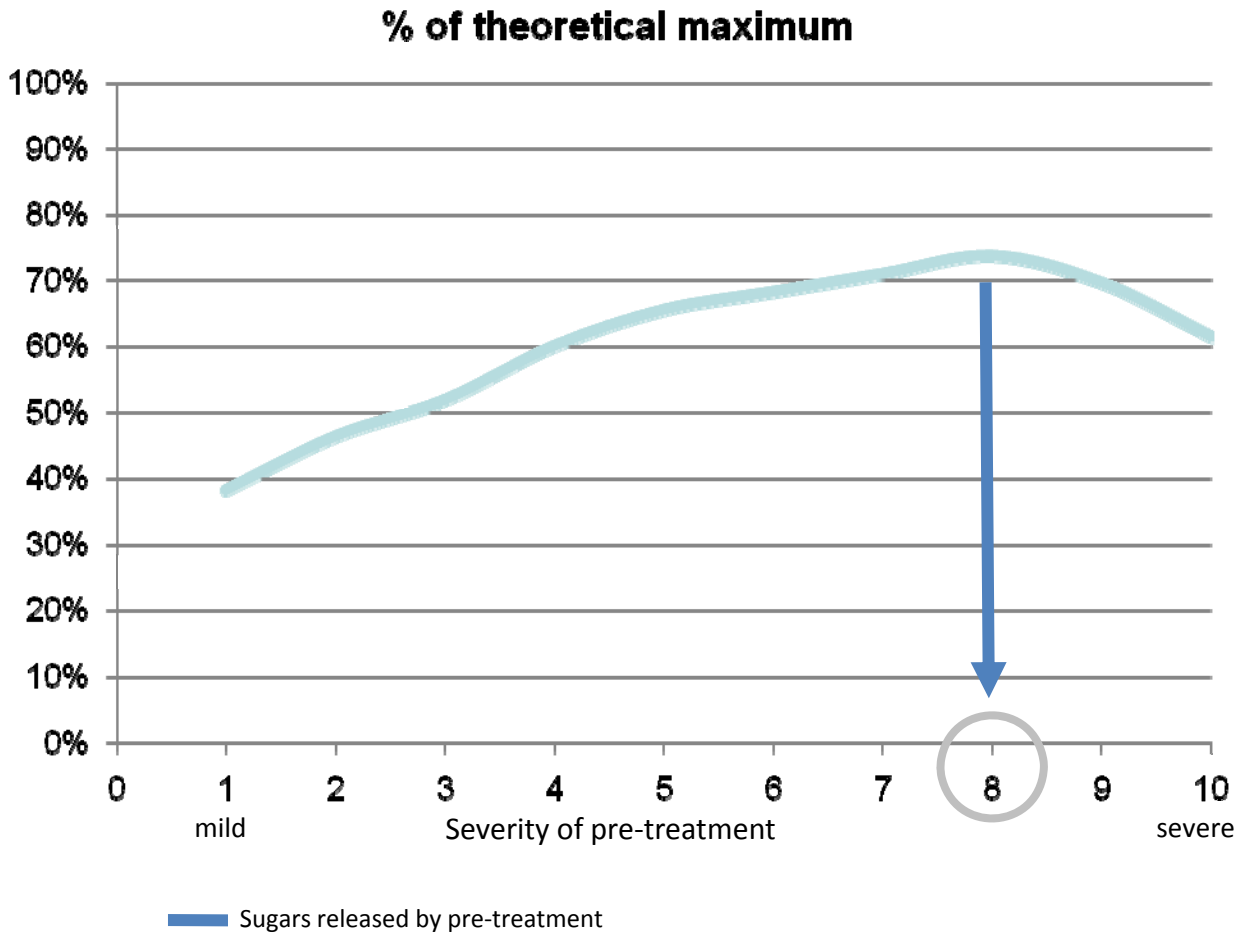
- Metabolically versatile (hexose, pentose, cellobiose, xylans)
- Rapid growth (eg μ_{glc} = 0.85 h^{-1}, μ_{xyl} = 0.60 h^{-1} aerobic 66^{o}C)
- Growth at ~70^{o}C allows ethanol removal by gas stripping
- Growth temperature compatible with typical process operations
- Facultative anaerobes allow

  1) aerobic biomass 2) anaerobic fermentation

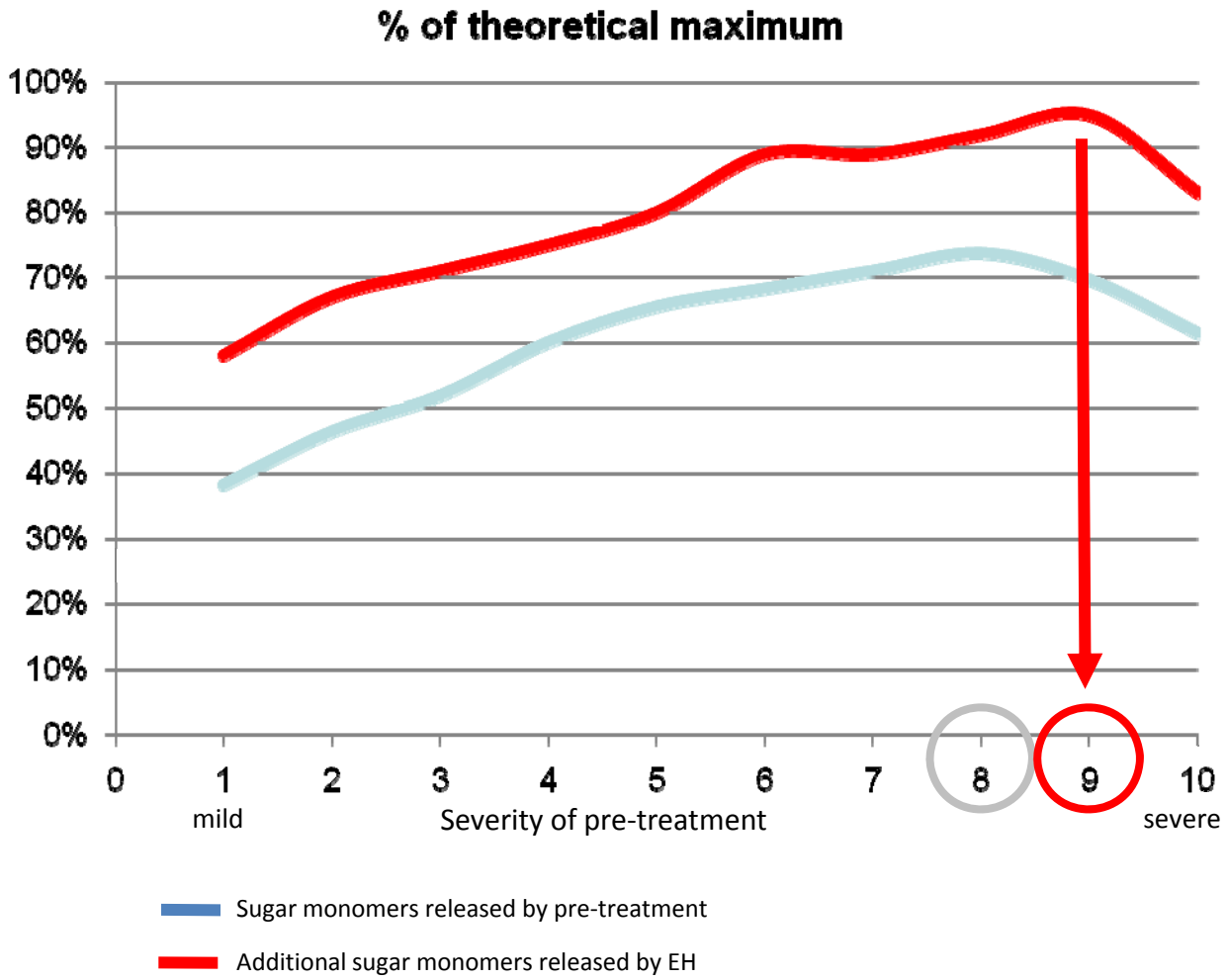
Disadvantages

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

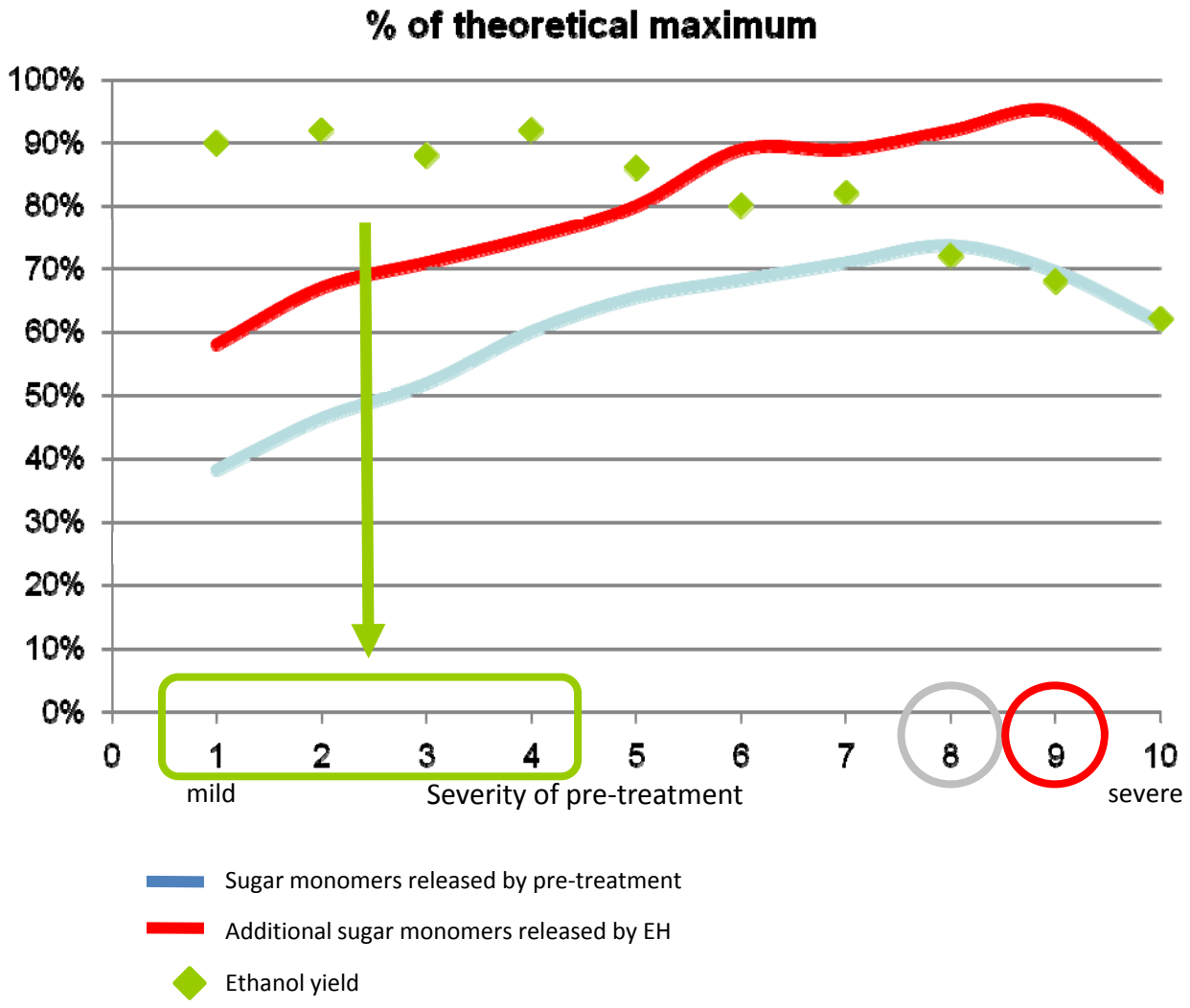
Initial pre-treatment studies



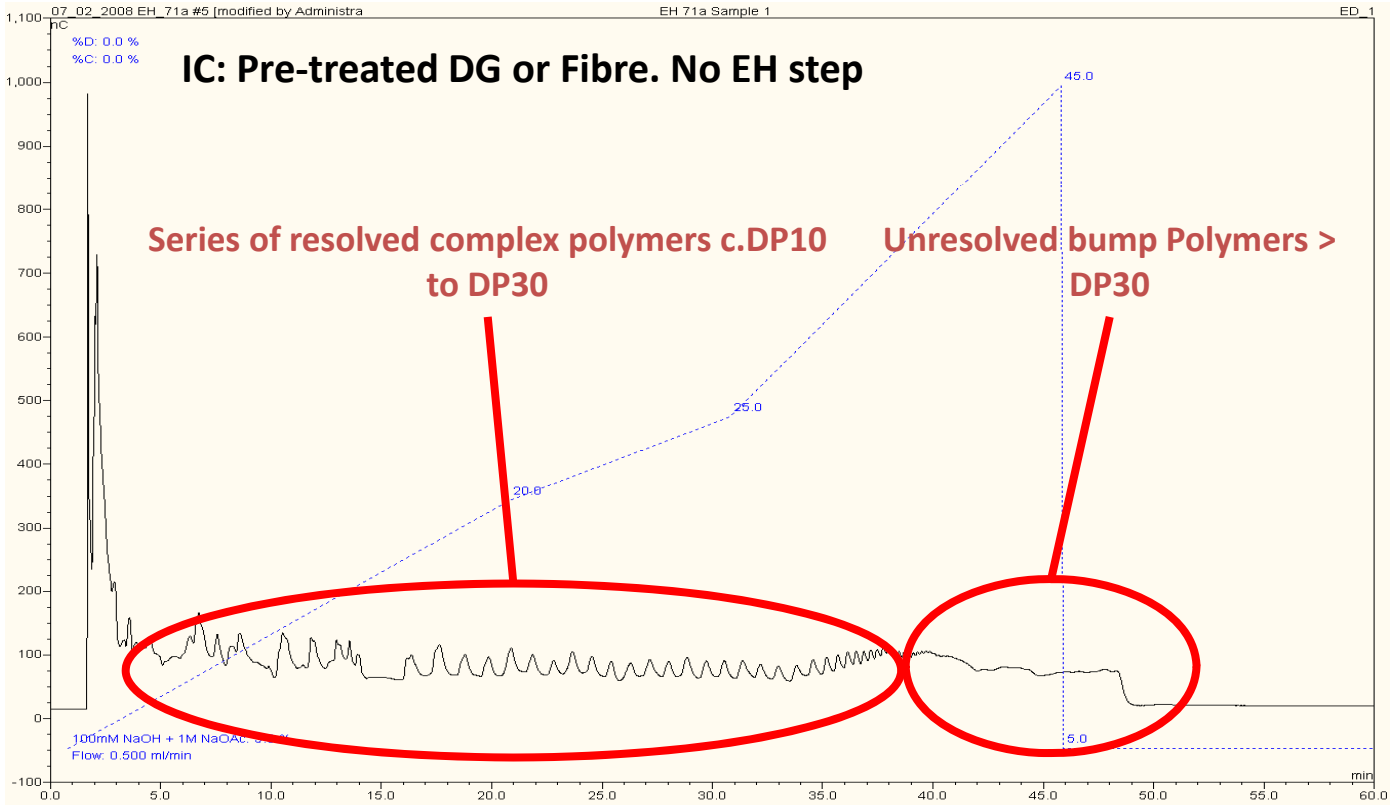
Following enzyme hydrolysis

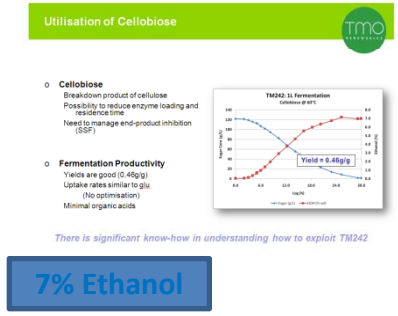


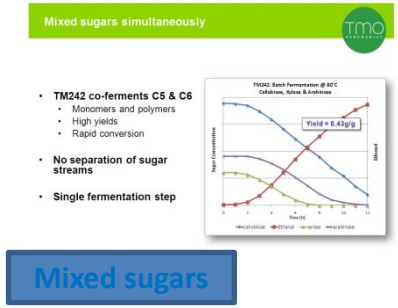
After Fermentation

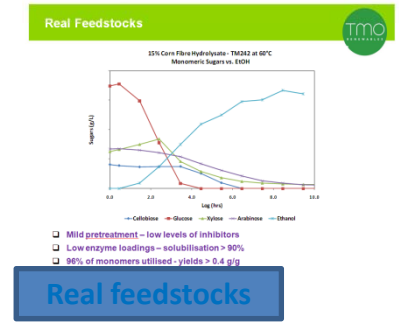


Utilisation of oligomers/polymers

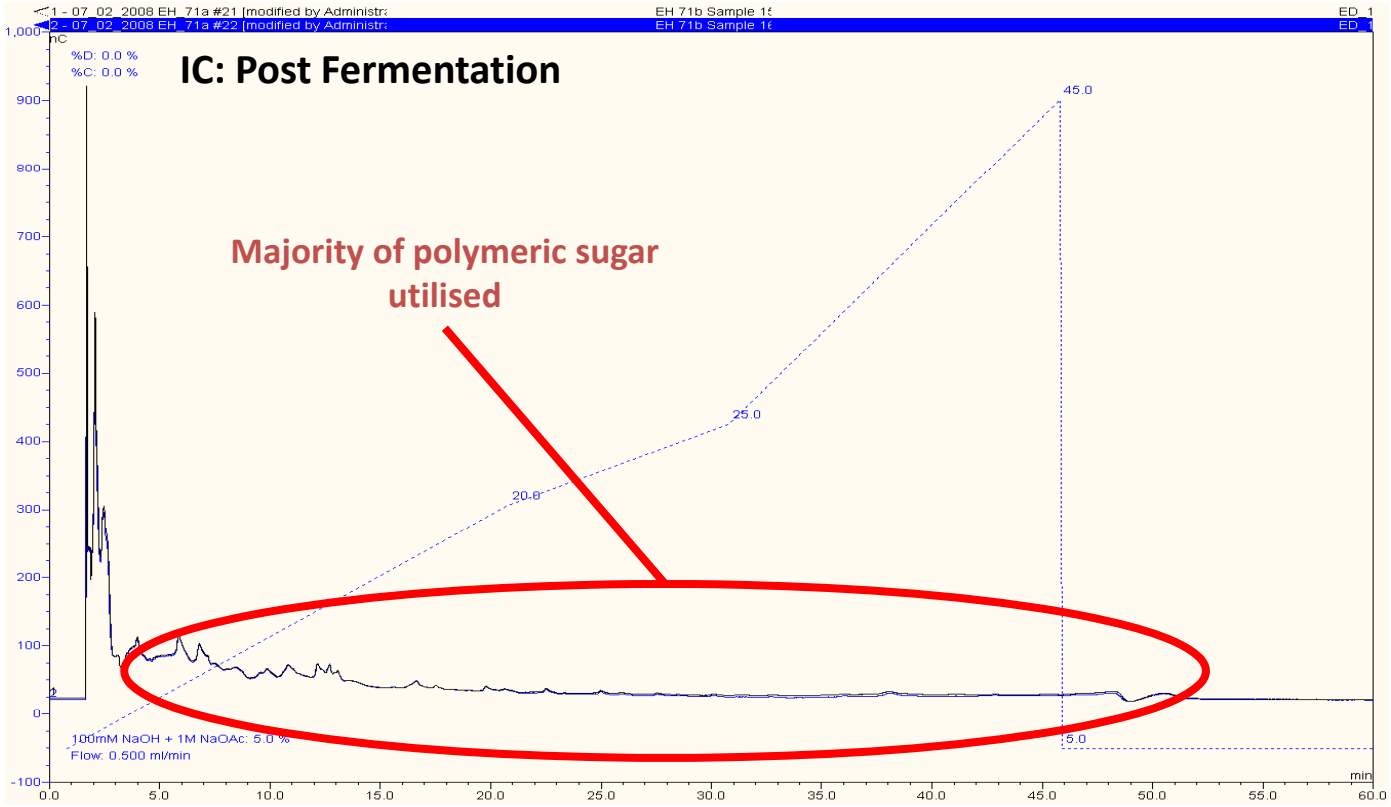


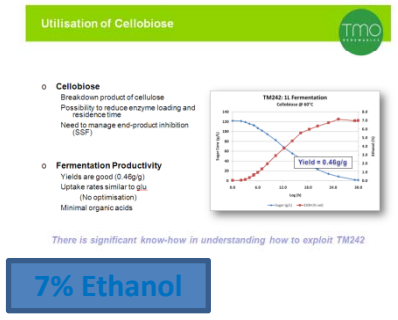


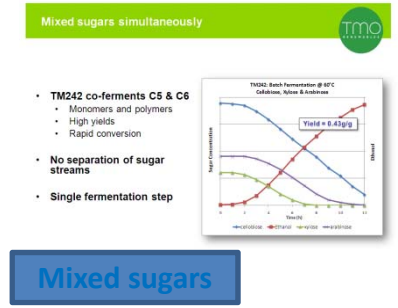


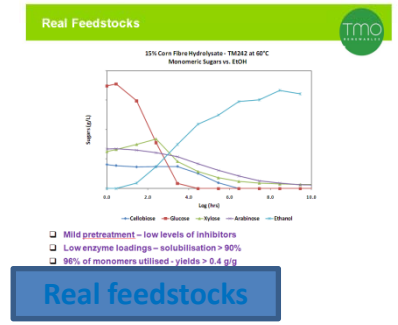


Utilisation of oligomers/polymers

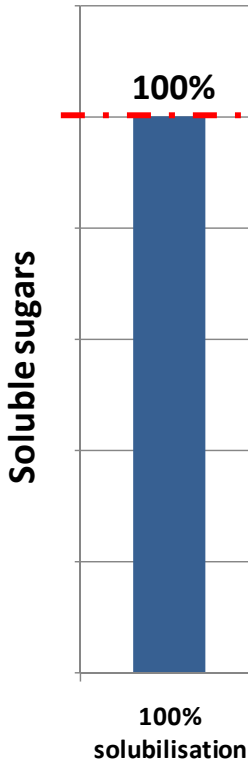


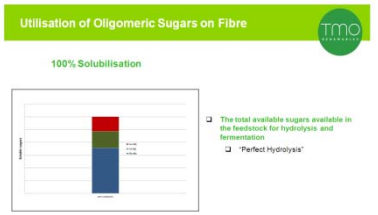


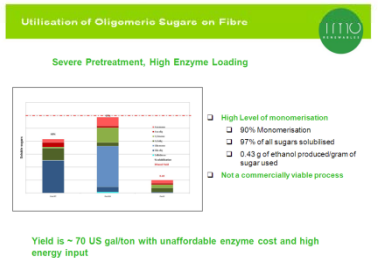


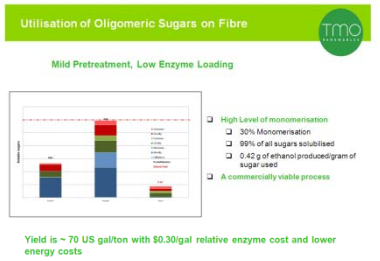


Saccharification strategy

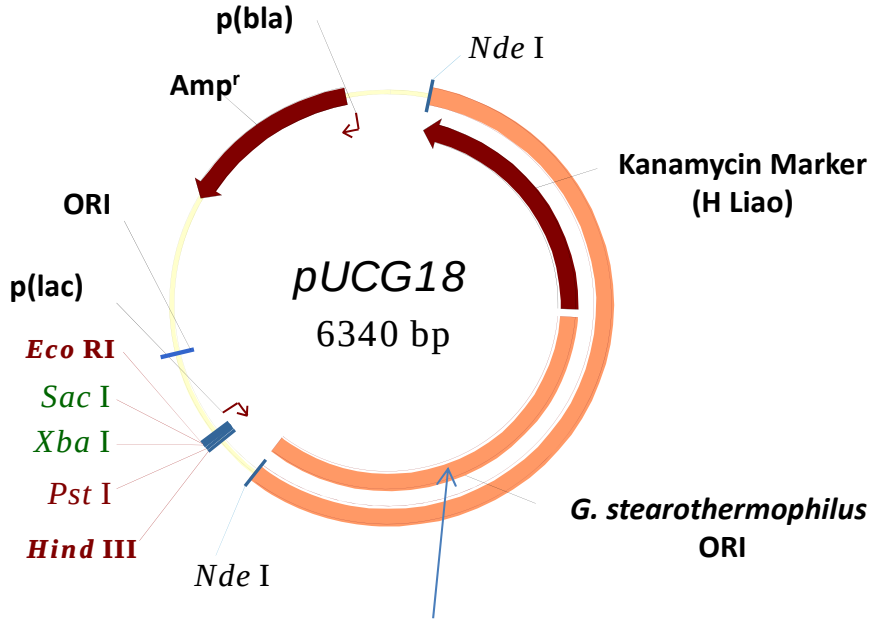






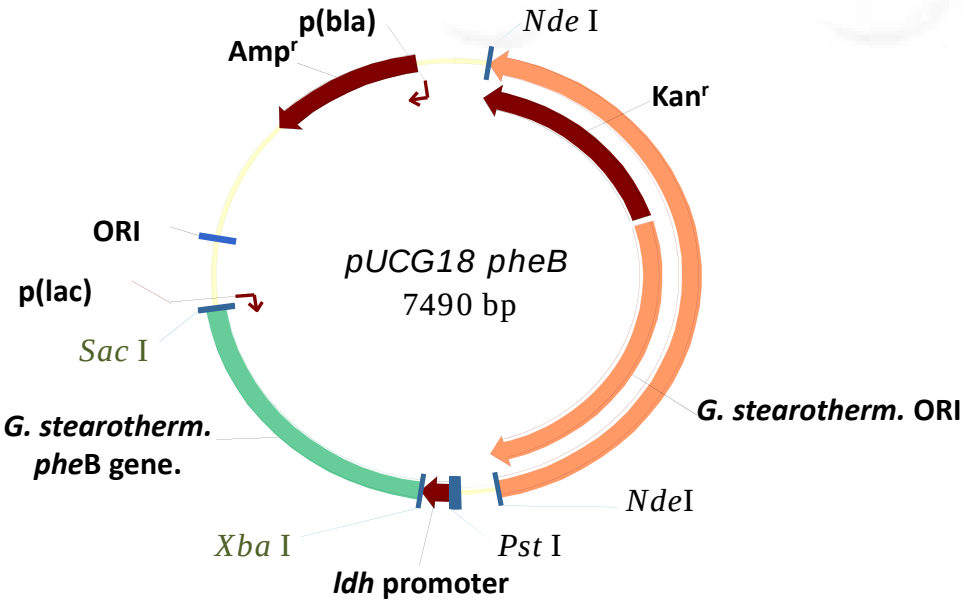


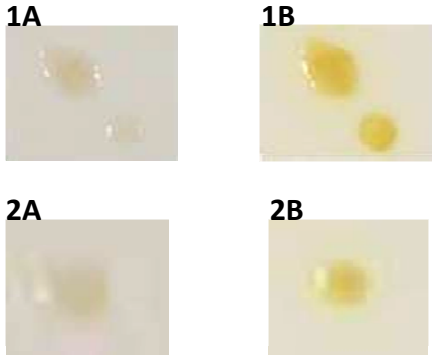
Shuttle vectors and reporter genes



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

Expressing superfolder GFP

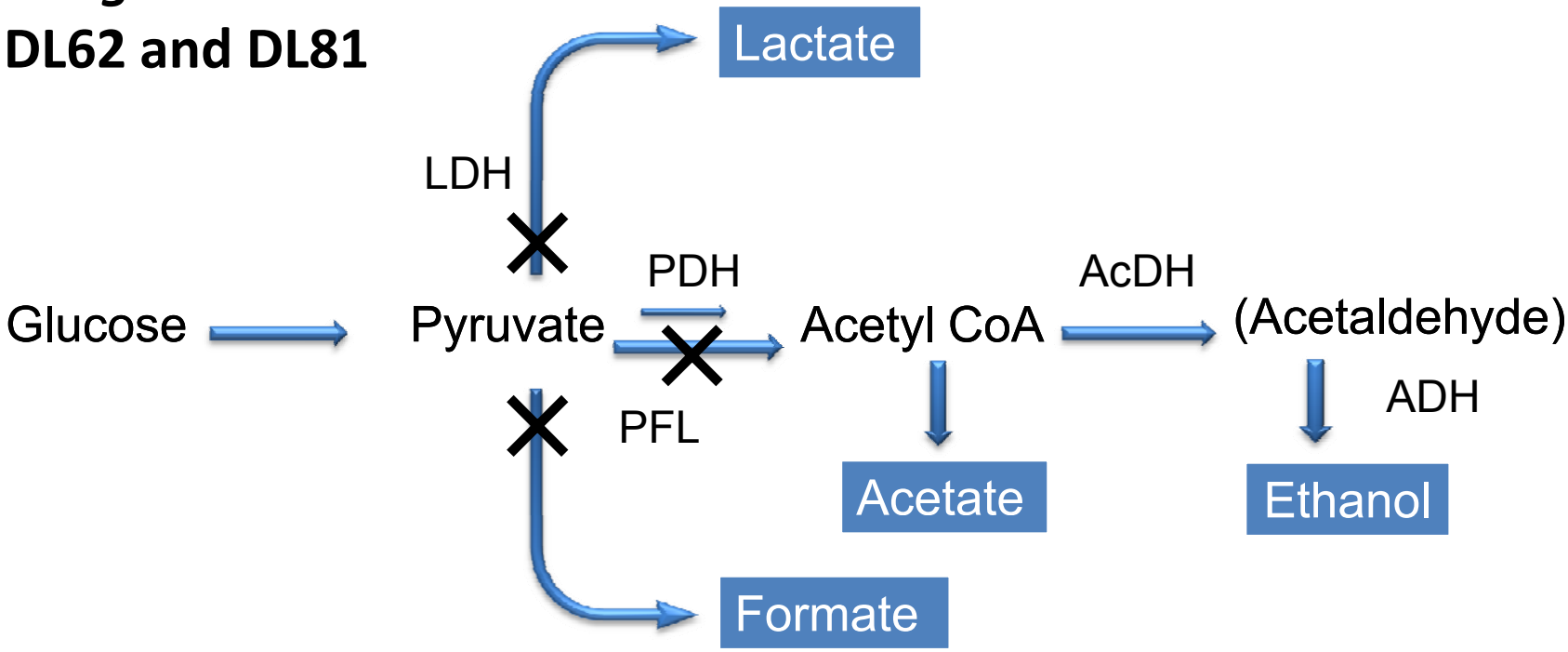




Catechol —C23O→ 2-hydroxymuconic semialdehyde

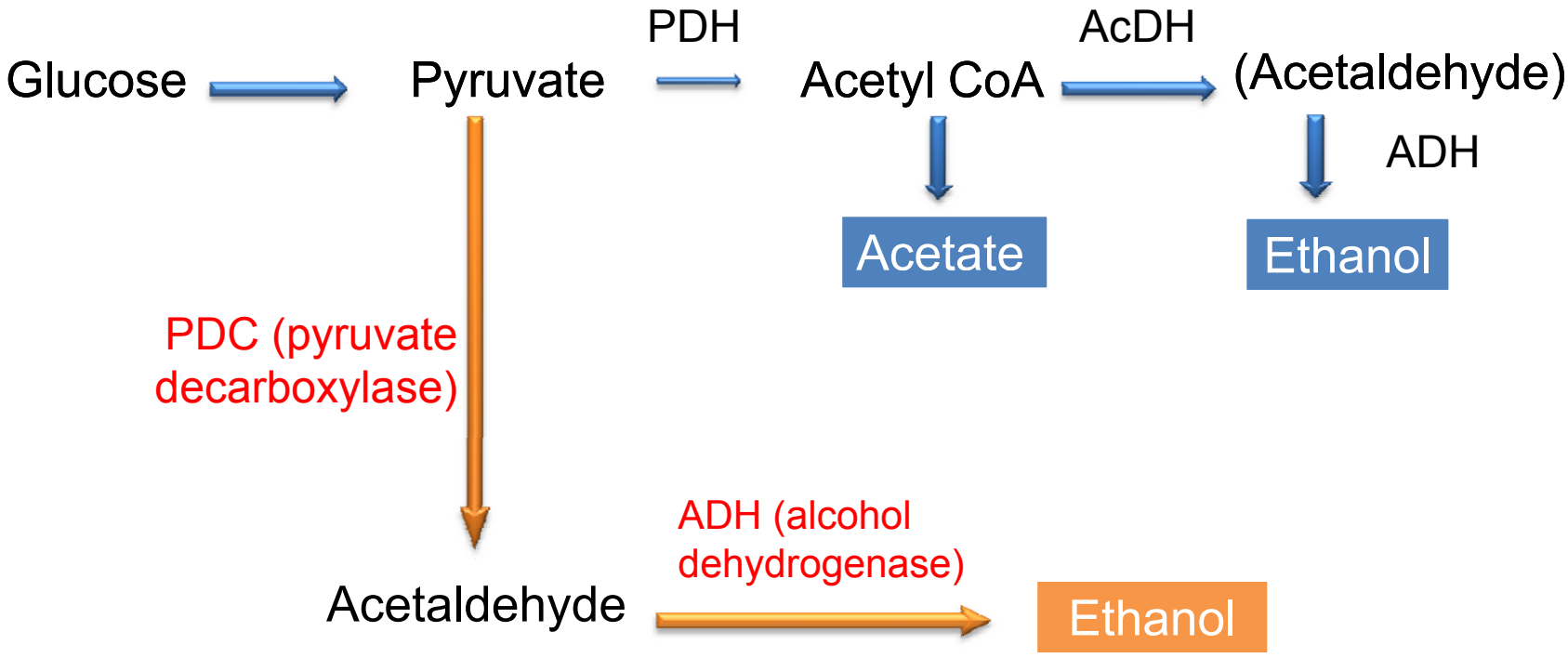
Ethanologenic Engineering Strategies

Fermentation pathways in
G. thermoglucosidasius
DL33, DL62 and DL81



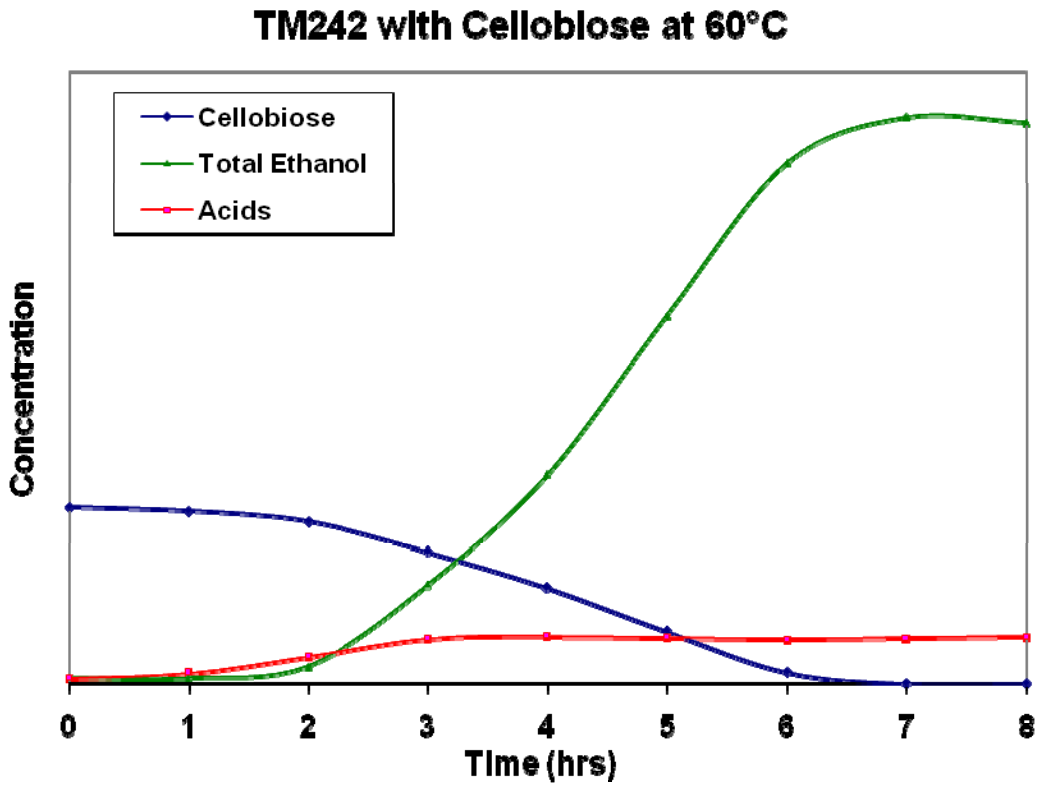
Ethanologenic Engineering Strategies

Fermentation pathways in
G. thermoglucosidasius
DL62



Growth of TM242 on cellobiose

Cellobiose - breakdown product from biomass hydrolysis



Typical Ethanol yield = 0.45 g/g sugar

(90% of theoretical)

TMO Cellulosic Ethanol Demonstration Facility

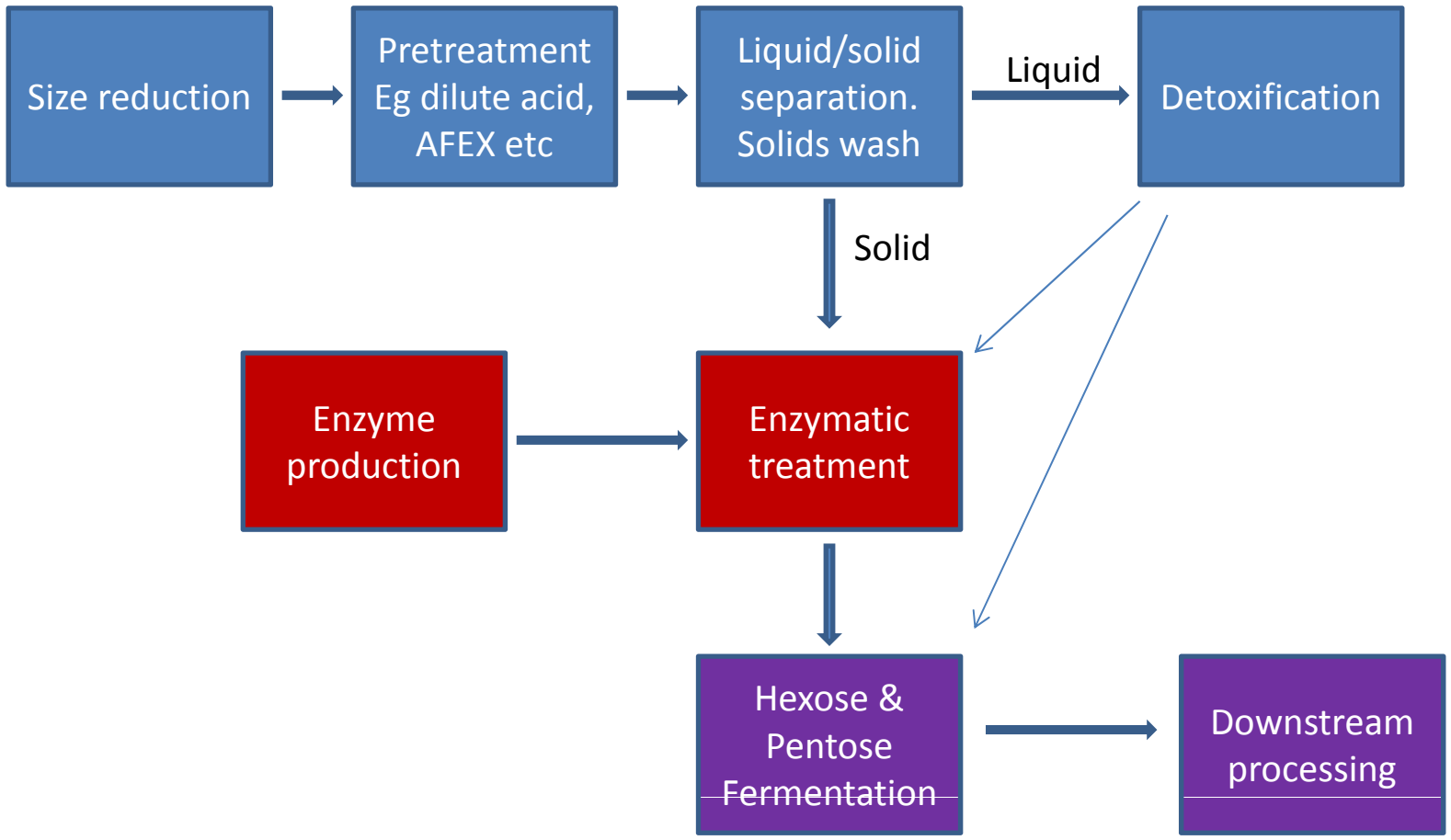
Cost: £7.8M
Pipework: 5km
Cabling: 50km
I/O: >2000

Flexible Capability
Multiple Feedstocks
High Solids Handling
24/7 Operation

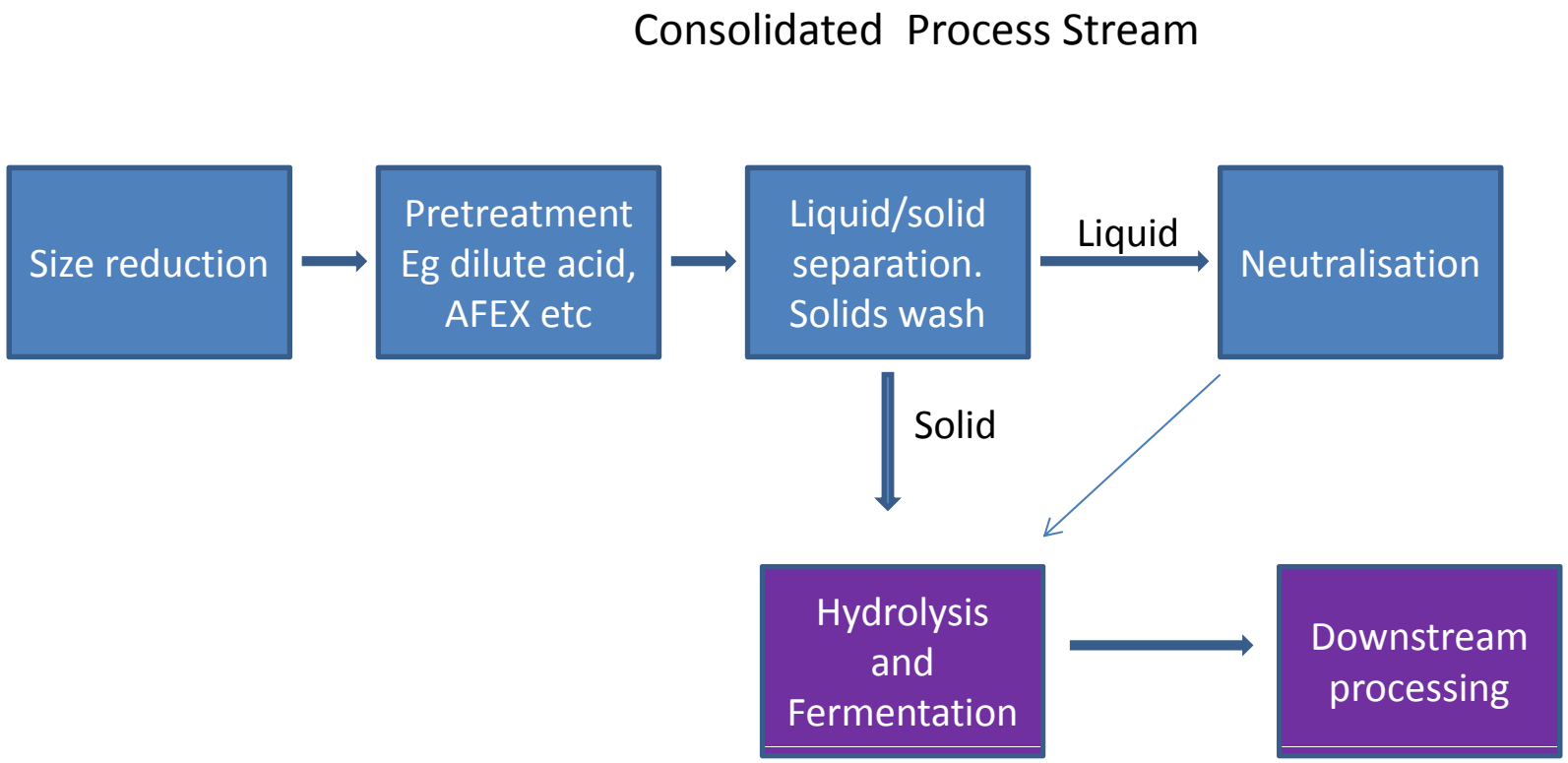
TMO RENEWABLES

Lignocellulosics - process diagram

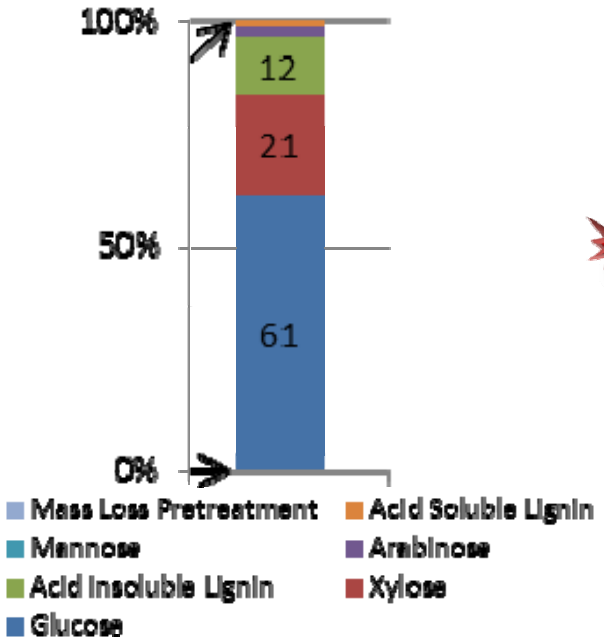
Typical Process Stream

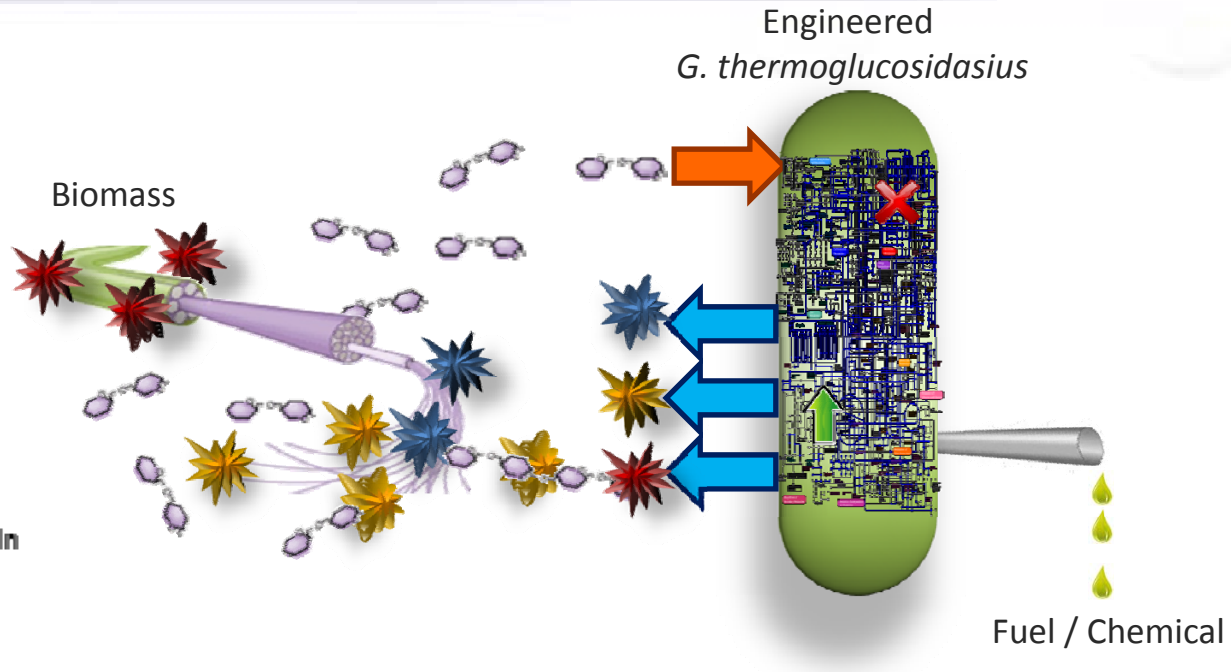


Lignocellulosics – consolidated bioprocess



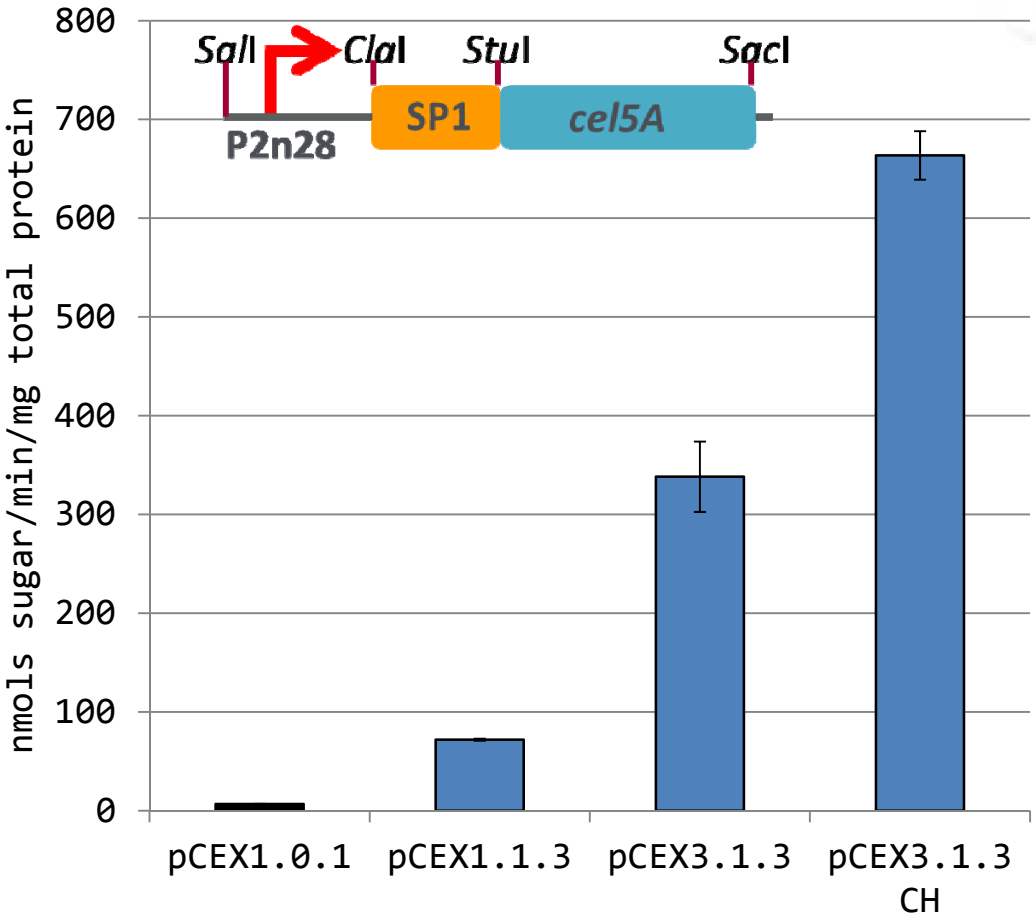
Consolidated Bioprocessing with *G. thermoglucosidasius*





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

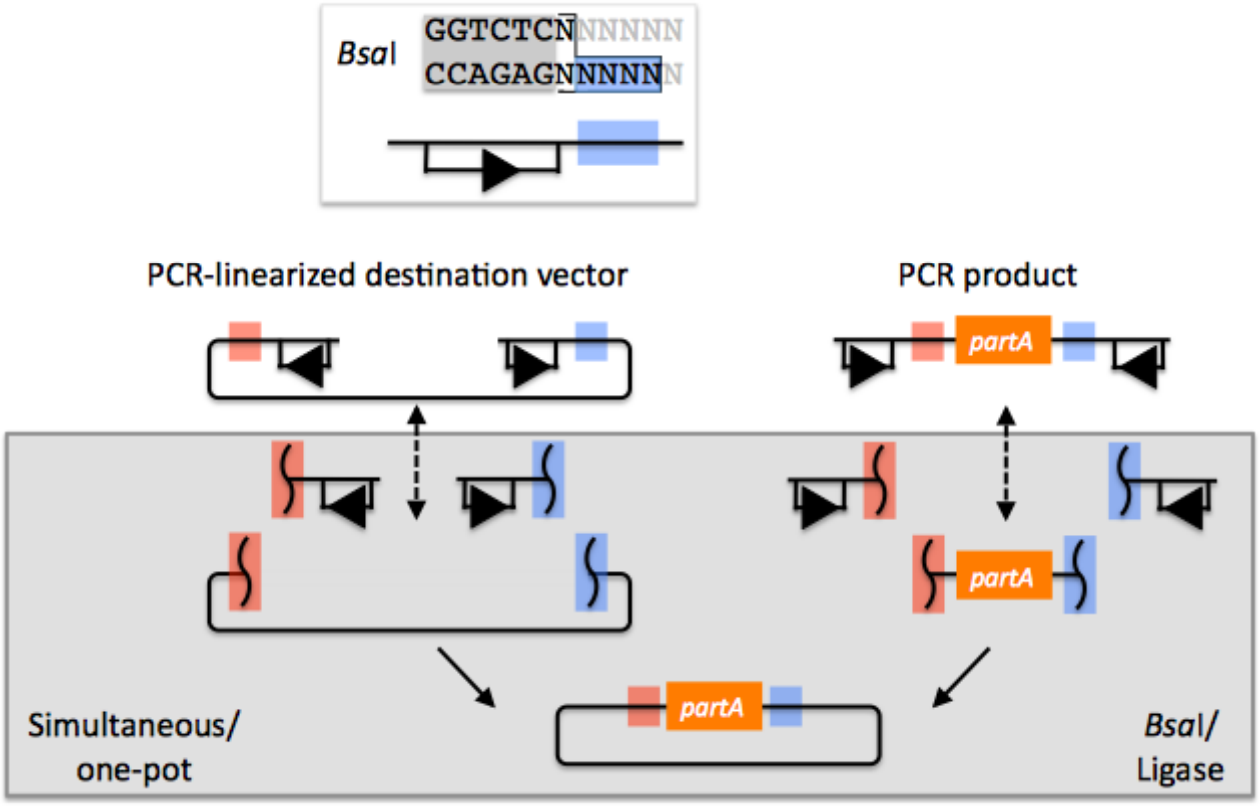
Improvements in GH activity



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

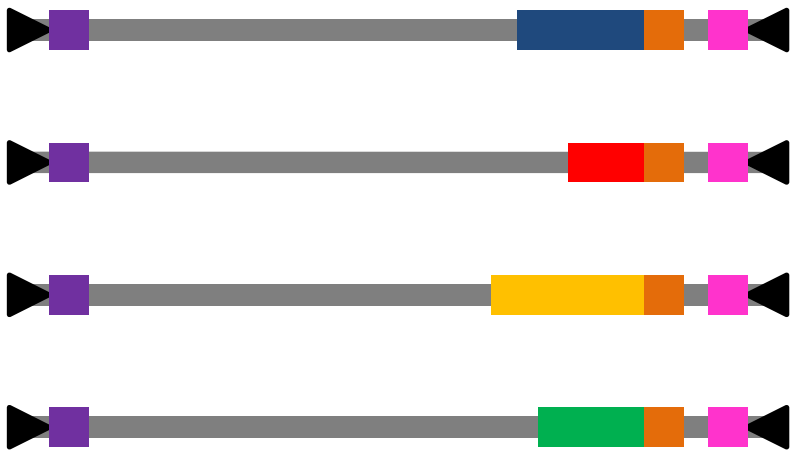
Assembling pCEX variants

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



Destination vectors

- Generate linearized product which *Bsa*I will cut in the *Stu*I and *Sac*I sites.



*Stu*I - AGGCCT

*Sac*I - GAGCTC

*Bsa*I - GGTCTC

GGTCTCGAGCTC
CCAGAGCTCGAG

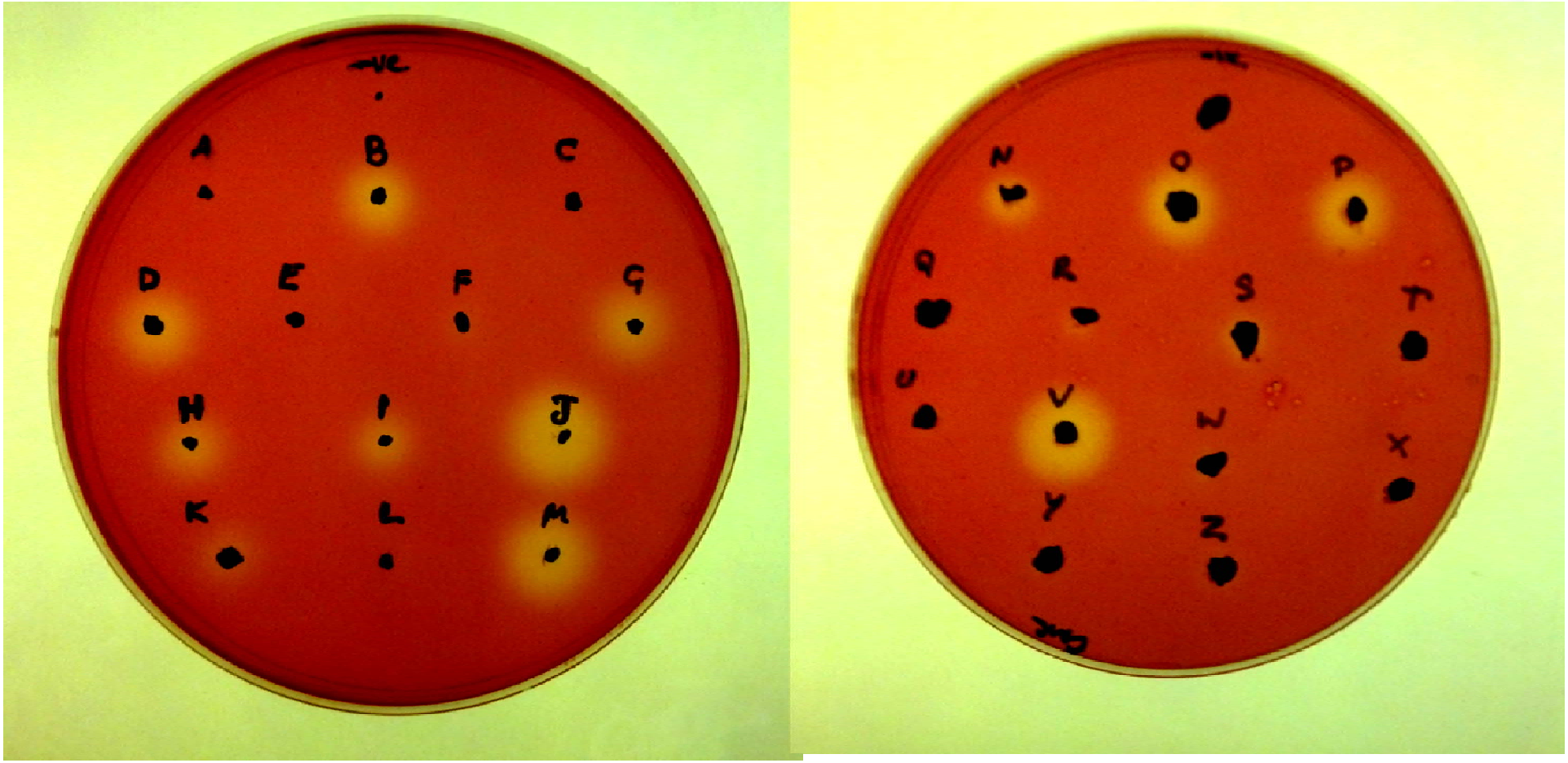
AGGCCTGAGACC
TCCGGACTCTGG

+ *Bsa*I

GGTCTCG...AGCTC
CCAGAGCTCGA...G

A...GGCCTGAGACC
TCCGG...ACTCTGG

Screening of signal sequences

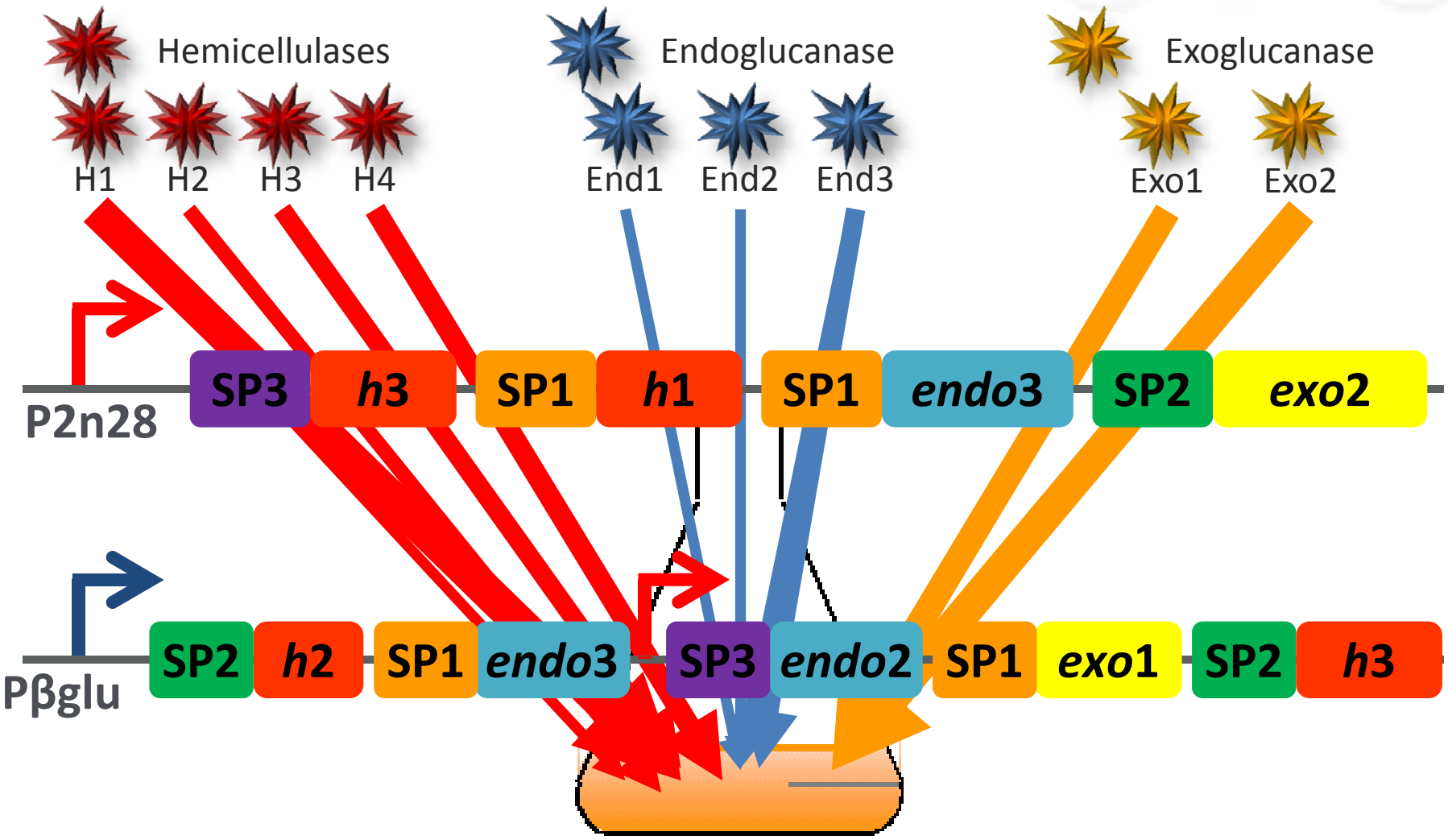


Preliminary GH activities

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

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

Assessing recombinant GH activity

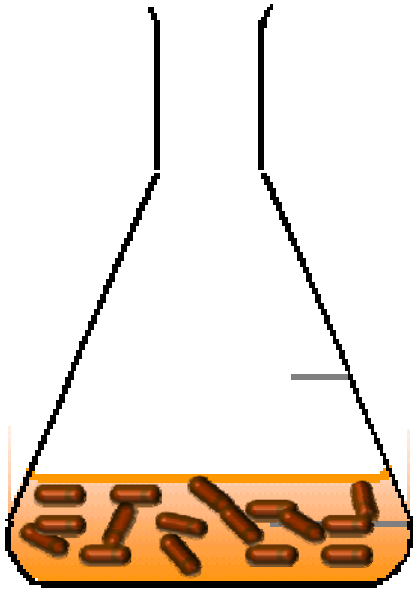


Evaluating CBP – *M. giganteus*

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

- GH enzymes were expressed in *G. thermoglucosidasius*.
- Strains were grown in 2TY media to and OD above 2.0 (OD600nm of 1 equates to ~0.25g/L cells)
- Saccharification measured as - mass loss of insoluble *M. giganteus* after incubation for 48hours @ 60°C (originally 285mg).



A

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

B

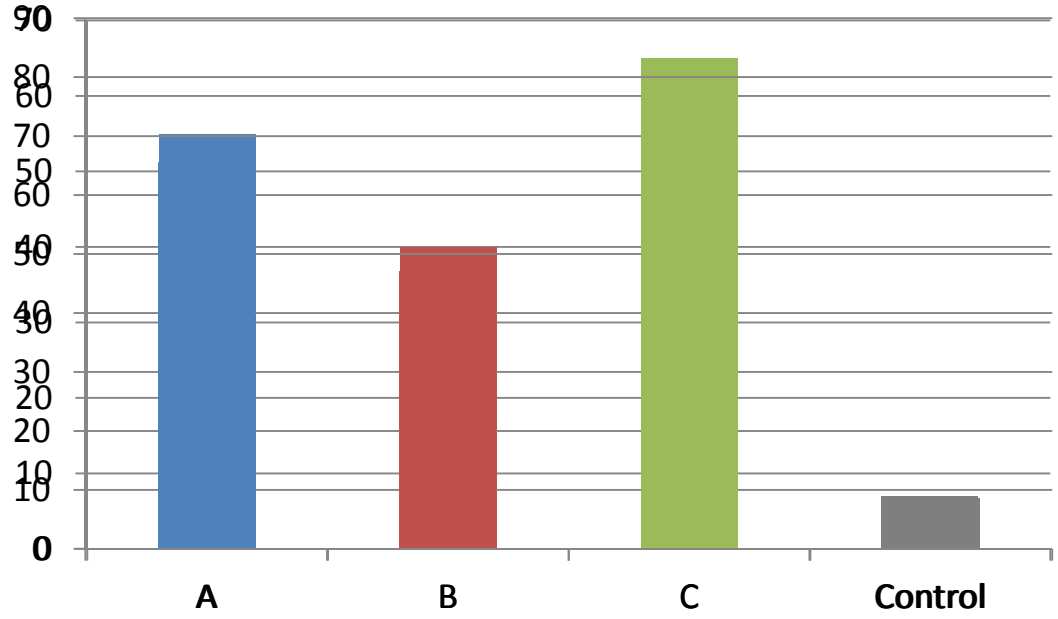
α-L-arabinofuranosidase
T. thermosaccharolyticum
Xylanase (GH11)
T. thermosaccharolyticum
Xylanase (GH10)
C. saccharolyticus
Endoglucanase (GH9)
C. saccharolyticus
Exoglucanase (GH48)
T. fusca YX

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

CBP – Mass loss

- % of Avicel (cellulose) mass lost 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*

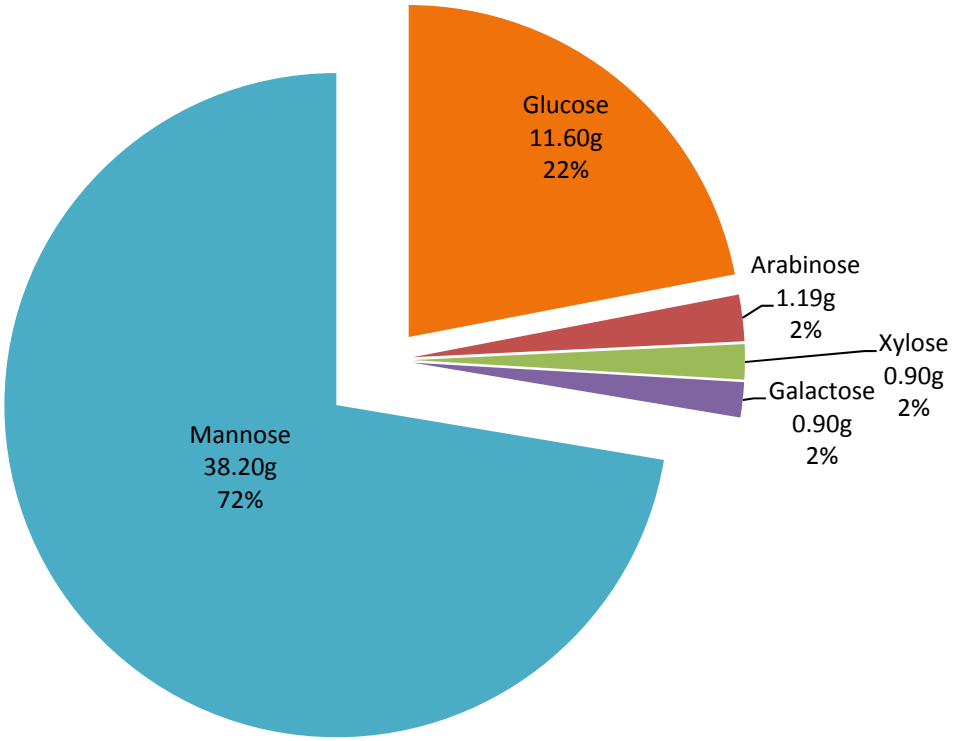
B

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

C

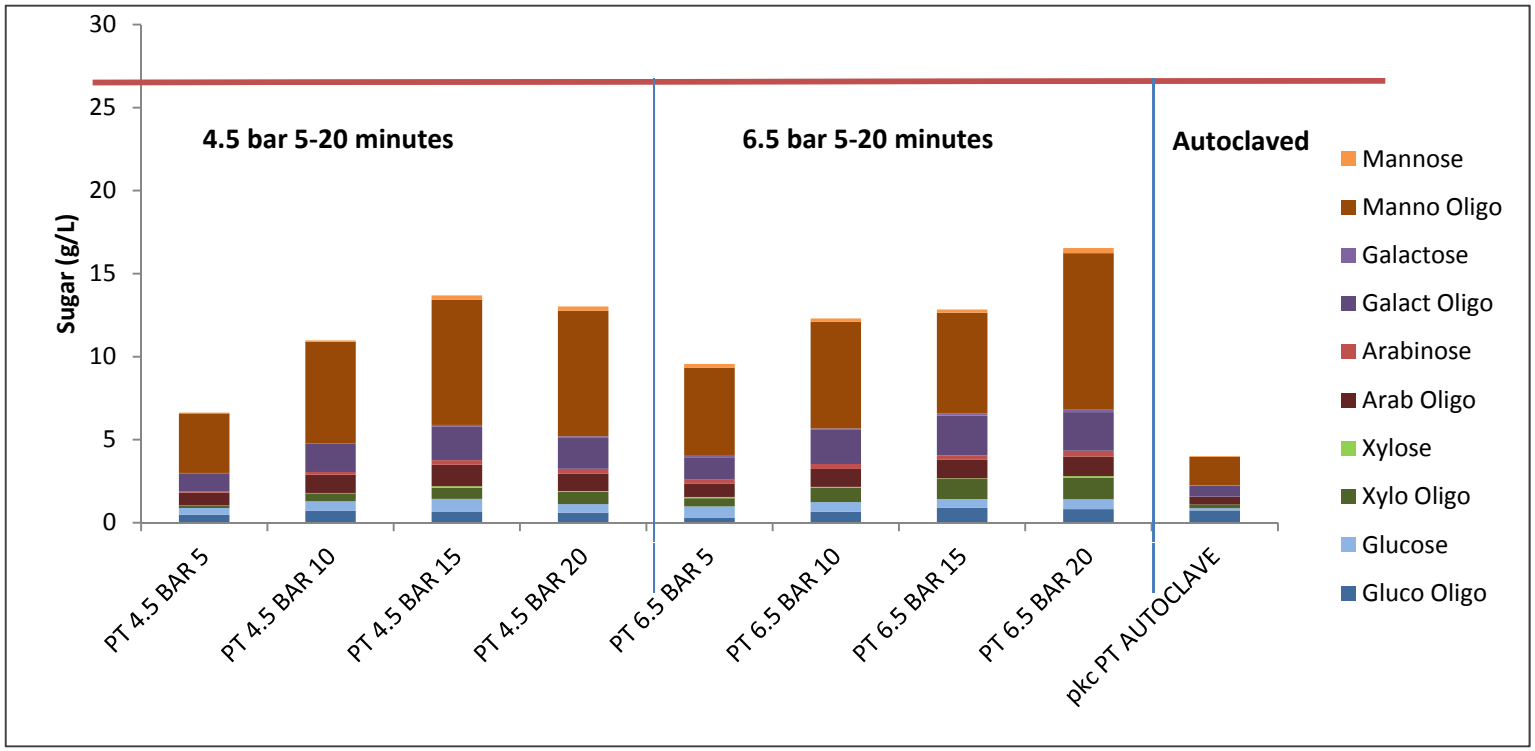
- α-L-arabinofuranosidase *T. thermosaccharolyticum*
- Xylanase (GH11) *T. thermosaccharolyticum*
- Xylanase (GH10) *C. saccharolyticus*
- Endoglucanase (GH9) *T. maritima*
- Exoglucanase (GH48) *T. fusca YX*
- Acetylxylan esterase *C. thermocellum*

Carbohydrate composition of PKC



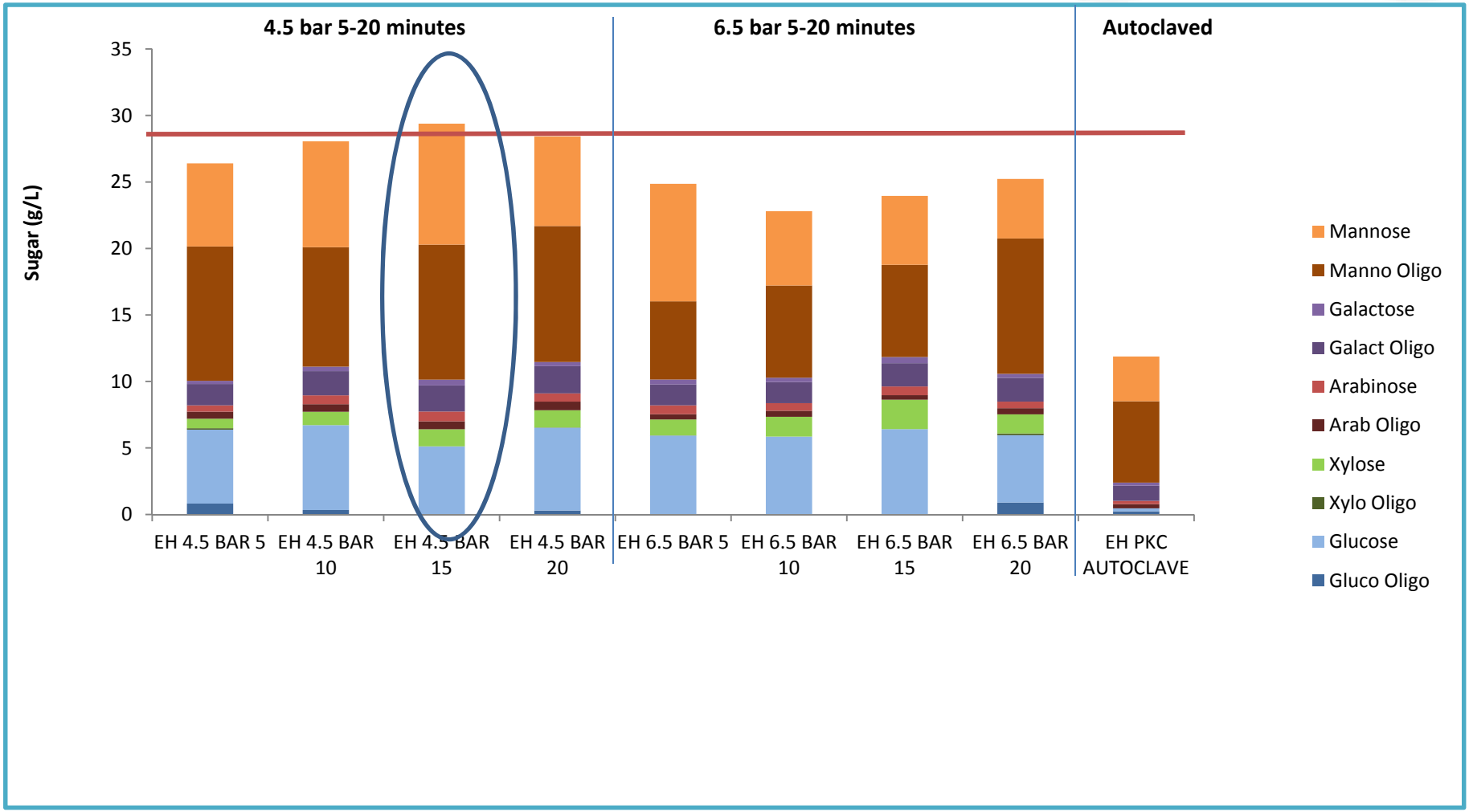
- Mainly Mannose and Cellulose (mannan and glucan) in PKC
- Possibility of producing biofuels from this biomass
- Enzyme required for hydrolysis are cellulase, mannanase + mannosidase

Steam Pre-treatment of PKC (5% DS)



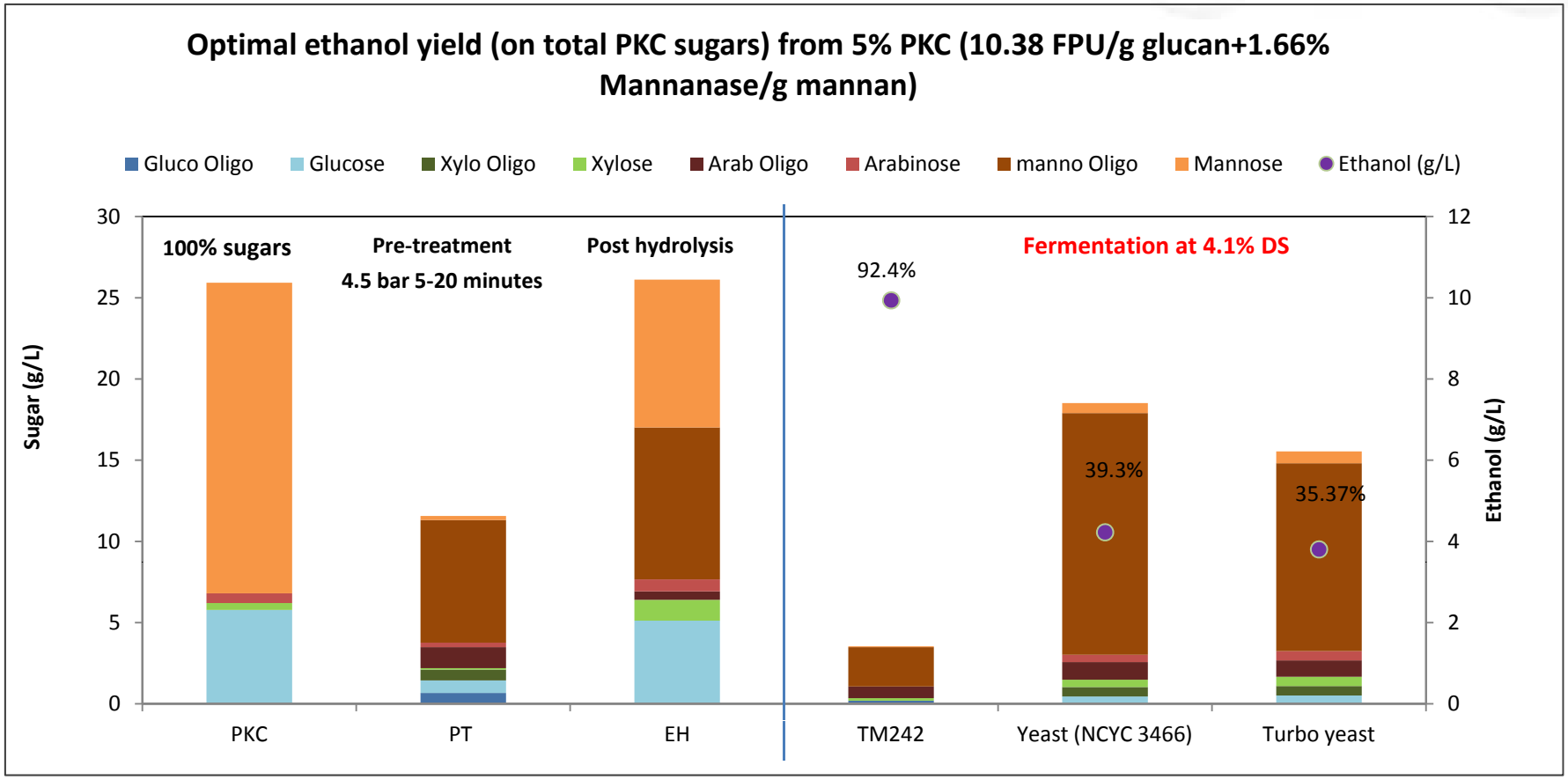
- Various pre-treatments (PT) were carried out for 5-20 minutes released mainly oligomeric sugars (soluble sugars shown).
- Increasing sugar release with increasing time of PT.

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.

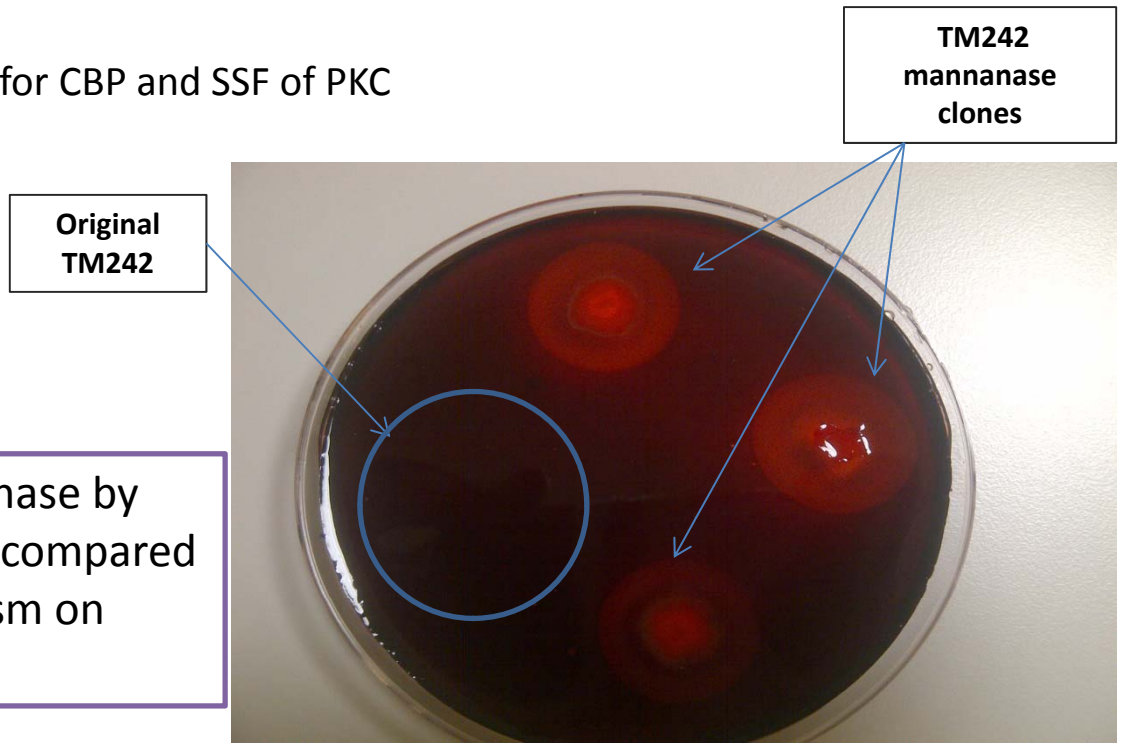
Production of ethanol from 5% PKC



- 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



Production of mannanase by clones of *Geobacillus* compared to the original organism on mannan agar plates

Thank you

- Jeremy Bartosiak-Jentys & Ali Hussein[1] - CBP and SigP
- Chris Ibenegbu, Charlie Bennett[1], Marisa Raita [1,2] - PKC
- Steve Martin & Kirstin Eley[3]

[1]Dept of Biology & Biochemistry, University of Bath

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

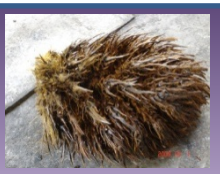
[3] TMO Renewables

Any Questions?

Palm Kernel Cake (PKC) Production

Palm Tree

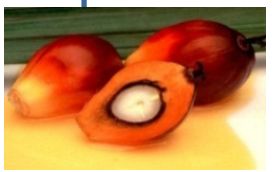
Fresh Fruit Bunches (FFB)



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

Cracking

Palm Kernel Cake (PKC) 6%

Palm Kernel Shell (PKS)

Crude Palm Kernel Oil

Oil palm tree (25 years)

-To start bearing bunches 2½–3 years after field planting

-After harvesting round 10–15 days per month

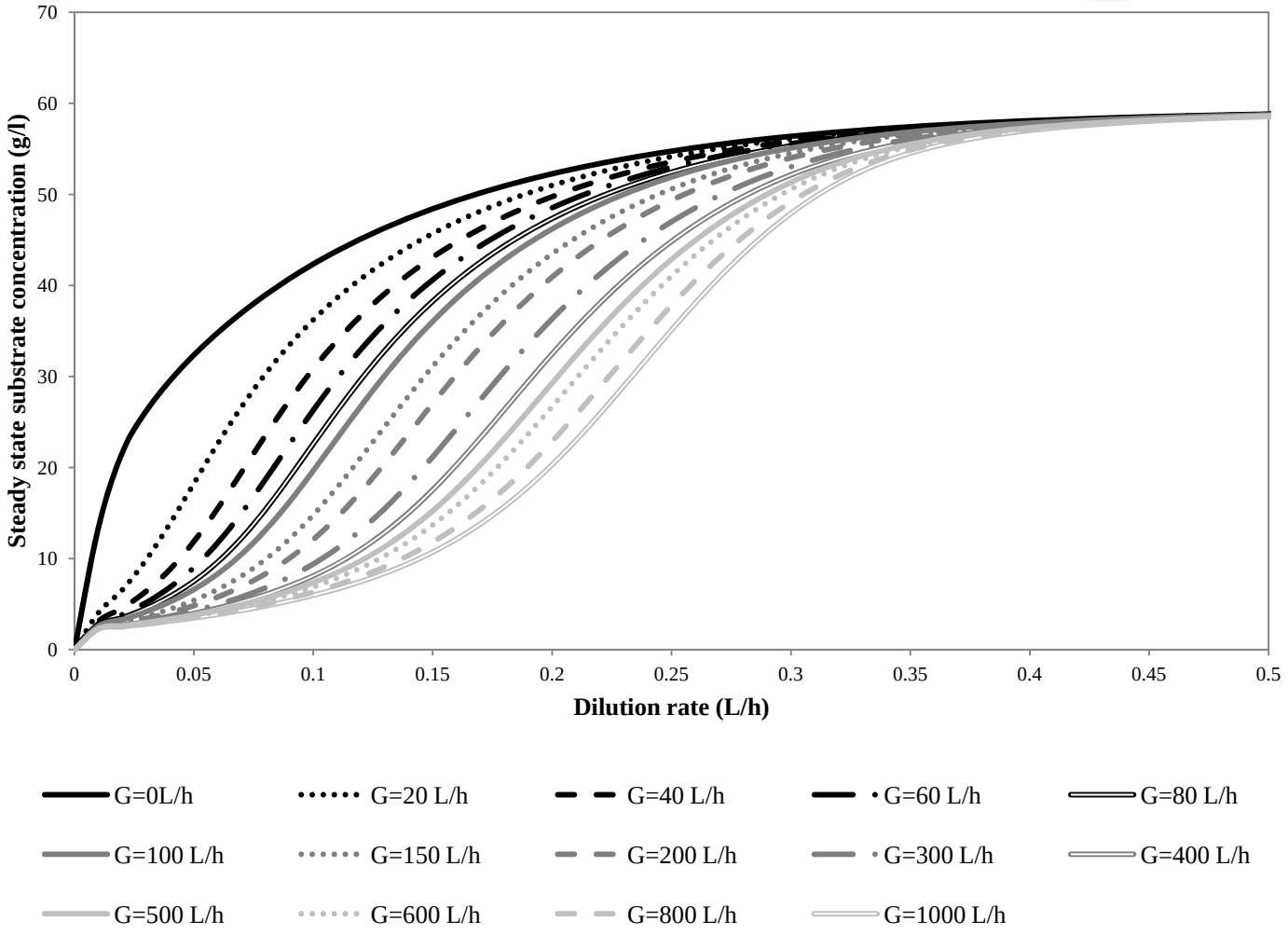
High Solids Loading CBP

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

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

- Recommendation is to run a fed batch process.

Modelling gas-stripping requirement for continuous culture



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