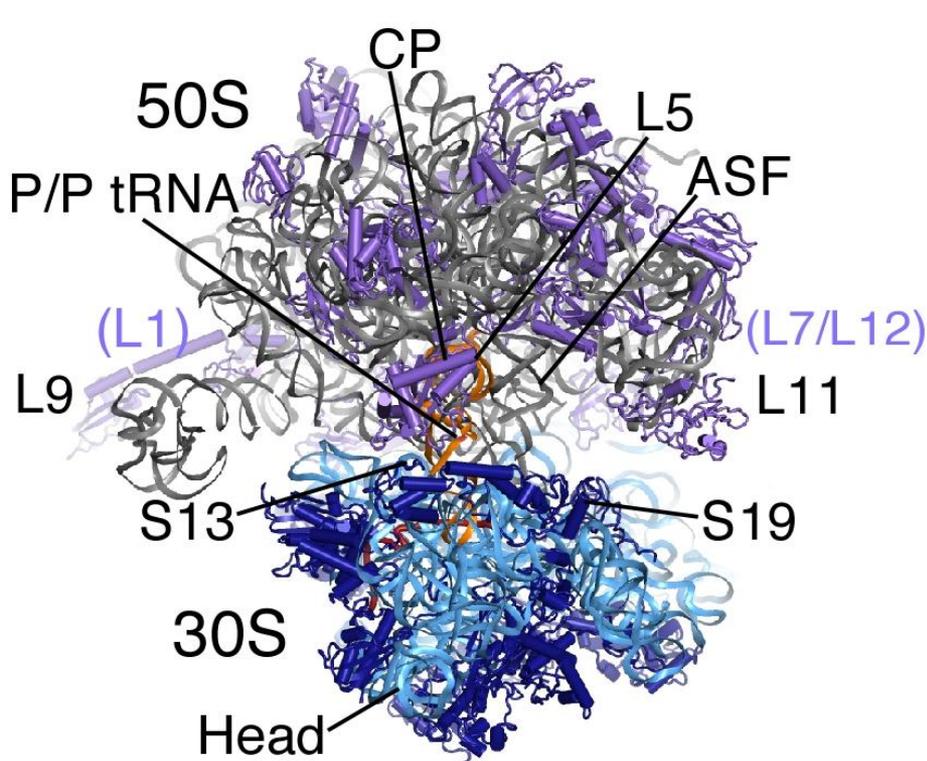




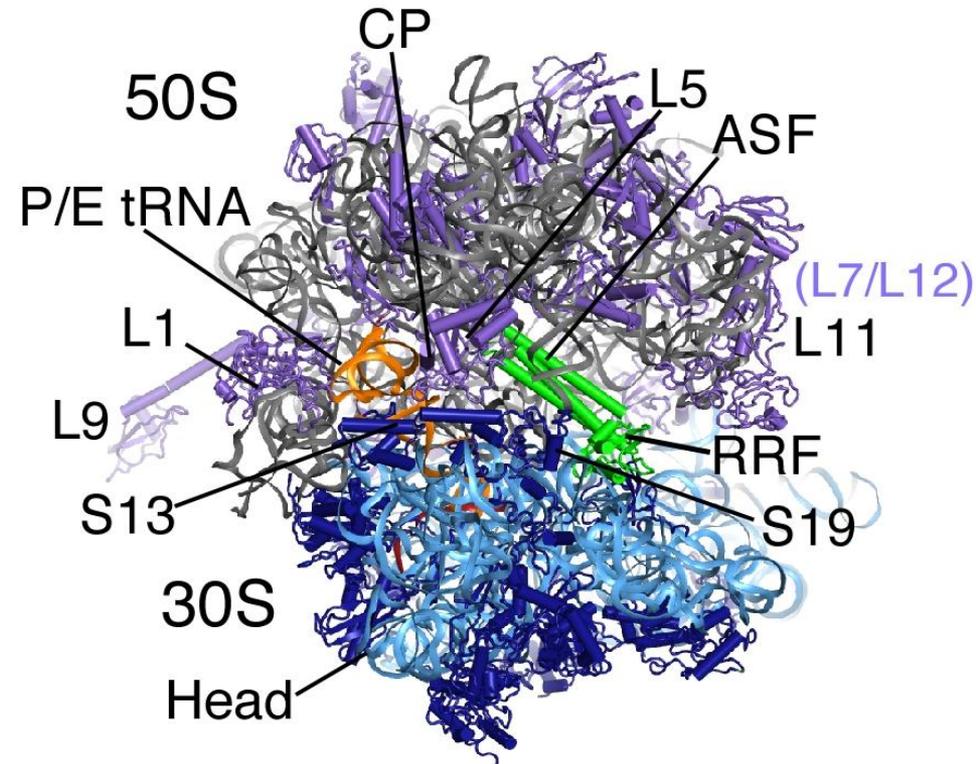
A TALE OF TWO FUNGI: IMPROVING BIOFUEL PRODUCTION FROM PLANT BIOMASS

Jamie Cate
BBEST, 8/17/2011

Crystal structures of the *E. coli* 70S ribosome



Unrotated, P/P tRNA



Fully rotated, P/E tRNA, RRF

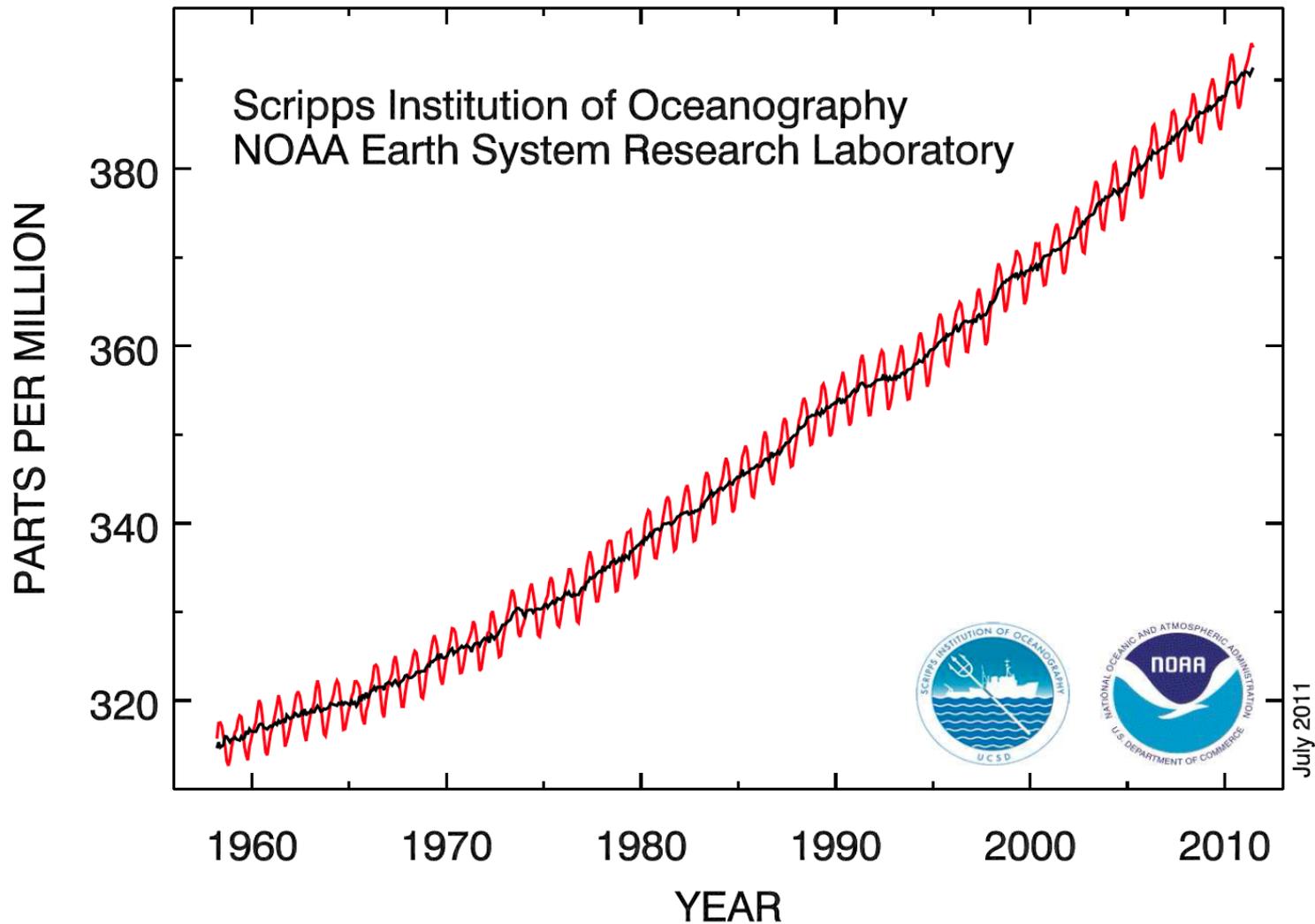
Atmospheric CO₂ Readings at Mauna Loa, Hawaii



Keeling *et al.*, Scripps Oceanographic Institute

Atmospheric CO₂ concentration increasing

Atmospheric CO₂ at Mauna Loa Observatory



Carbon-free power in the 21st Century

“Stabilizing CO₂ at 1990 levels...will require **10 TW** of carbon-free primary power *by 2018*, equal to the total 1990 primary power of the world economy.”

| | |
|----------------|-----------------------------|
| 1990 levels | 350 ppm CO ₂ |
| Pre-industrial | 270-280 ppm CO ₂ |

“Doubling of pre-industrial atmospheric CO₂ requires this much carbon-free power *by 2035*.”

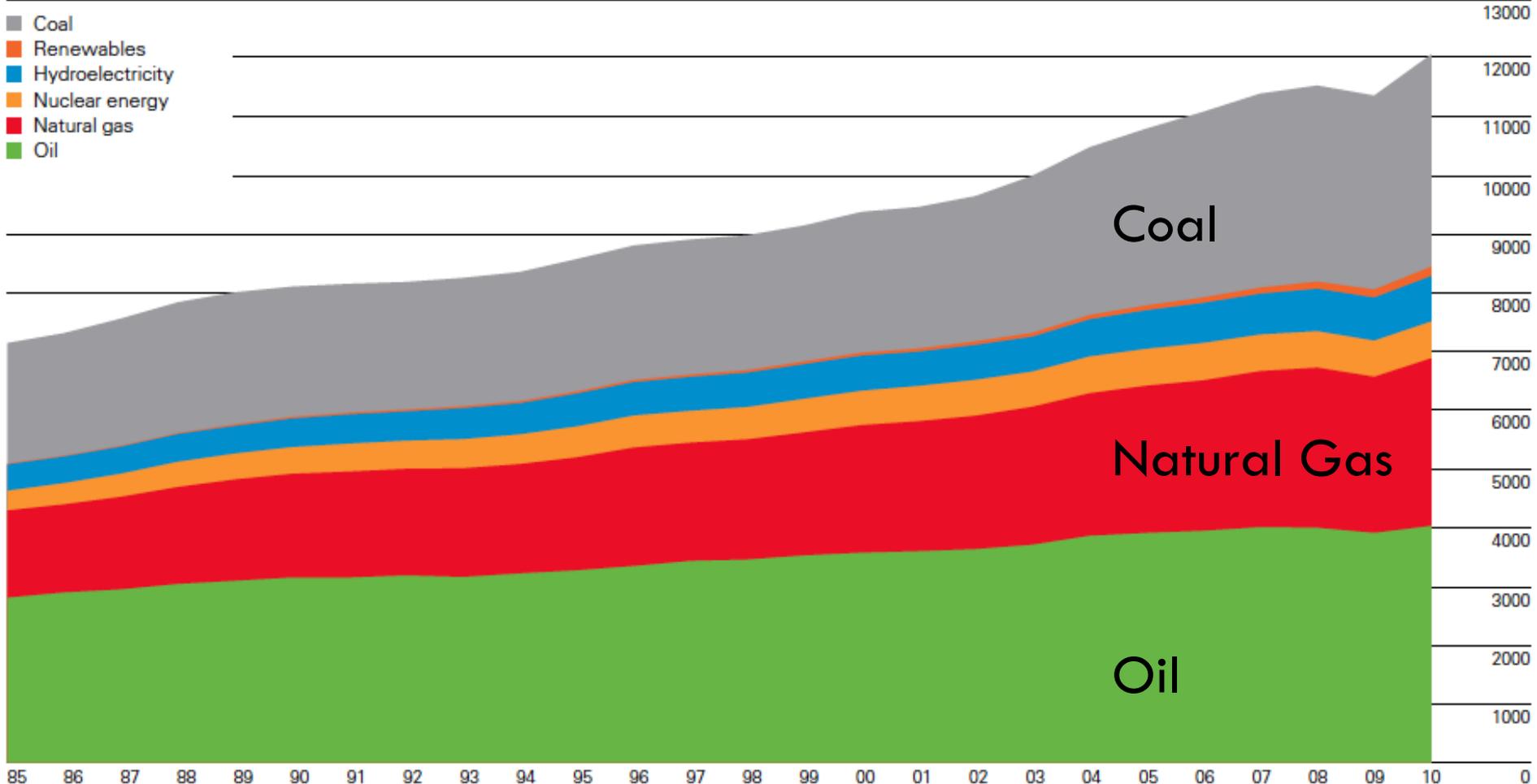
550 ppm CO₂ is higher than has been seen in many millions of years.

M.I. Hoffert *et al.*
Nature (1998) **395**, 881-ff.
cf. J. Hanson *et al.* (2008)
The Open Atm. Sci. J. **2**, 217-ff.

Oil dominance in world energy use

World consumption

Million tonnes oil equivalent

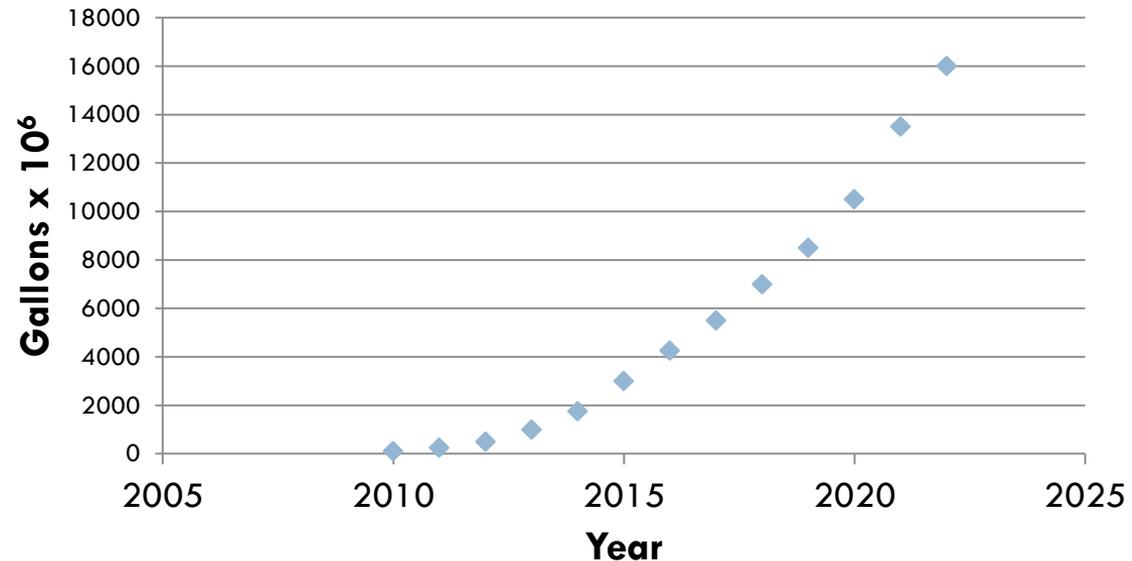


Energy Independence and Security Act

16 billion gallons of cellulosics must be used by 2022

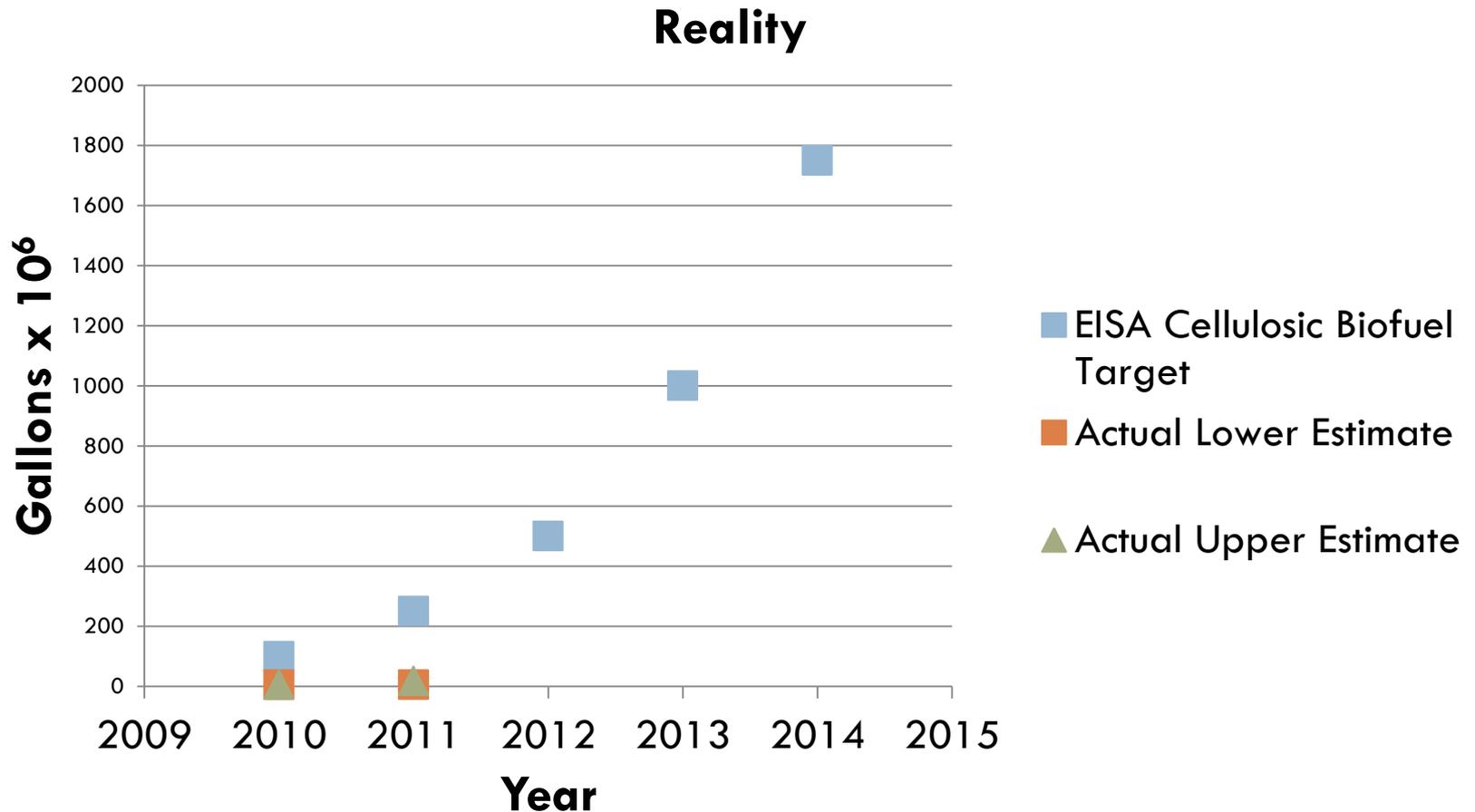


Cellulosic Ethanol Target



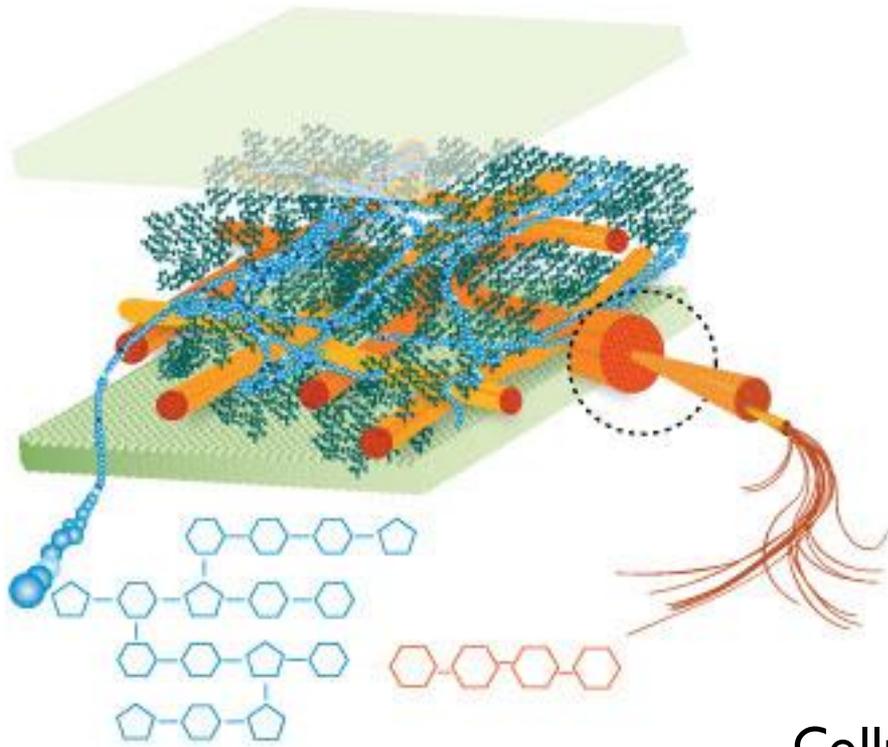
Energy Independence and Security Act

16 billion gallons of cellulosics must be used by 2022

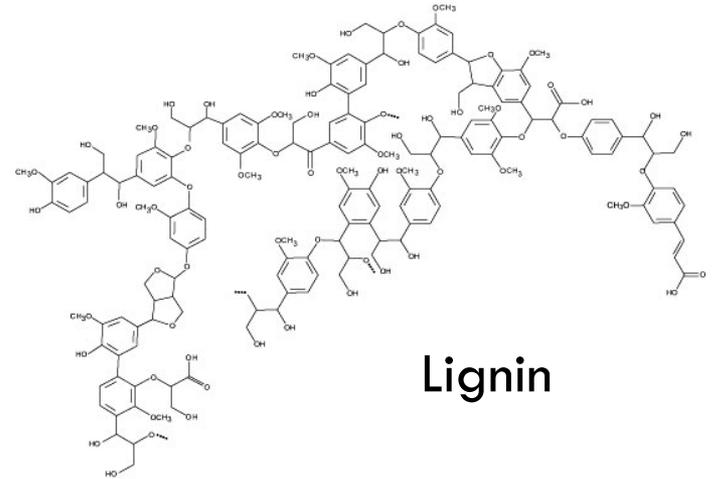


The Plant Cell Wall

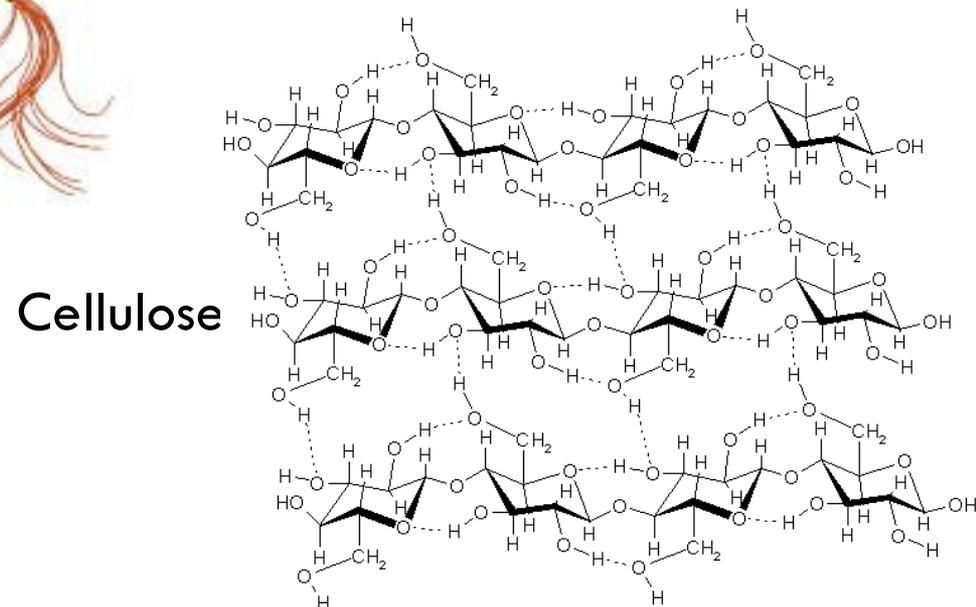
An abundant source of *recalcitrant* sugar



Hemicellulose

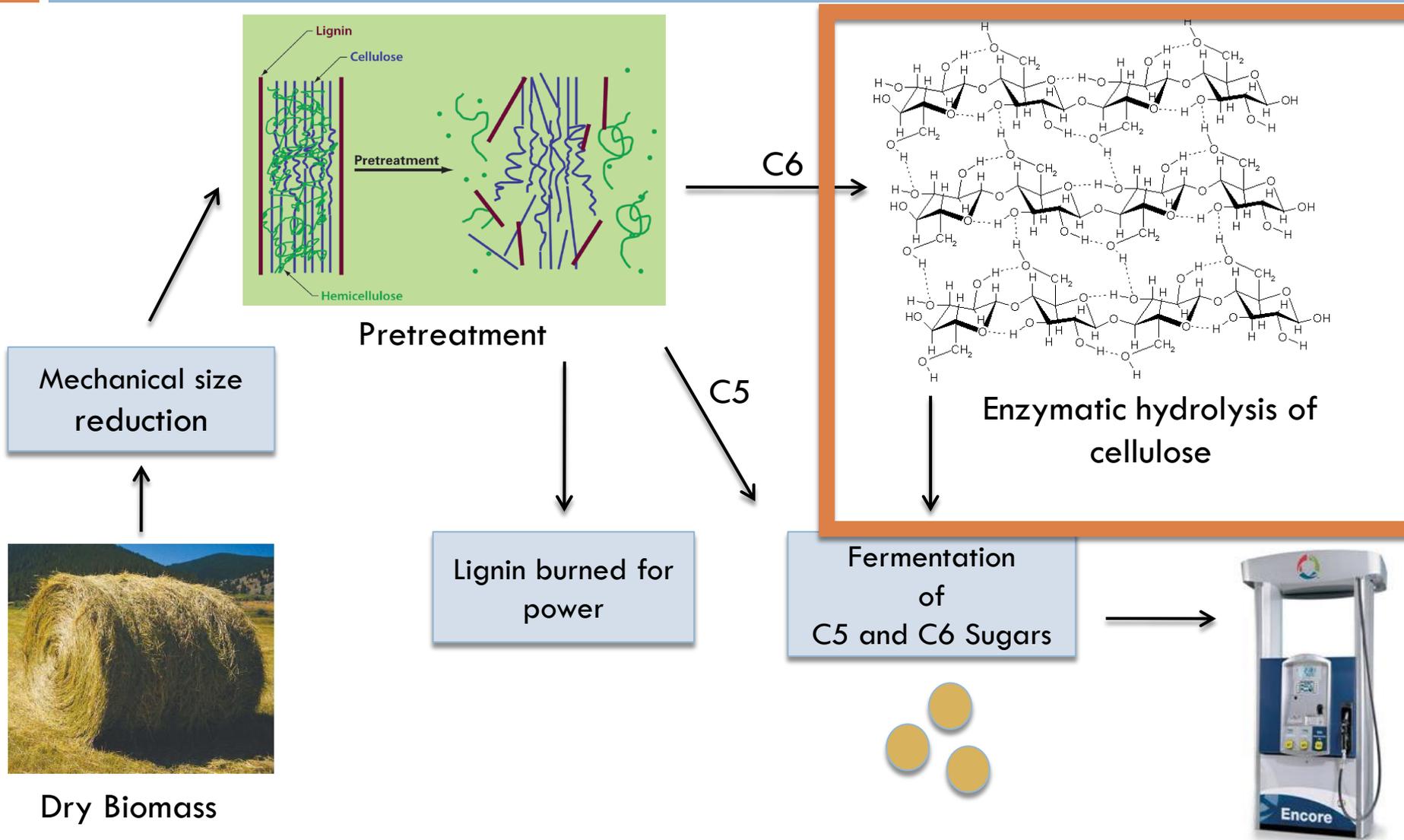


Lignin



Cellulose

Making expensive cellulosic biofuels



Fungal plant cell wall degradation

Brown rot



Postia placenta

Soft rot



Trichoderma reesei

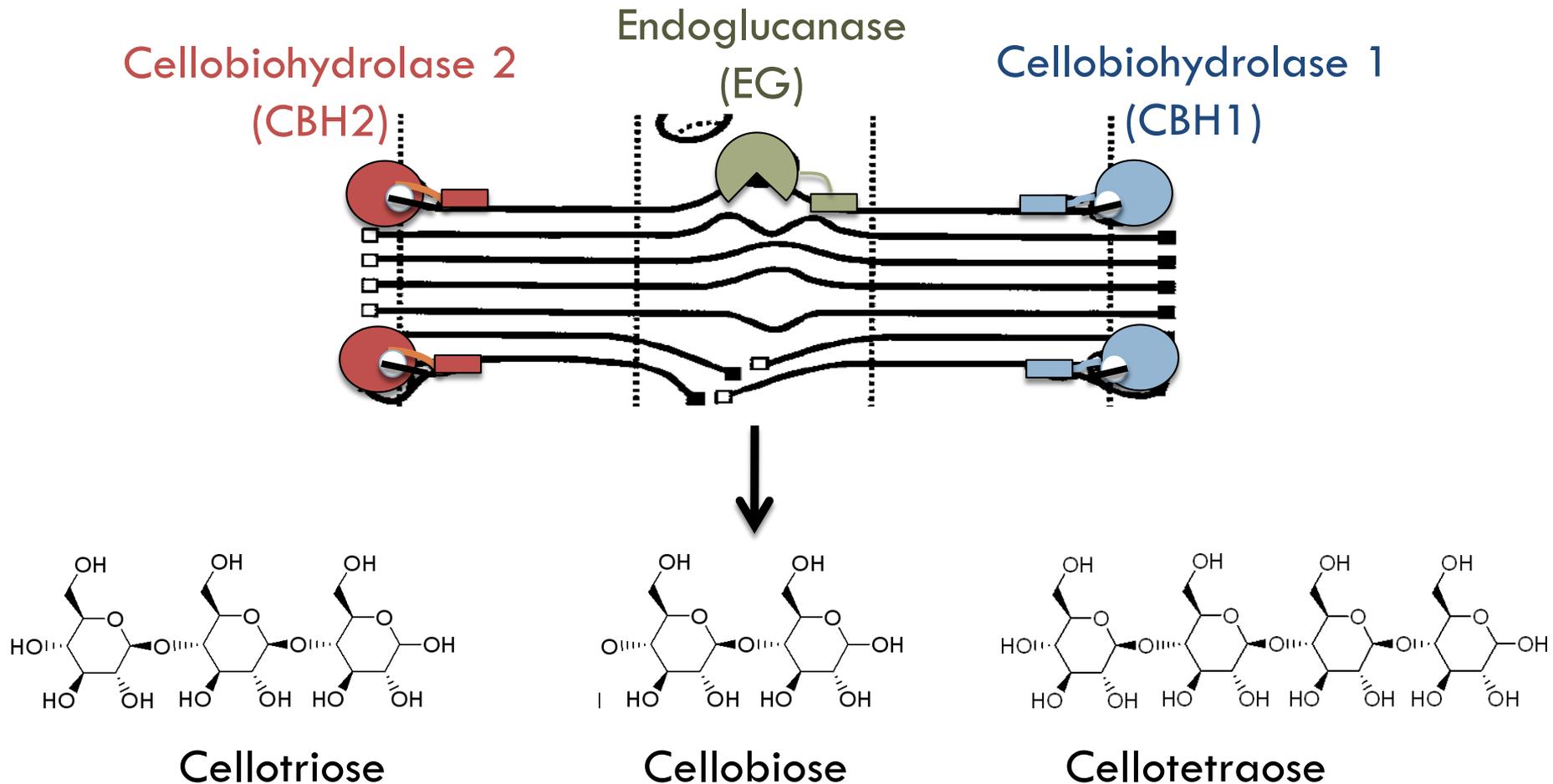
White rot



Phanerochaete chrysogenum

Fungal cellulose hydrolysis

Trichoderma reesei cellulase system



cf L. Lynd *et al.* (2002) *MMBR* **66**, 506-ff.

Neurospora crassa: a model biomass degrader



N. tetrasperma on *Ulex europaeus* in Surrey, England. Credit: Martin Bidartondo



N. crassa in the lab. Credit: Susan Jenkins

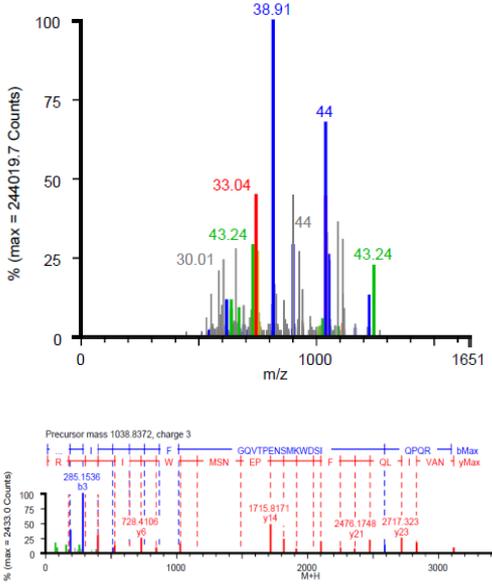
Grows on plant cell walls and pure cellulose

Mature model organism, well developed tools, large scientific community

Cellulases first described in 1977 by Eberhart *et al.*

A systems-level study of biomass degradation

Secretome Proteomics



Shotgun proteomics of culture broth from cellulose and *Miscanthus* cultures

Whole genome Microarrays/ RNASeq



Miscanthus giganteus



sucrose



pure cellulose

Growth on salts media with various carbon sources

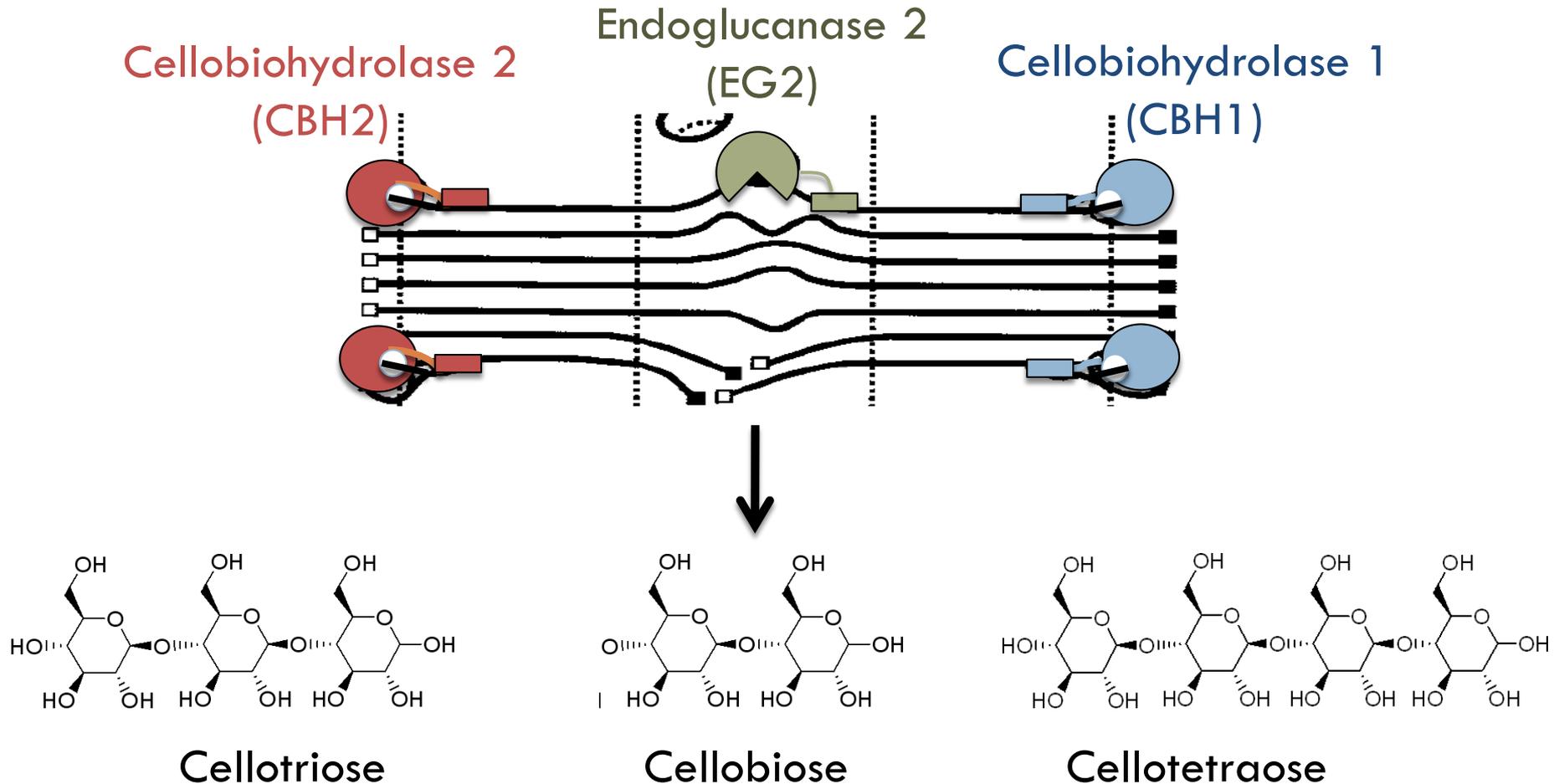
Gene Deletions & Phenotyping



Delete secreted proteins, check growth on cellulose

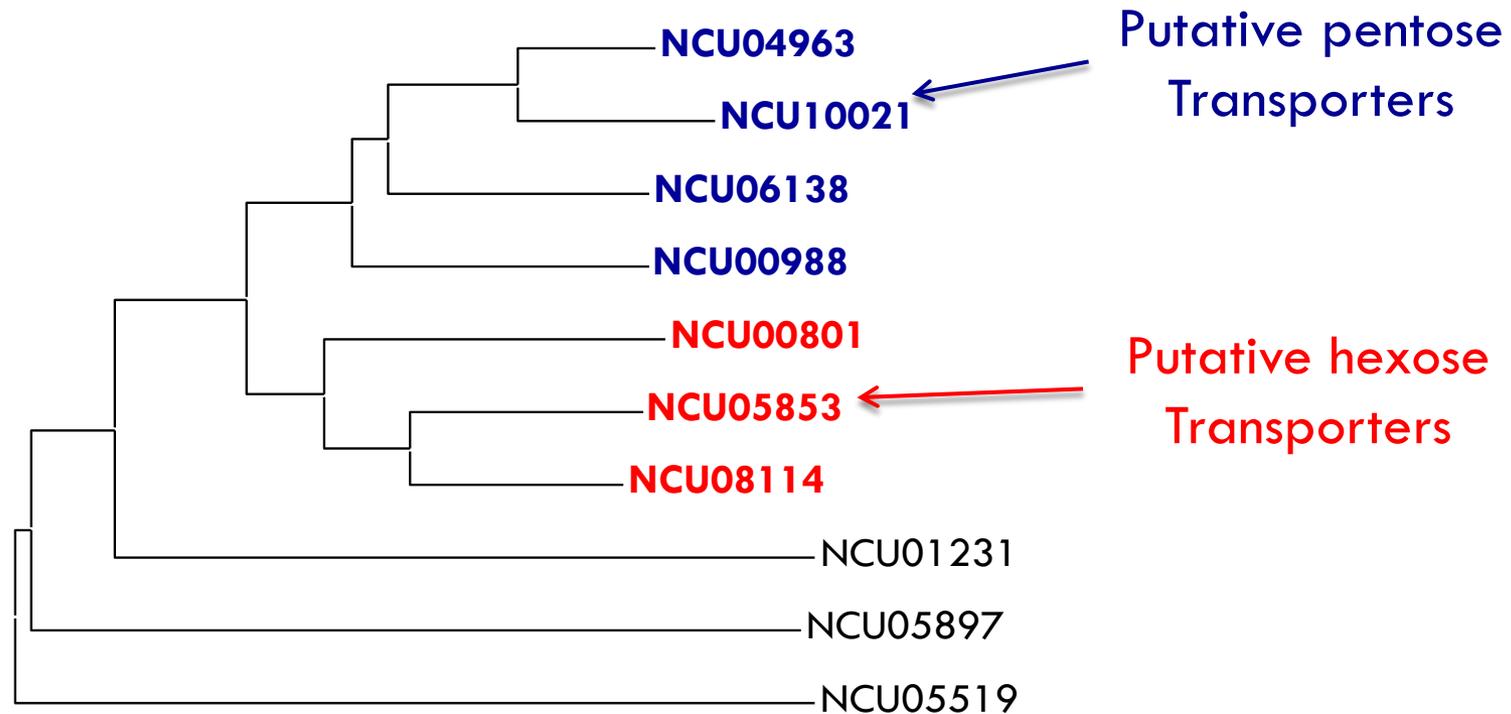
Fungal cellulose hydrolysis

Neurospora crassa cellulase system

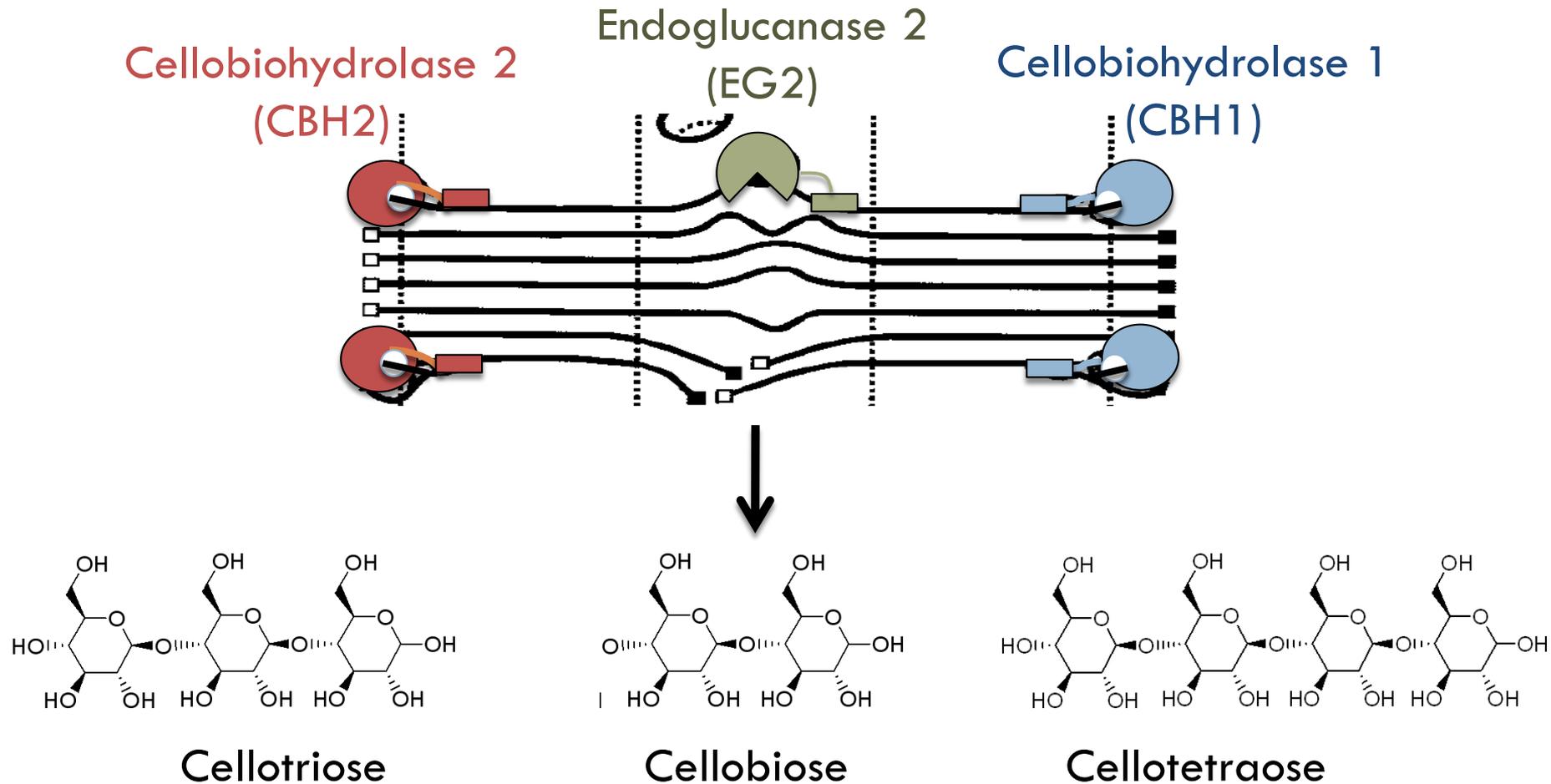


cf L. Lynd *et al.* (2002) *MMBR* **66**, 506-ff.

10 transporters upregulated on cellulose

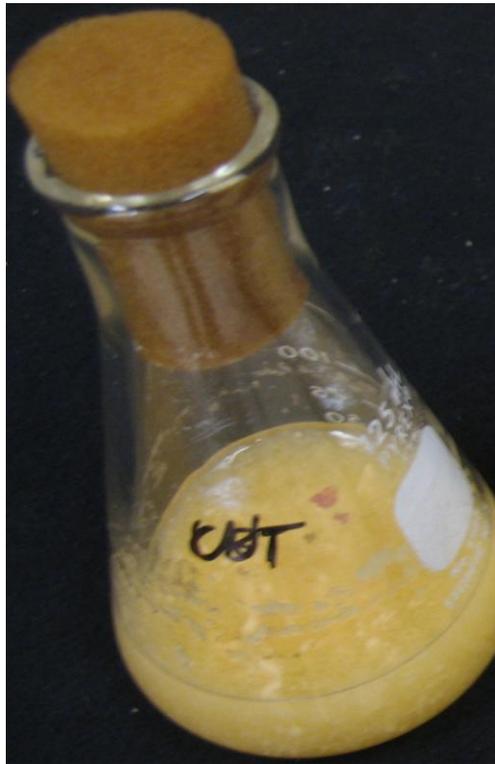


Are these transporters of cellulose hydrolysis products?

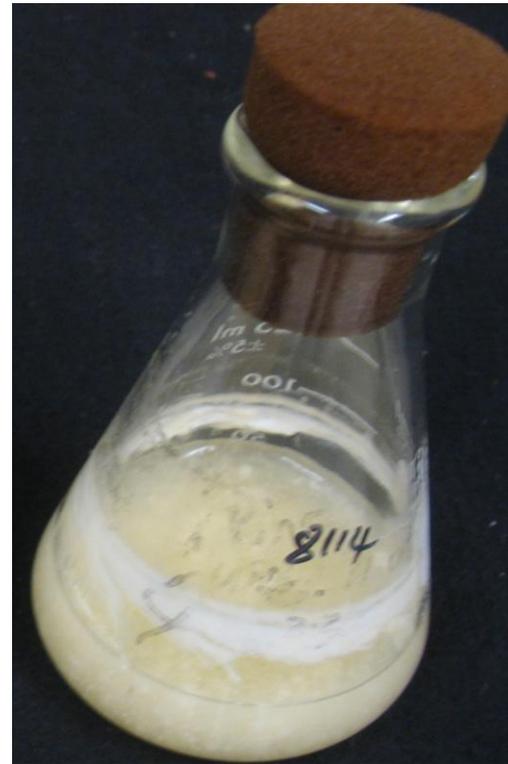


Transporter KO grows poorly on cellulose

3 days of growth on Avicel, a model cellulose



Wild type



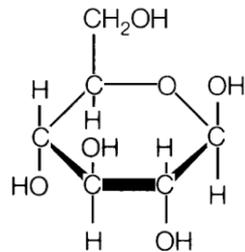
NCU008114 knockout

S. cerevisiae sugar utilization

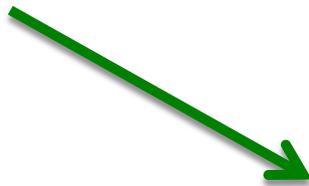
Yeast has been used to produce ethanol for ~7000 years

But *not* for cellulosics

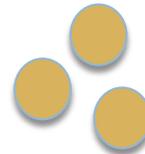
Cannot naturally consume the products of fungal cellulases



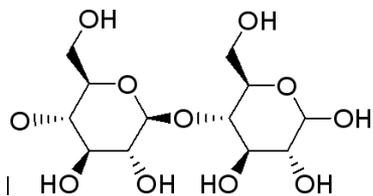
Glucose



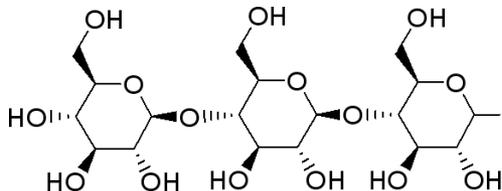
Yeast



Ethanol

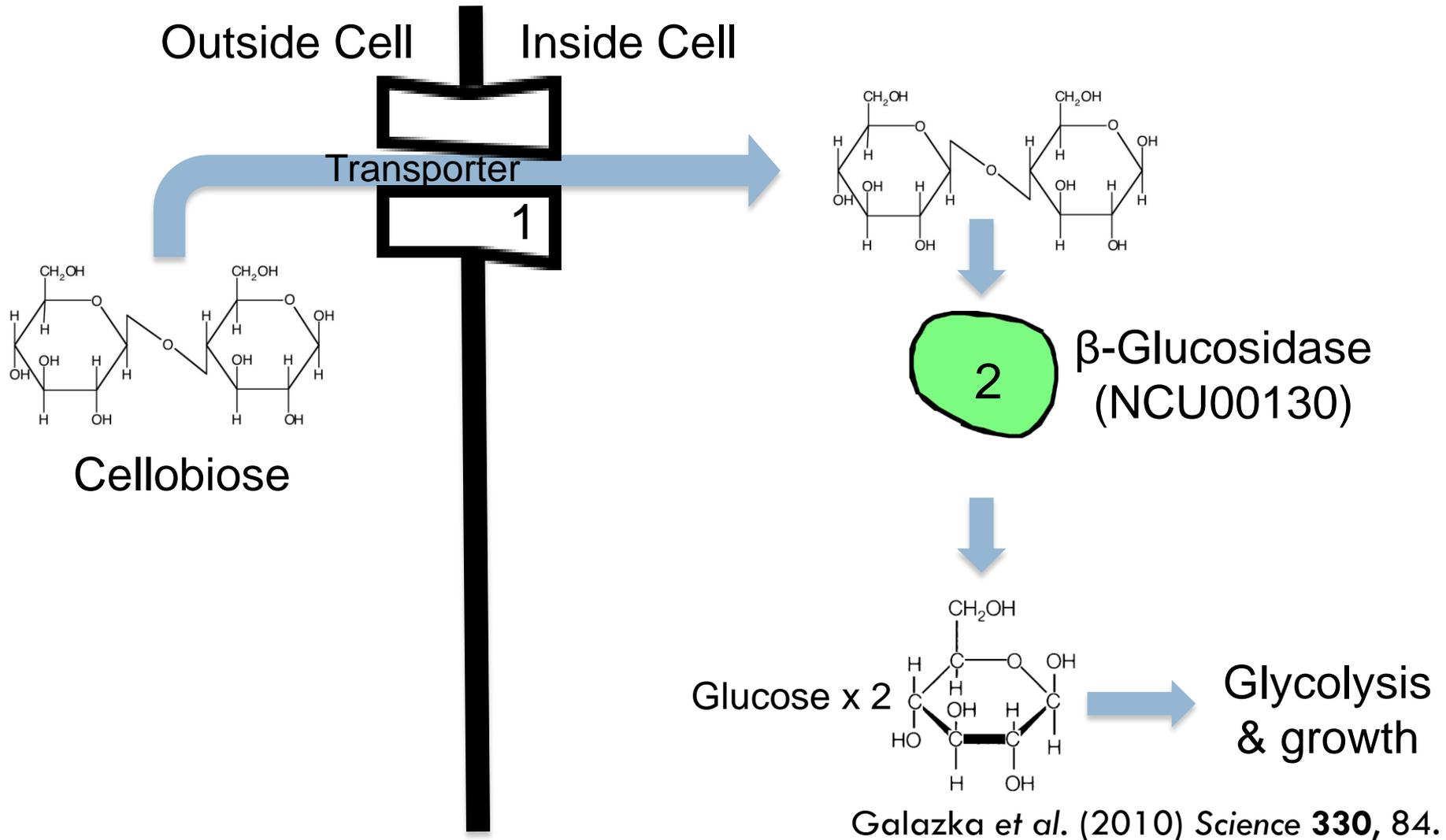


Cellobiose



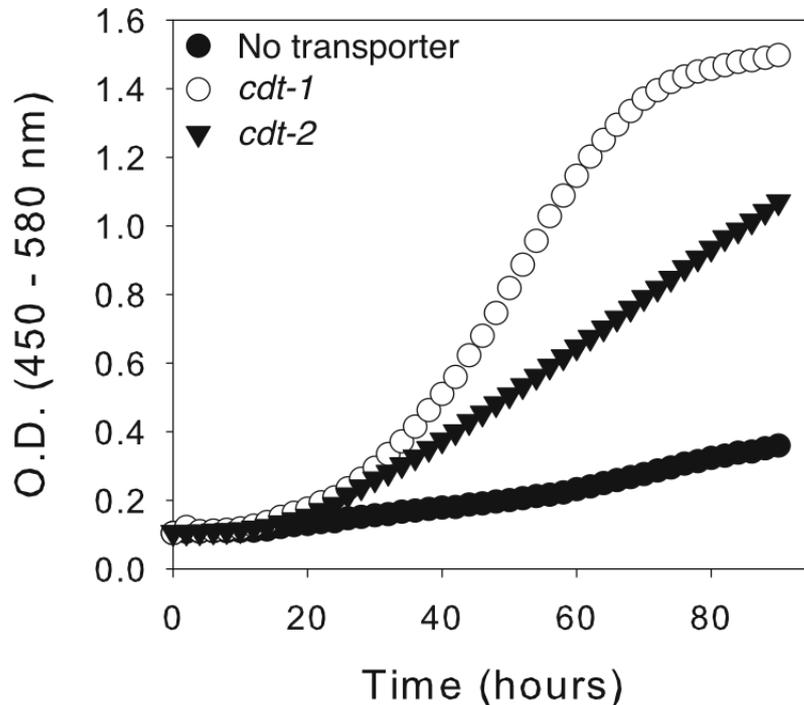
Cellotriose

Screening for cellodextrin transporters in yeast

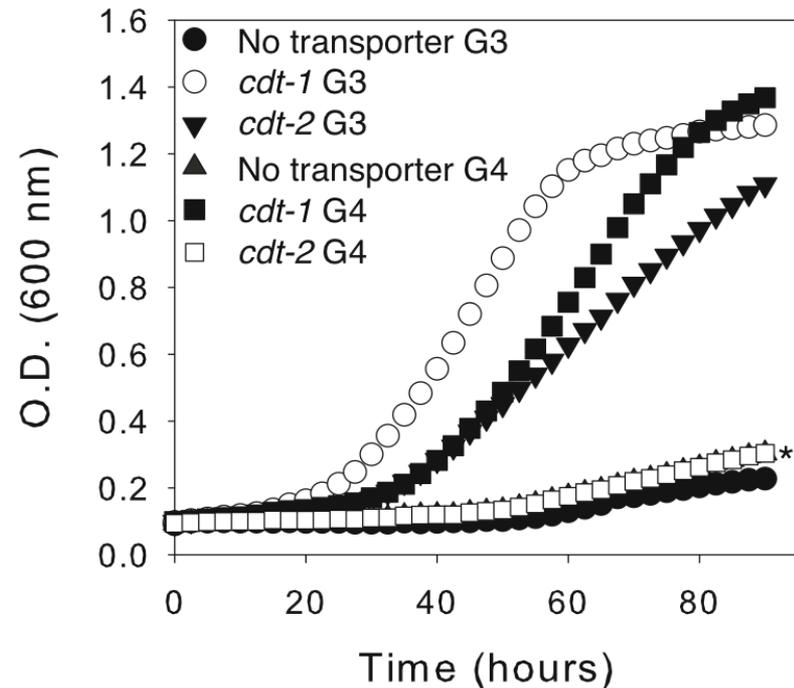


Two strains metabolize cellobiose

Growth on 2% cellobiose

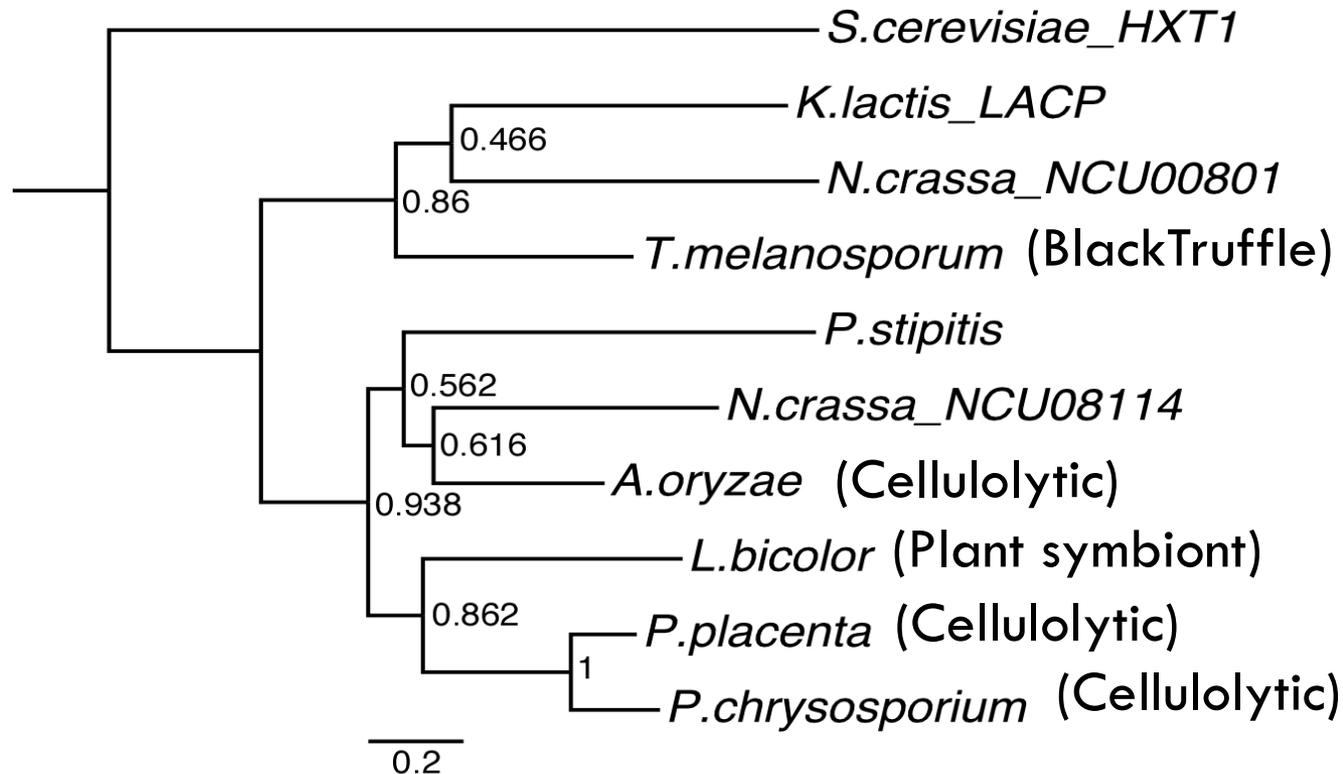


Growth on cellotriose, -tetraose



All strains express NCU00130 (β -glucosidase)

Transporters are used by fungi during interactions with plant walls

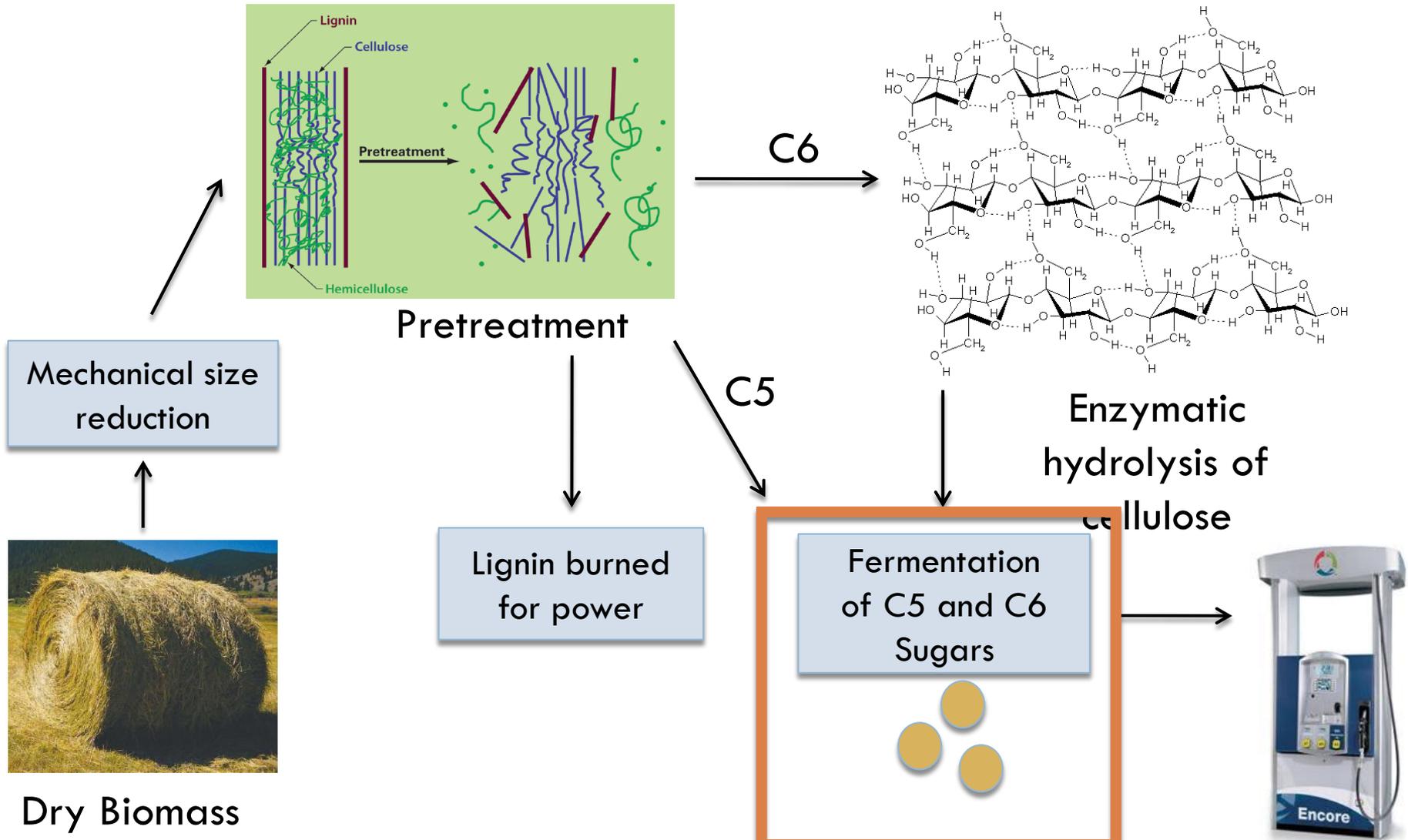


Cellodextrin transporters reported to be upregulated by fungi during catabolism of plant walls or cellobiose, or during symbiosis with plants.

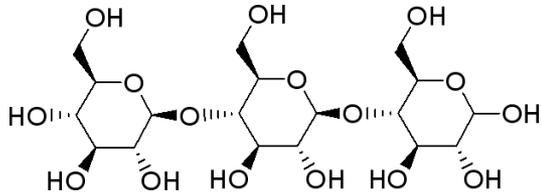


APPLICATION OF TRANSPORTERS

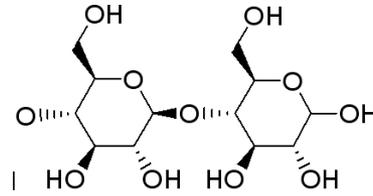
Making expensive cellulosic biofuels



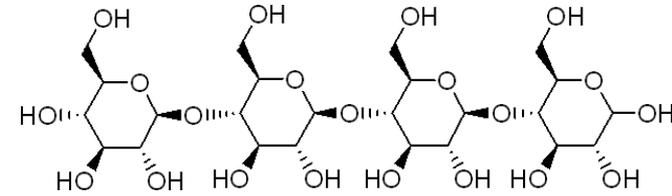
Fungal cellulases are supplemented with β -glucosidase



Cellotriose

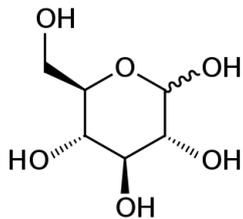


Cellobiose



Cellotetraose

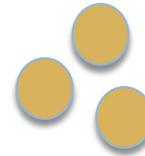
β -glucosidase



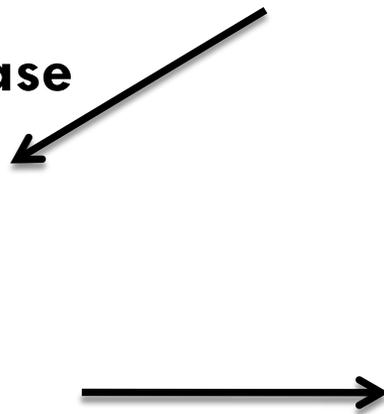
Glucose



Yeast

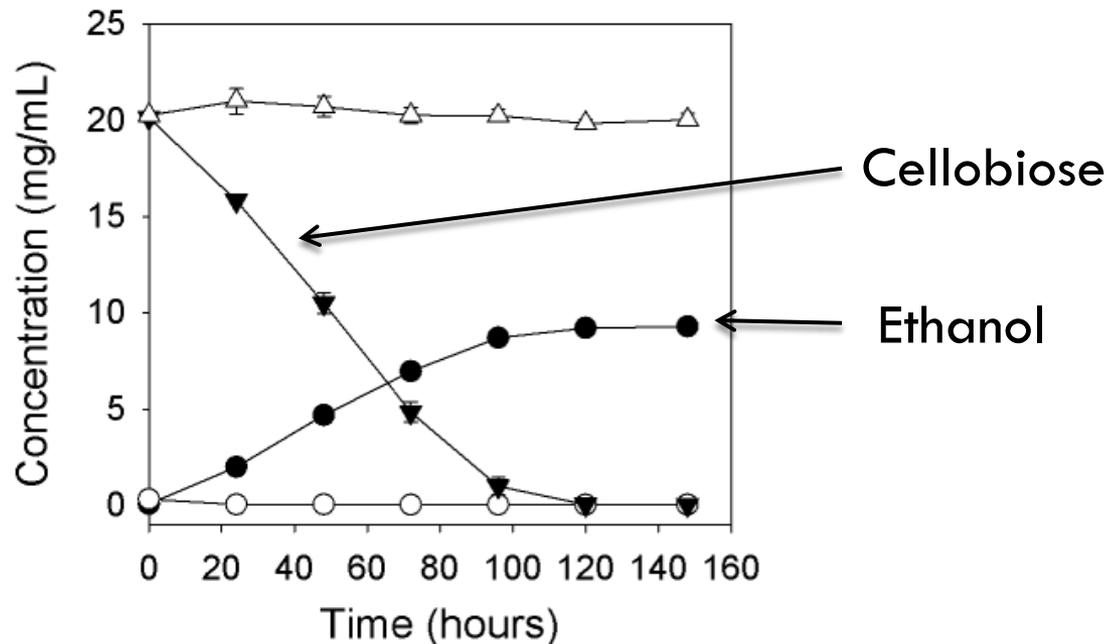


Ethanol



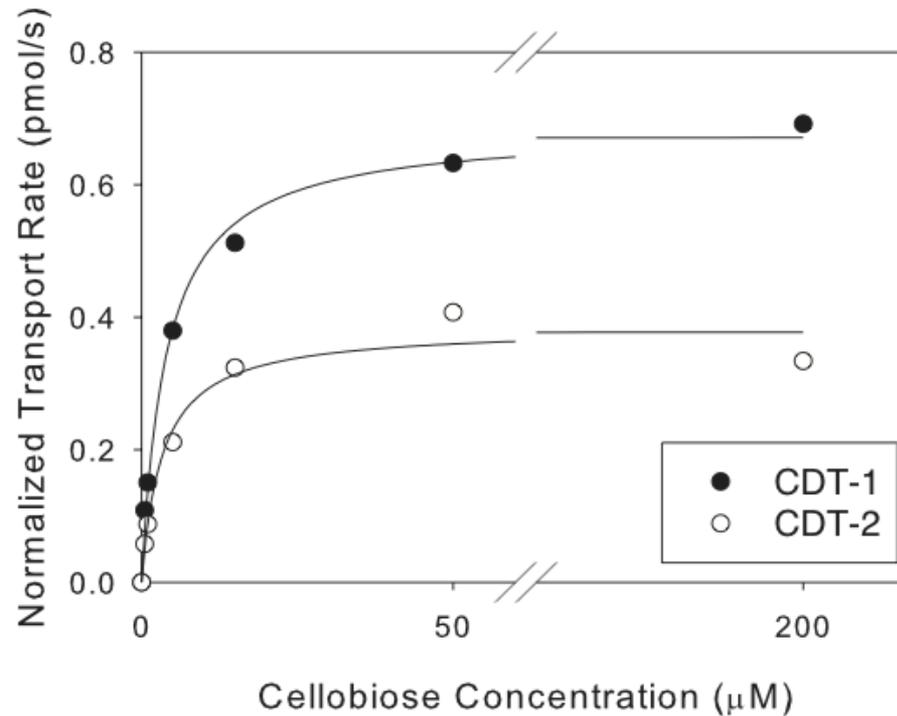
Fermentation of cellobiose

Yeast strain with cellodextrin transport system
ferments cellobiose to ethanol
86% of the theoretical yield



Strains express NCU00130 (β -glucosidase)

Transporters have high affinity for cellodextrins



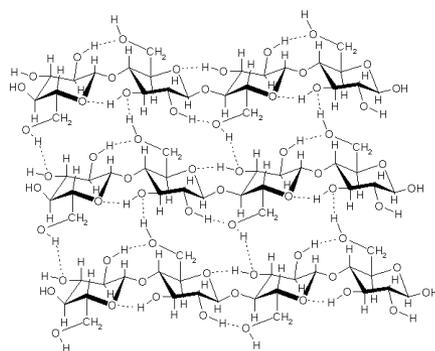
K_m of cellodextrin transporters is 3 – 4 μM

(compare to 100 – 1000 μM for β-glucosidases and 1000 – 10,000 μM for yeast hexose transporters)

Simultaneous Saccharification & Fermentation

Simultaneous Saccharification & Fermentation

(SSF)

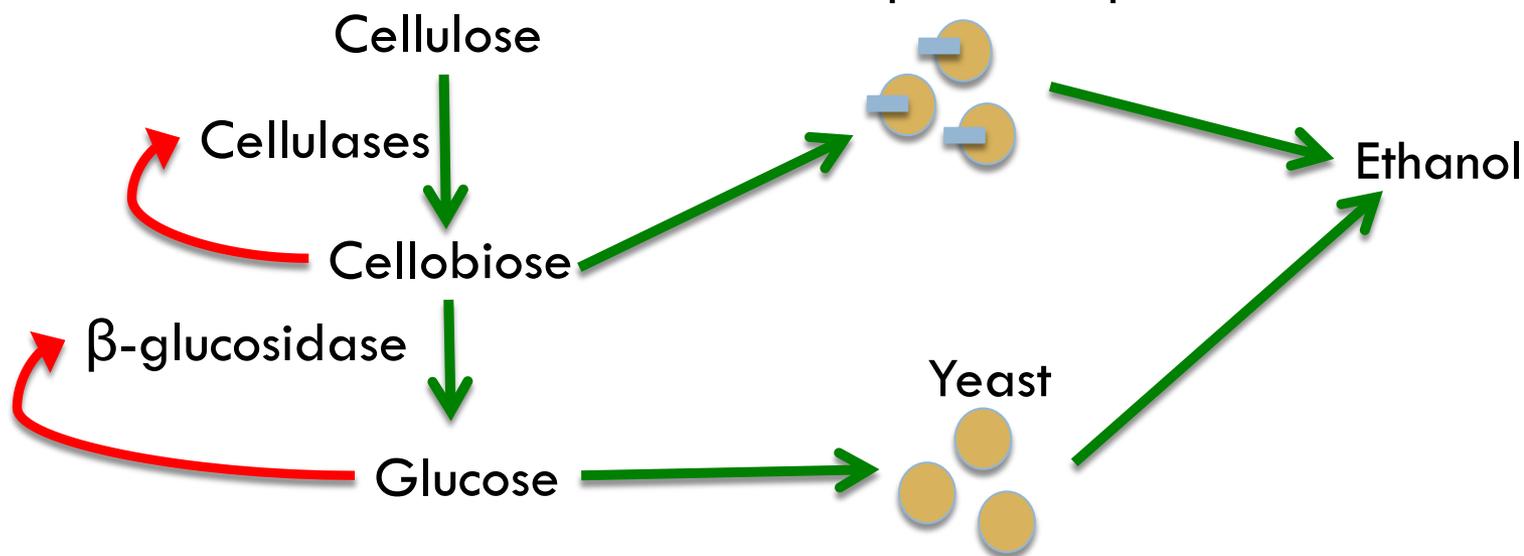


United States Patent [19]

Gauss et al.

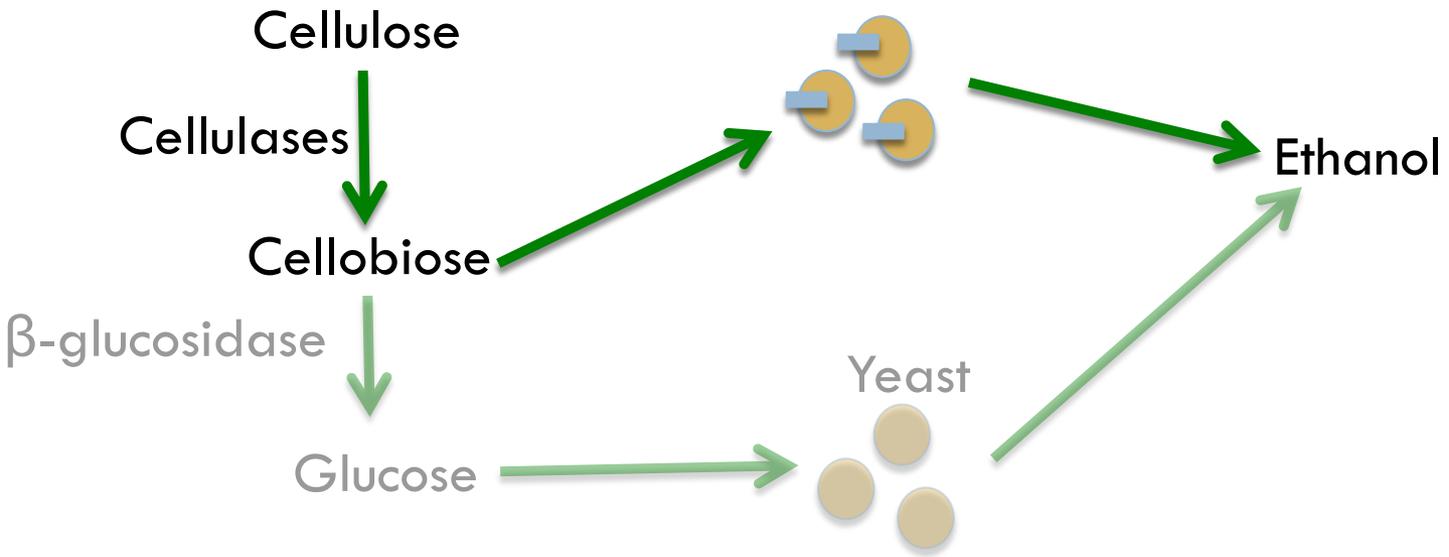
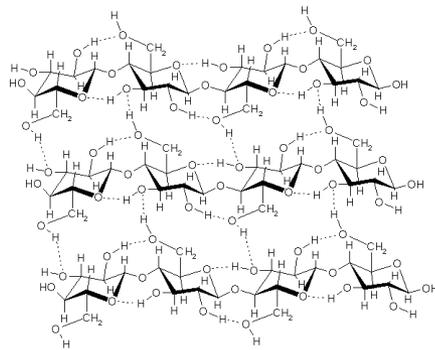
[54] MANUFACTURE OF ALCOHOL FROM CELLULOSIC MATERIALS USING PLURAL FERMENTS

Yeast plus transporter

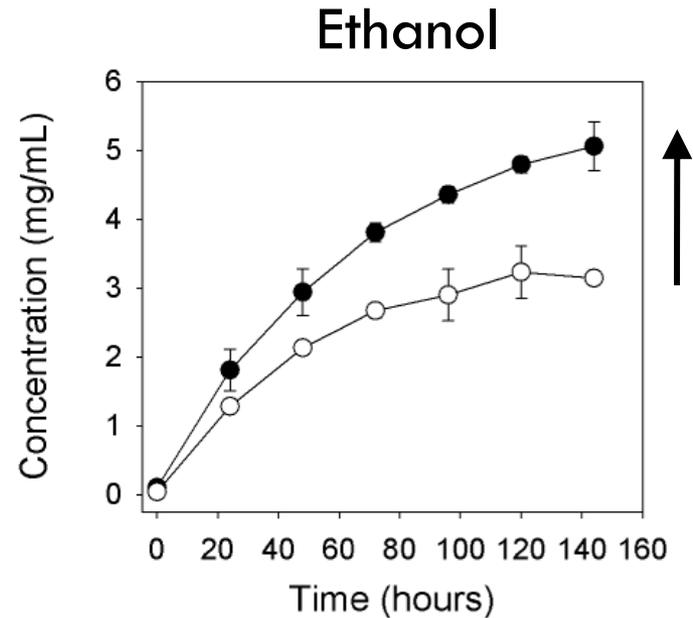
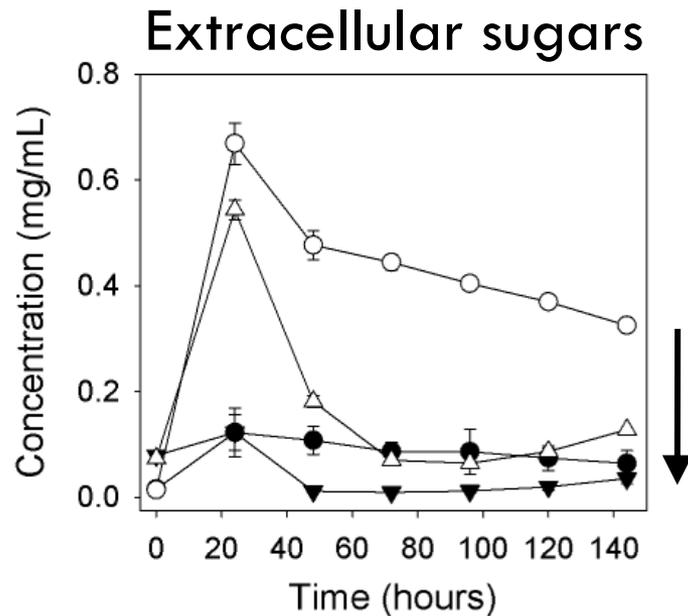
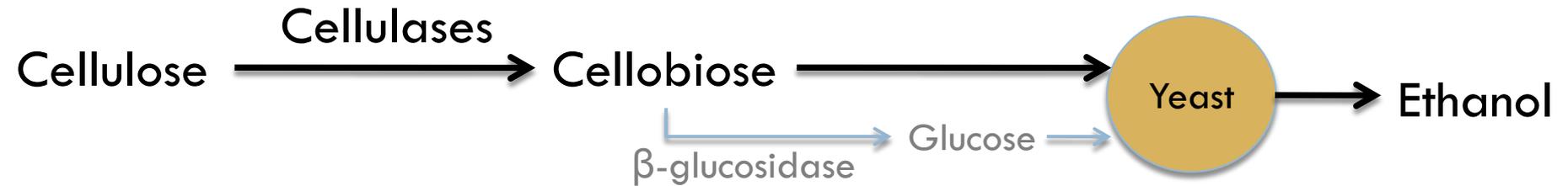


SSF could be enhanced by high affinity transport

Simultaneous Saccharification & Fermentation (SSF)

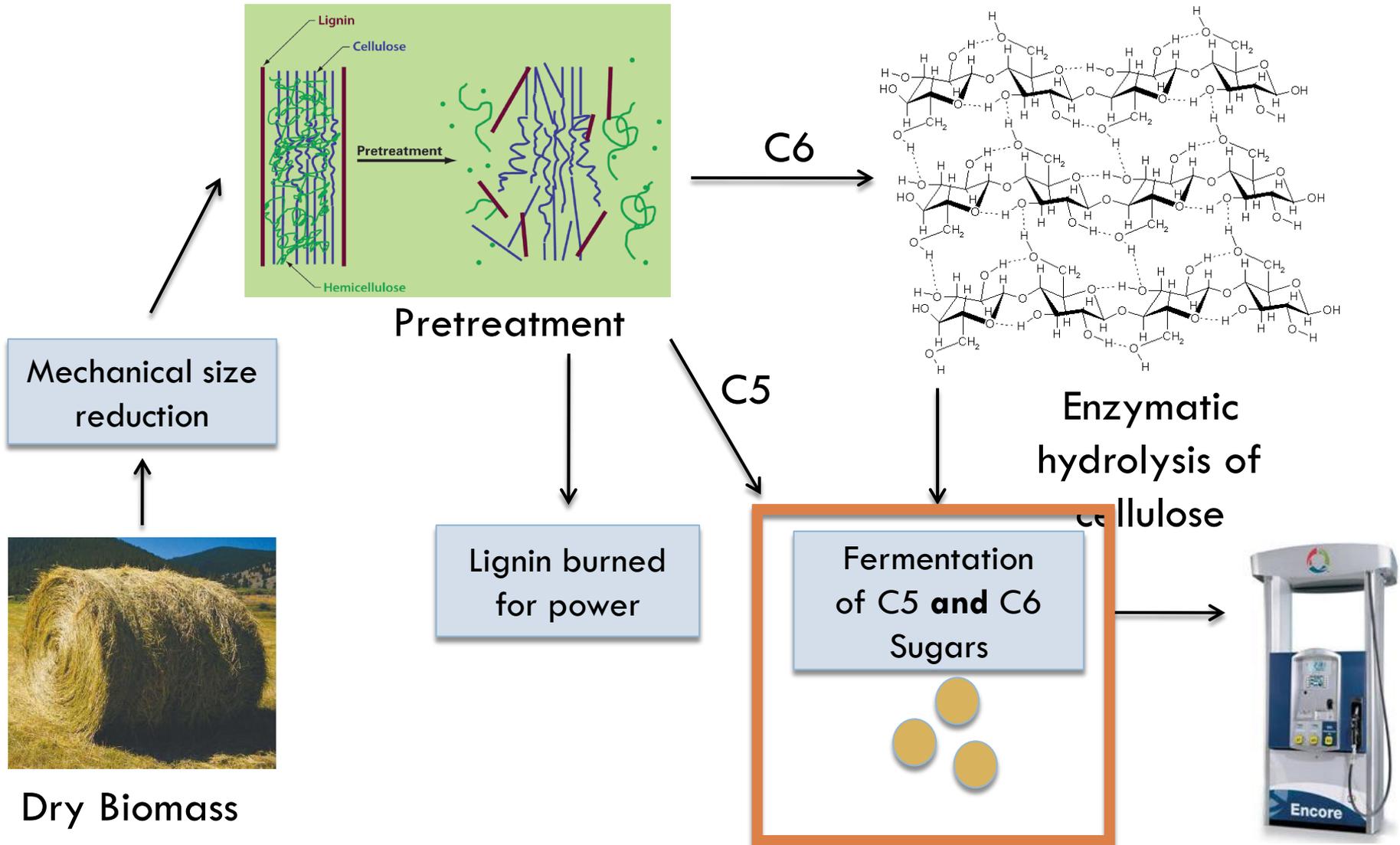


Simultaneous Saccharification & Fermentation

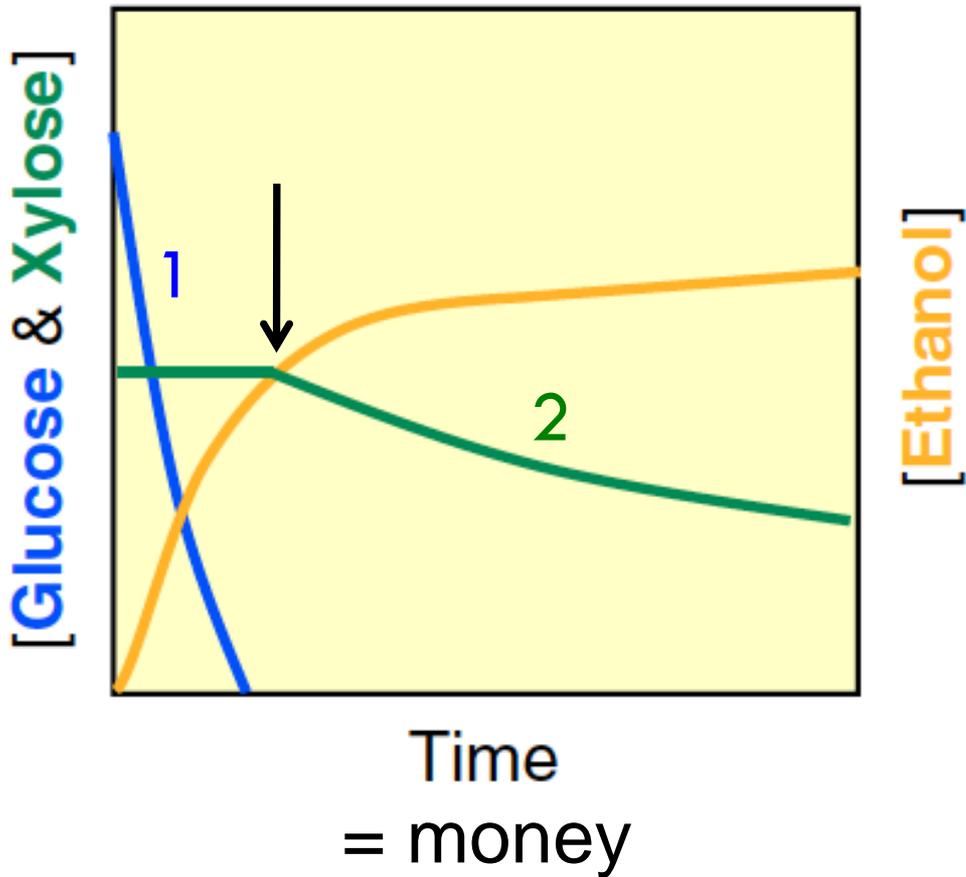


All strains express NCU00130 (β -glucosidase)

Making *less expensive* cellulosic biofuels



Simultaneous Saccharification & Co-Fermentation

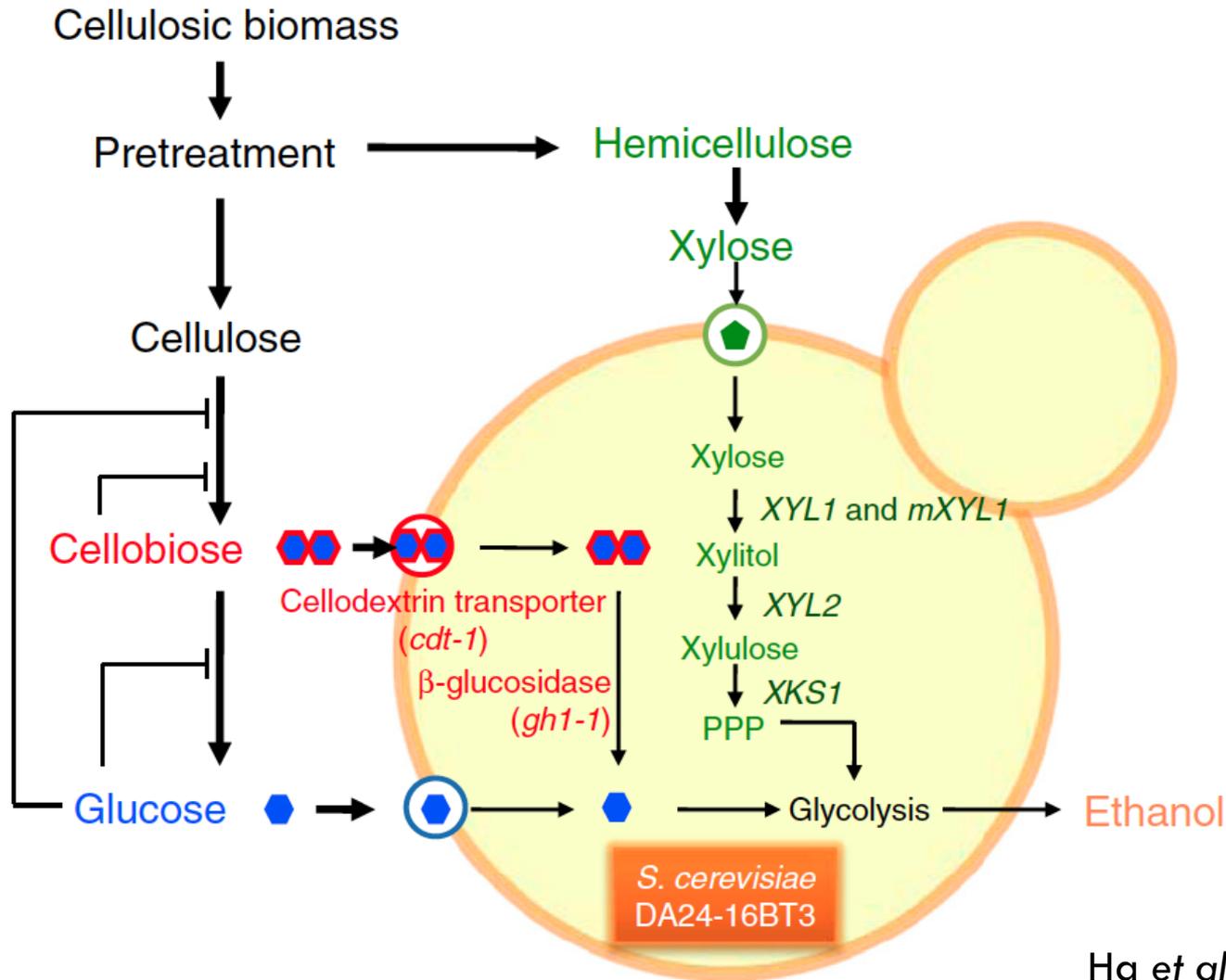


WT yeast cannot utilize xylose.

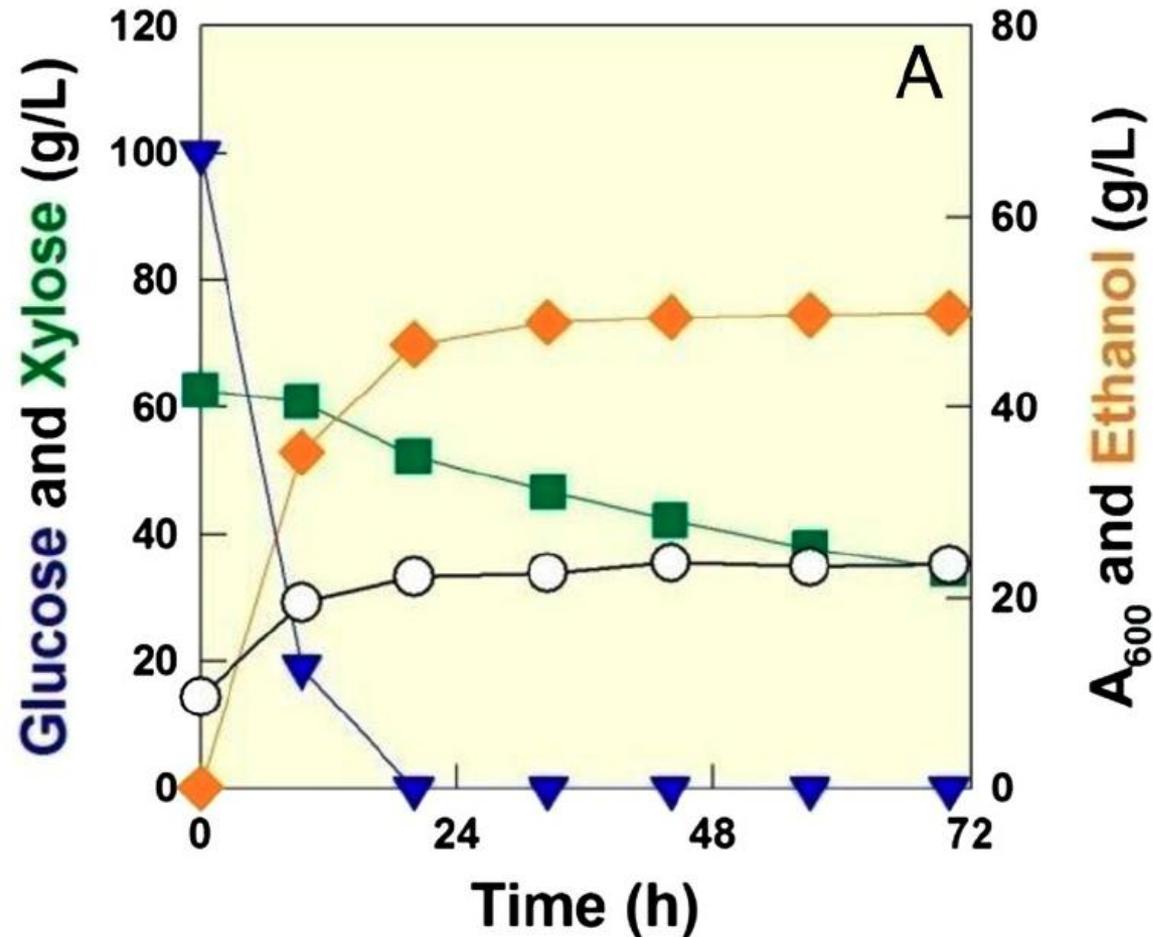
Engineered yeast exhibit diauxic growth.

Xylose fermentation starts when EtOH concentrations are already high (stress).

Cellulose and xylose co-fermentation

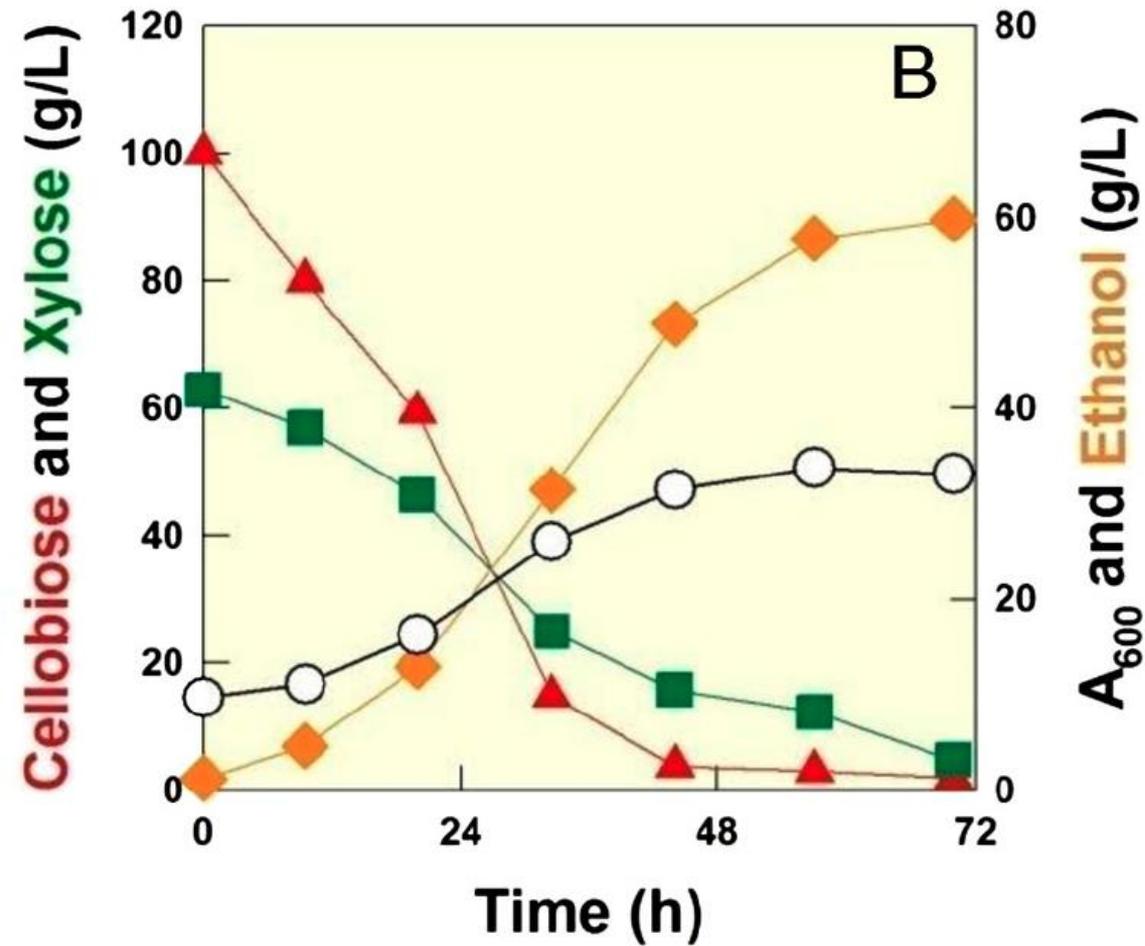


Cellodextrin Transport Improves Co-Fermentation



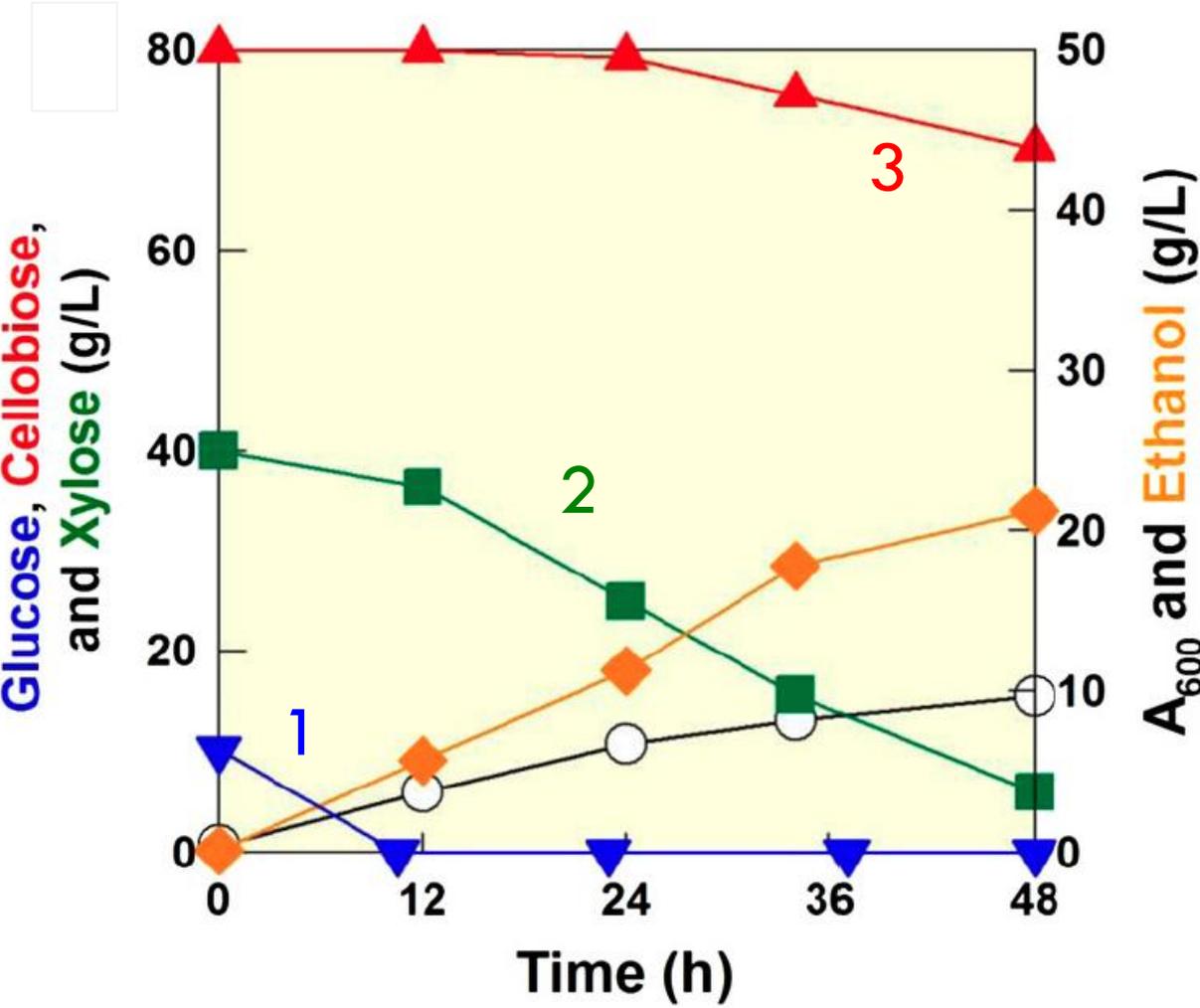
Engineered *S. cerevisiae*
DA24-16BT3

Cellodextrin Transport Improves Co-Fermentation



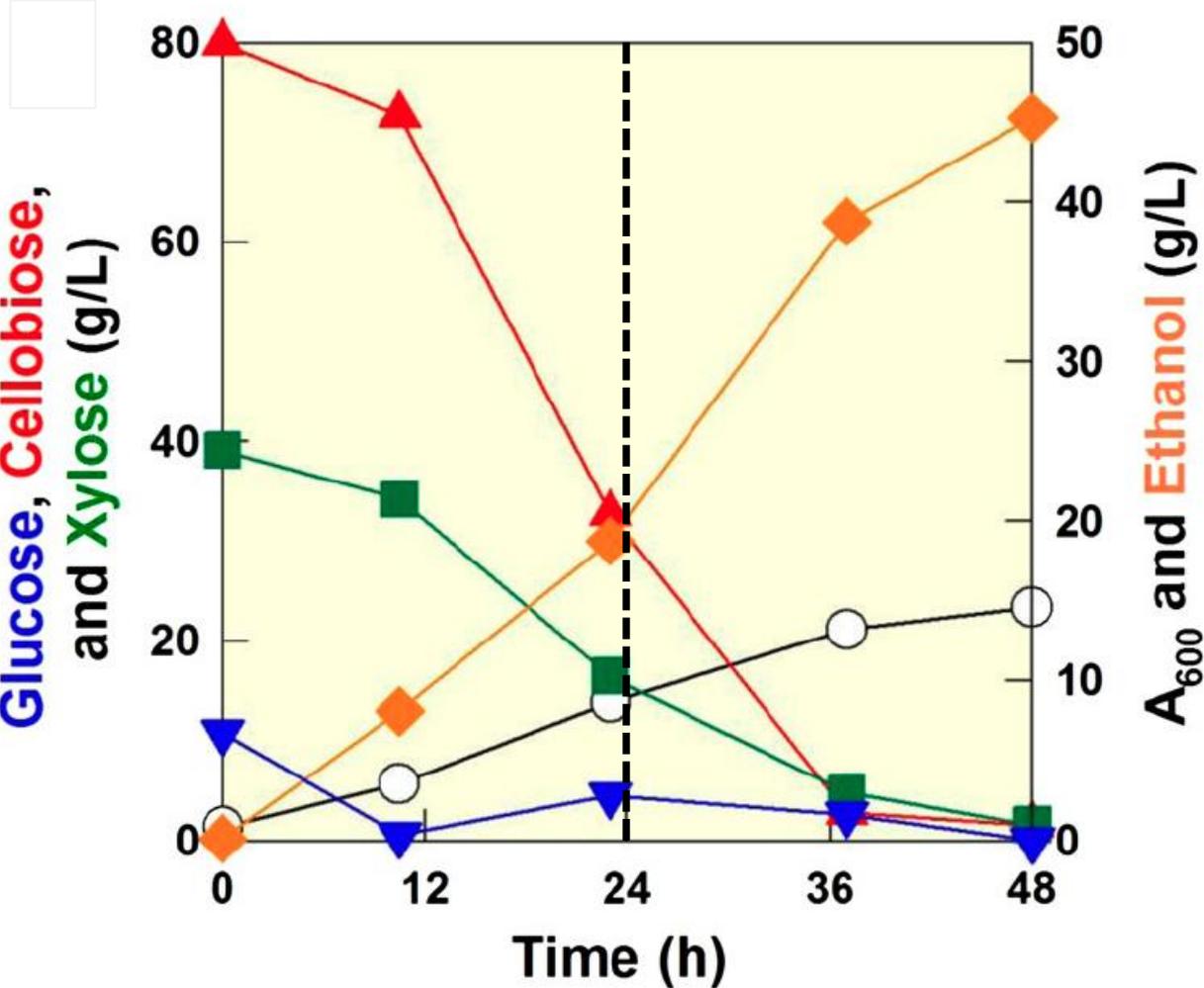
Engineered *S. cerevisiae*
DA24-16BT3

Cellodextrin Transport Improves Co-Fermentation



Pichia stipitis

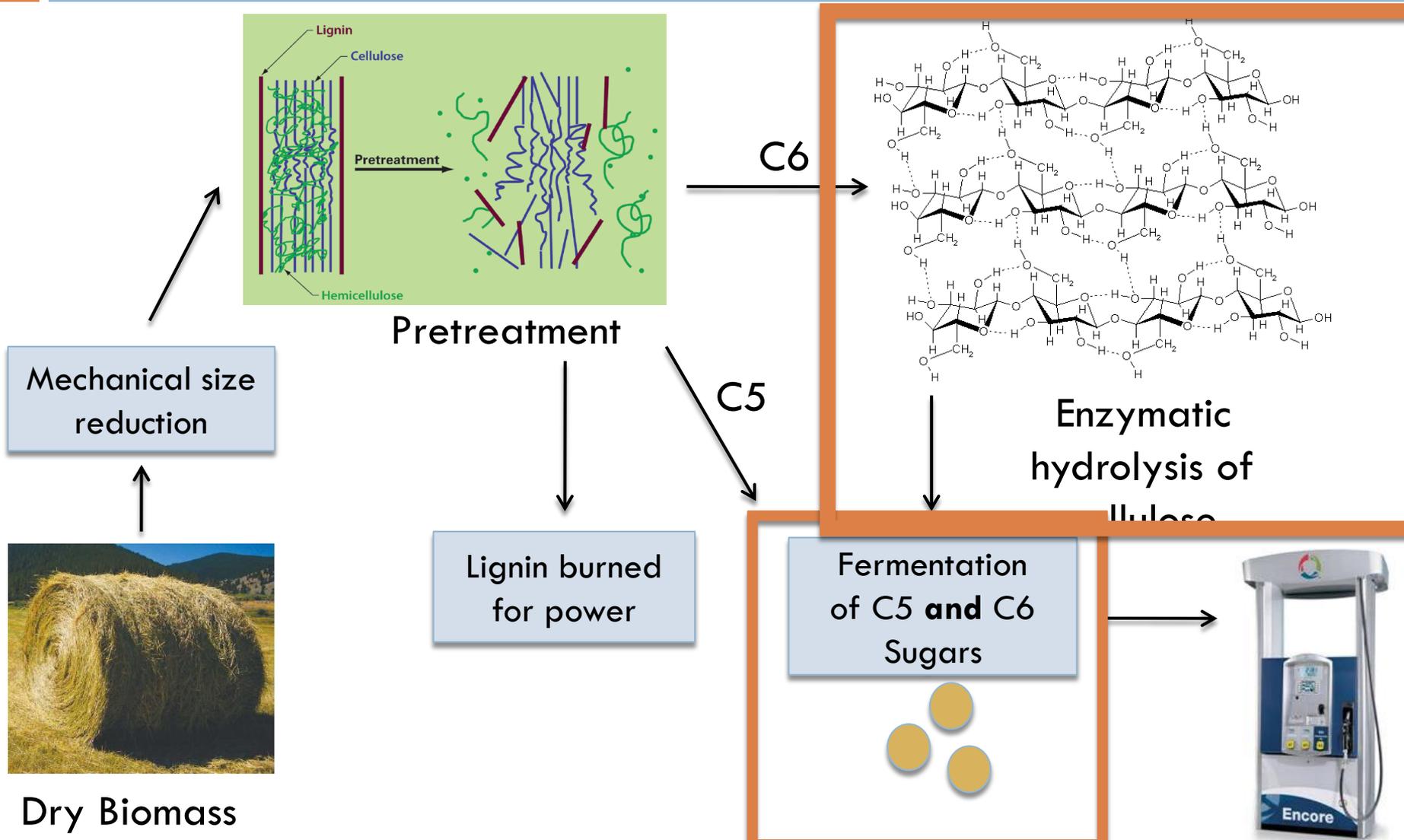
Cellodextrin Transport Improves Co-Fermentation



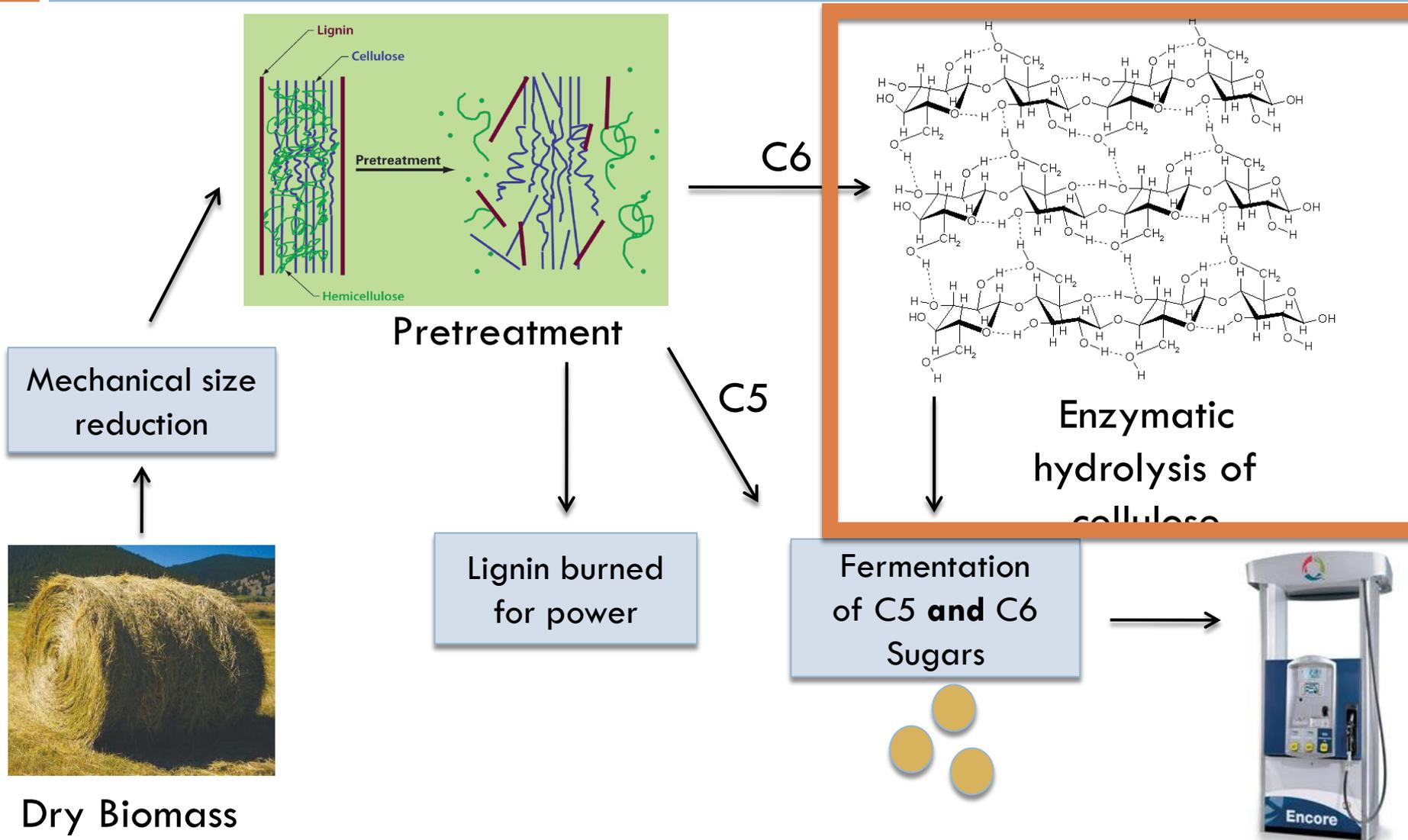
Engineered *S. cerevisiae*
DA24-16BT3

Industrial strain test:
Complete fermentation
in 24 hr.

Making *less expensive* cellulosic biofuels

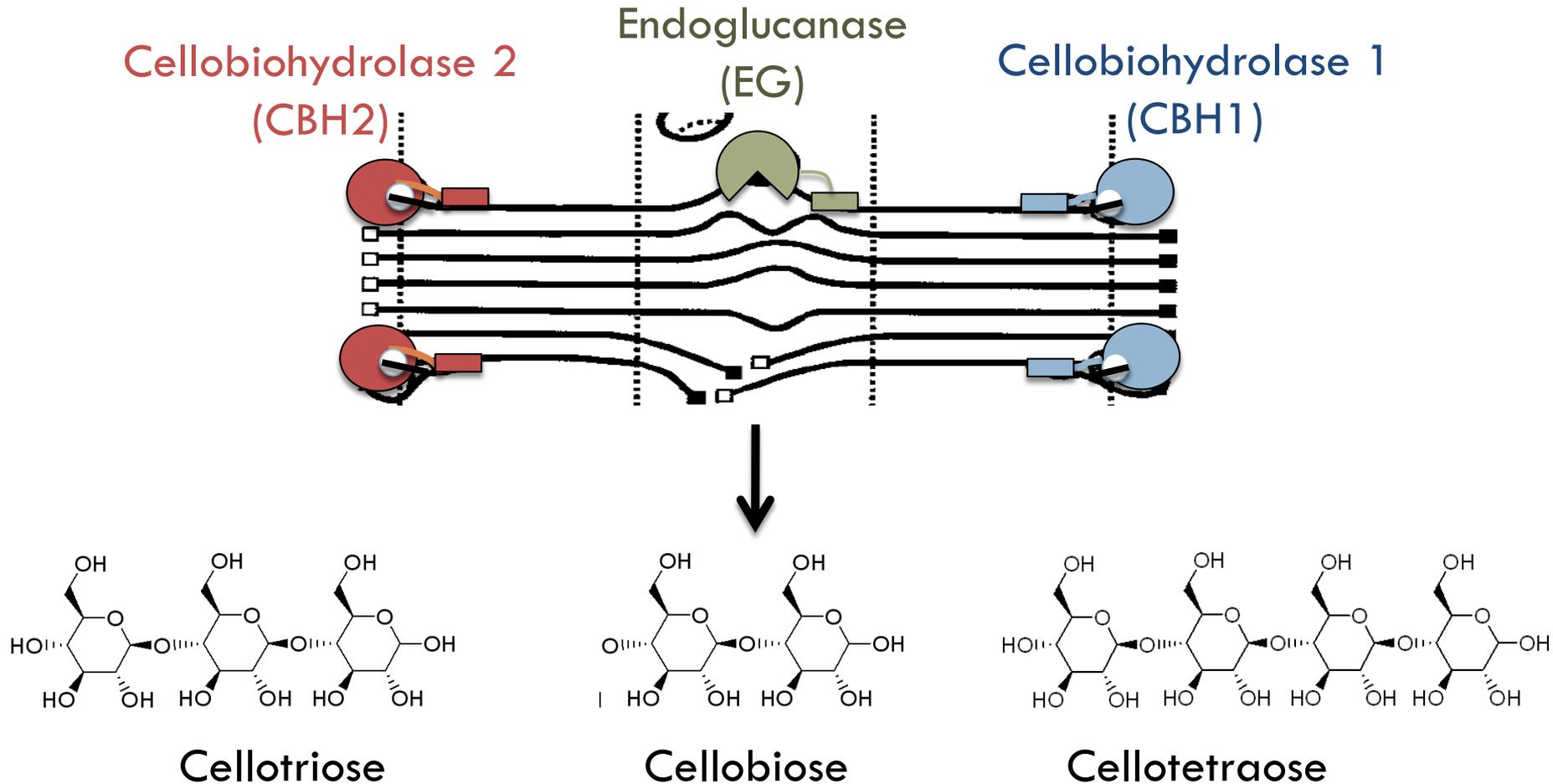


Making *less expensive* cellulosic biofuels

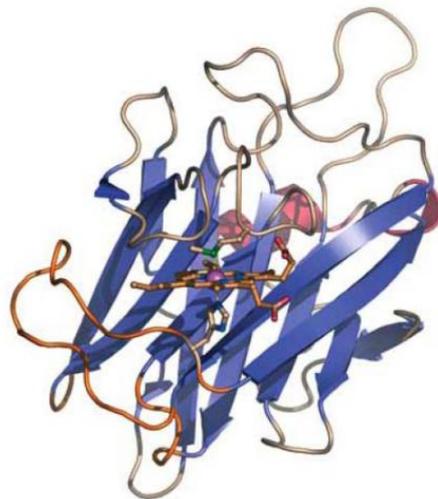
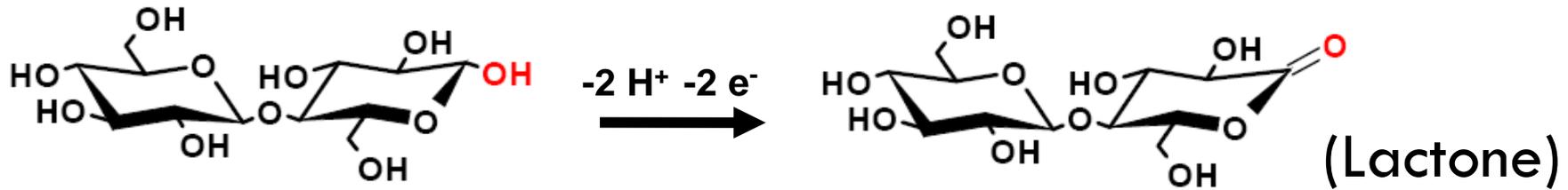


More lessons to learn from *N. crassa*, a story of two detectives

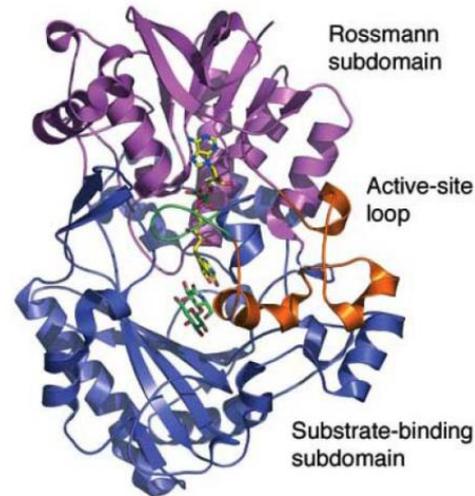
Trichoderma reesei cellulase system



Cellobiose dehydrogenases in fungi oxidize cellobiose. Why?



Heme Domain



Flavin Domain

X-ray structures from *P. chrysosporium*

Biological function of cellobiose dehydrogenase

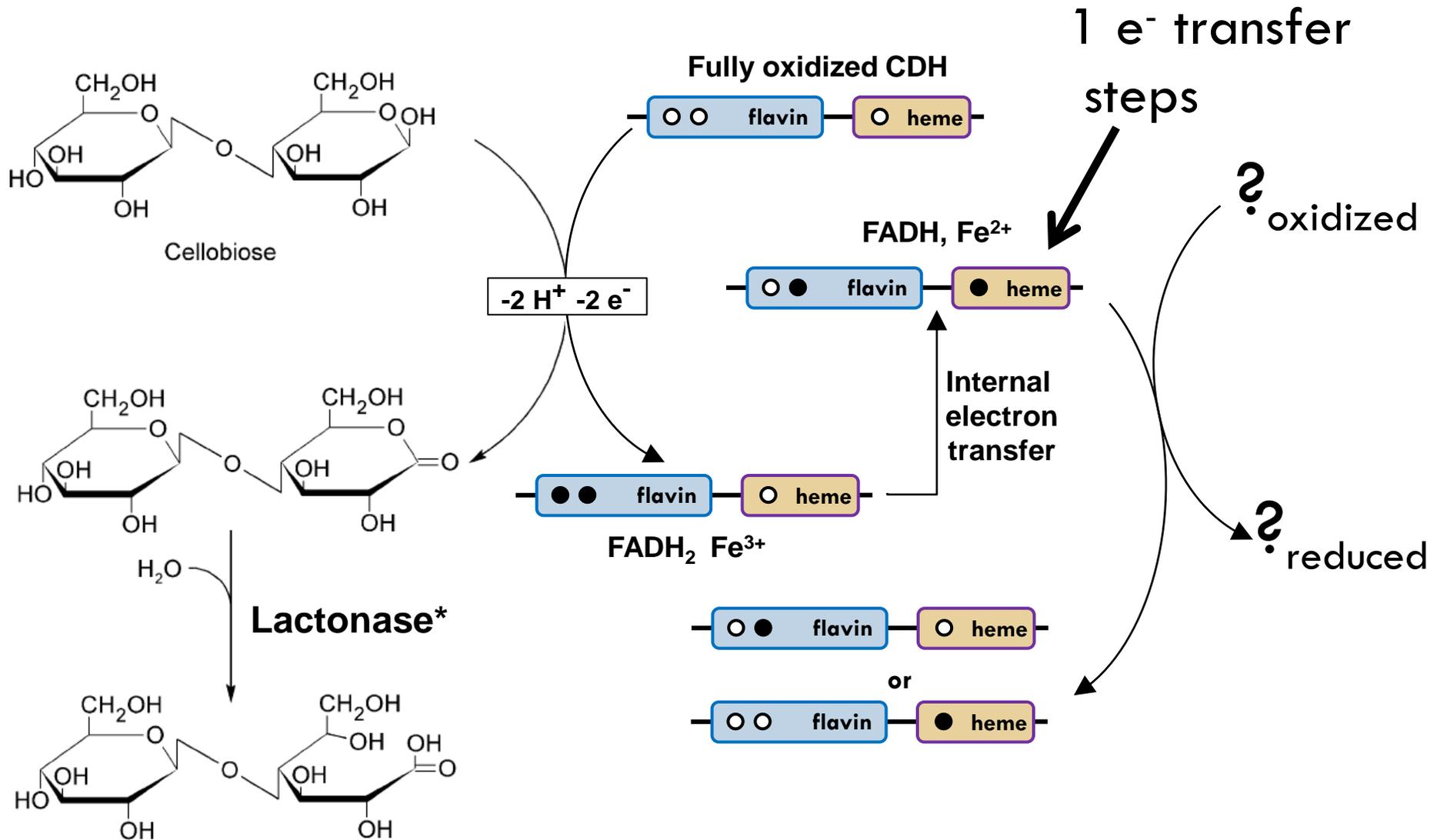
6.6. Biological Function

Over the years, there have been probably as many functions proposed for CDH as there have been workers involved, perhaps even more because some investigators have suggested several different functions.

Example: Fenton chemistry, OH^\bullet generation for cellulose breakdown.

RNA biochemist perspective: “The nuclear option.”

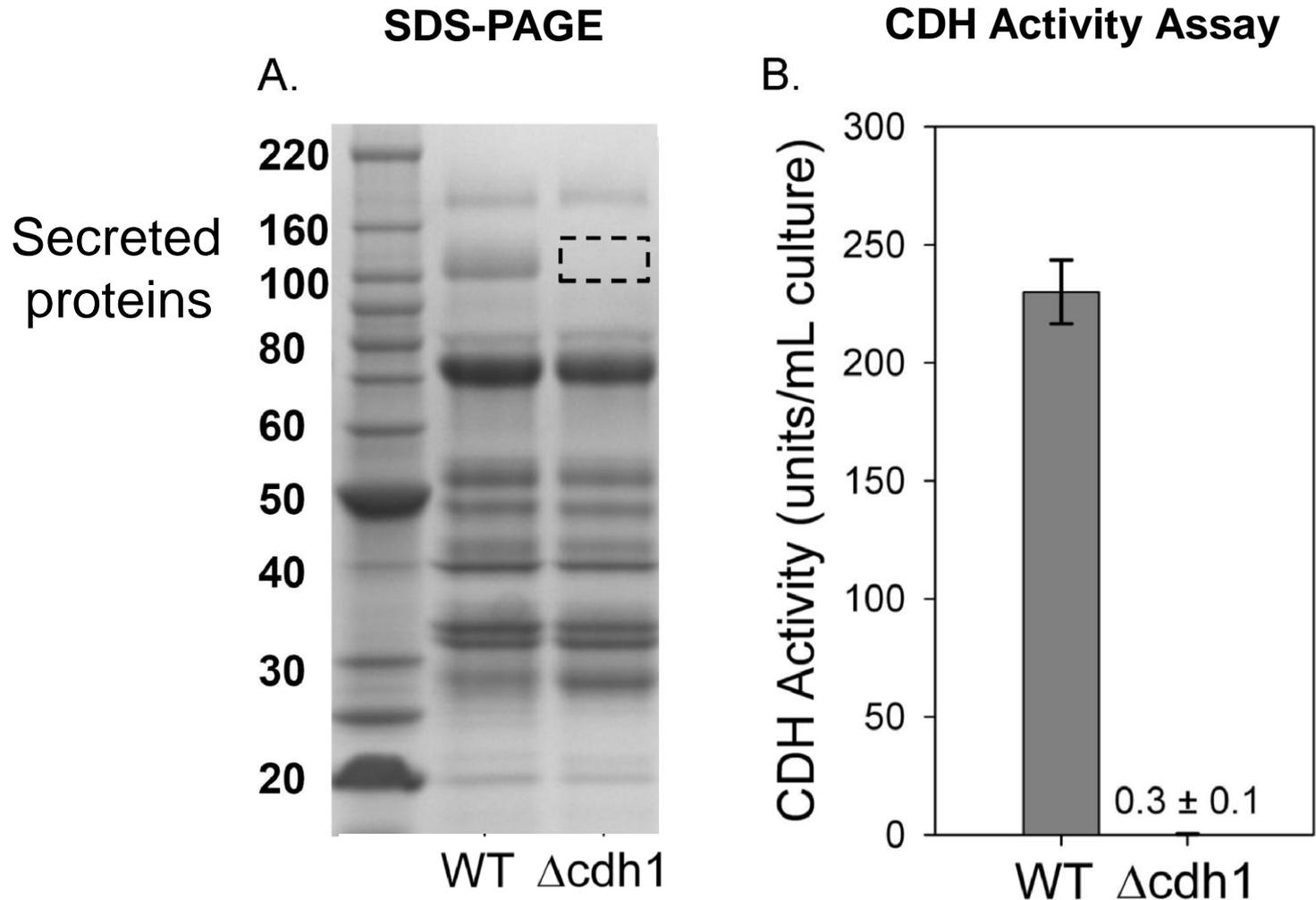
Where does cellobiose dehydrogenase (CDH) deposit the 2 electrons?



*Beeson, WT *et al.* (2011) *Appl. Env. Micro.* **77**, 650-ff.

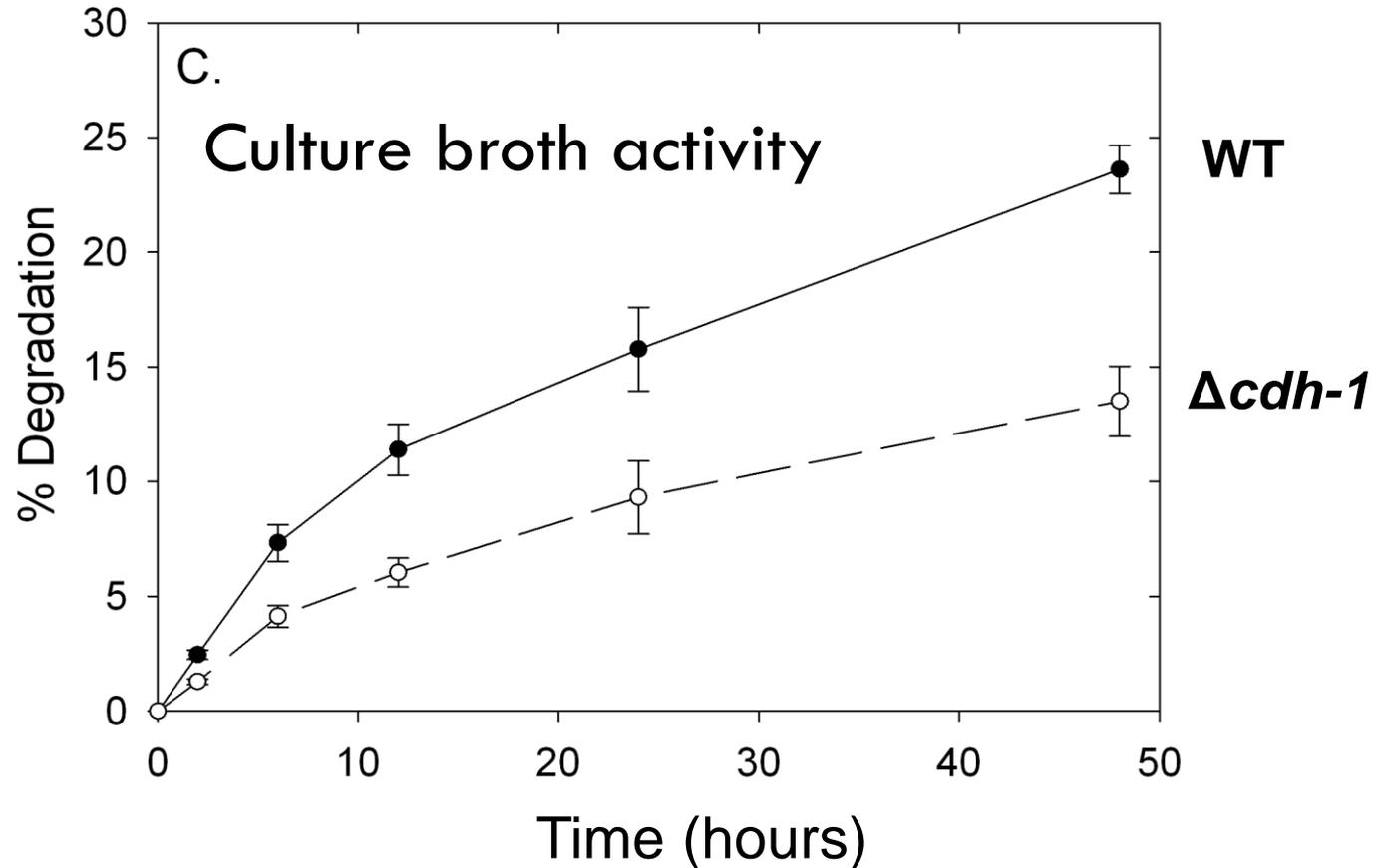
$\Delta cdh-1$ strain of *N. crassa*

WT and $\Delta cdh-1$ *N. crassa* strains were grown on Vogel's with 2% Avicel for 7 days.



CDH activity in the culture filtrate reduced ~800-fold

$\Delta cdh-1$ strain cellulase activity

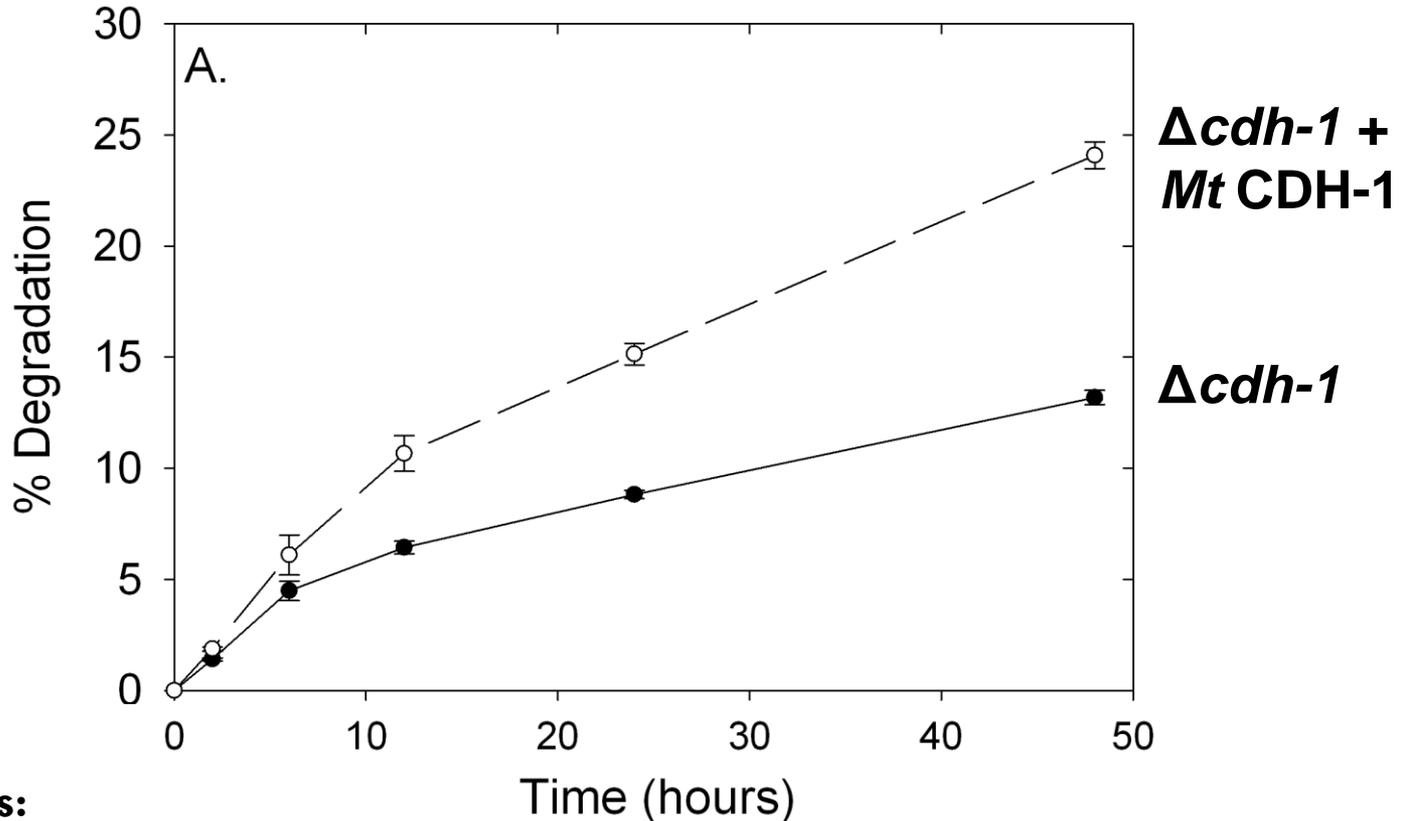
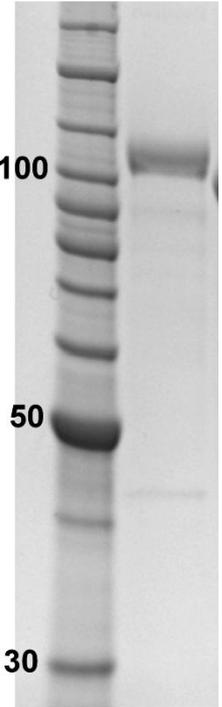


Assay conditions:

10 mg/mL Avicel
0.05 mg/mL culture filtrate
50 mM Na Acetate pH 5.0, 40 °C
Biological triplicate

Deletion of *cdh-1* reduces cellulase activity ~2x when added on an equal protein basis.

$\Delta cdh-1$ *in vitro* complementation with *M. thermophila* CDH-1



Assay conditions:

10 mg/mL Avicel

0.05 mg/mL culture filtrate

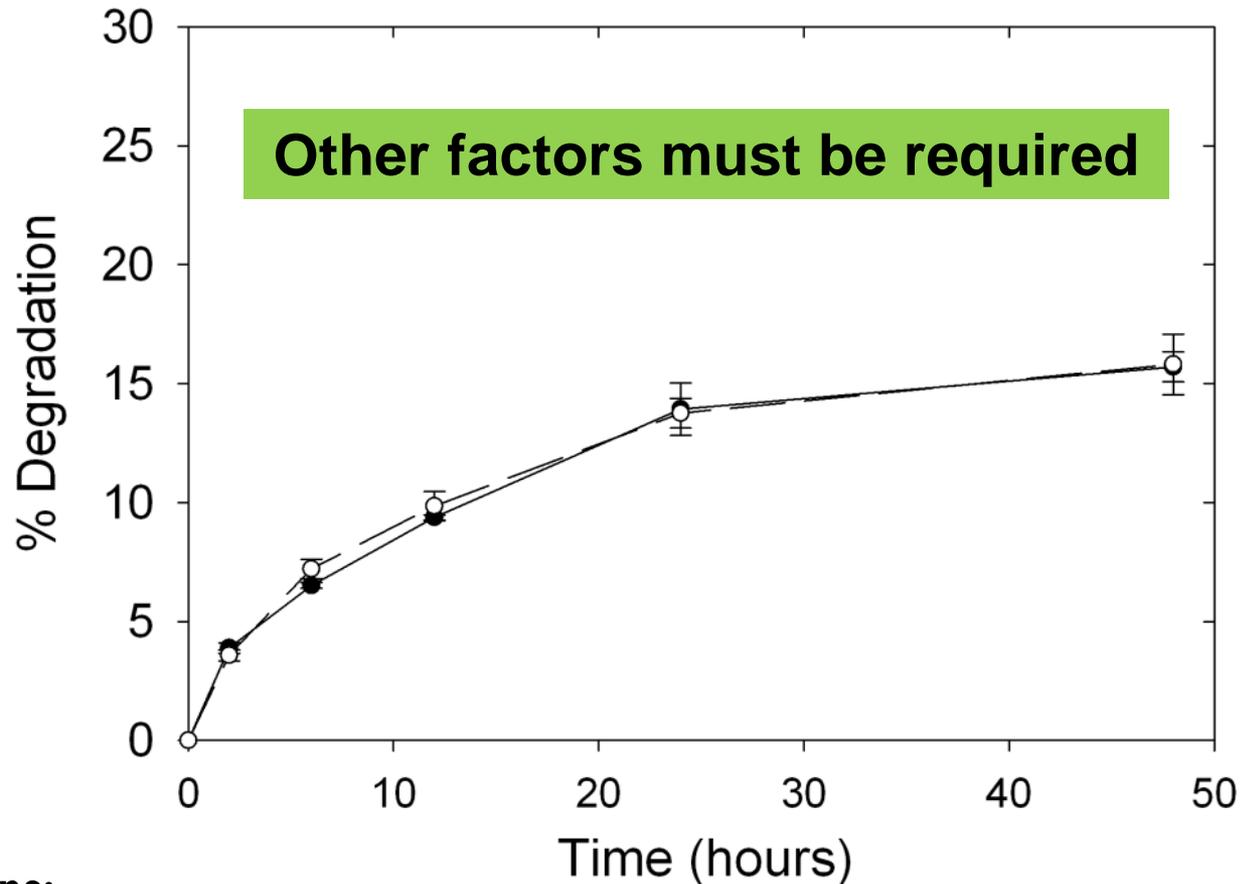
50 mM Na Acetate pH 5.0, 40 °C

200 μ g MtCDH-1 per g Avicel

Addition of purified *Mt* CDH-1 results in a 1.6 to 2.0-fold stimulation of cellulase activity.

CDH stimulation of pure cellulases

N. crassa core cellulases \pm *Mt* CDH-1



Assay conditions:

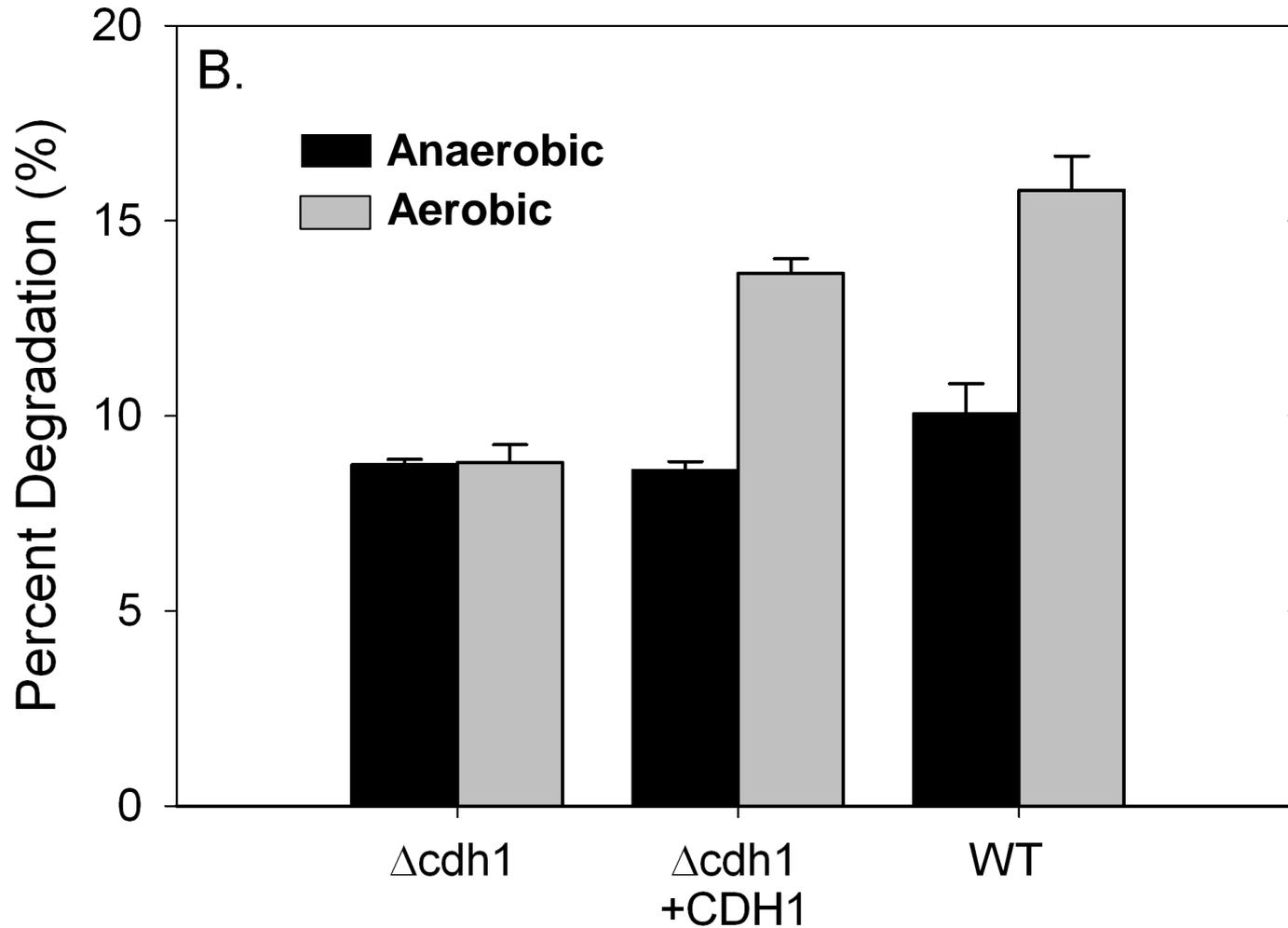
10 mg/mL Avicel

0.05 mg/mL core cellulase (CBH1, CBH2, EG, BG)

0.002 mg/mL *Mt* CDH-1

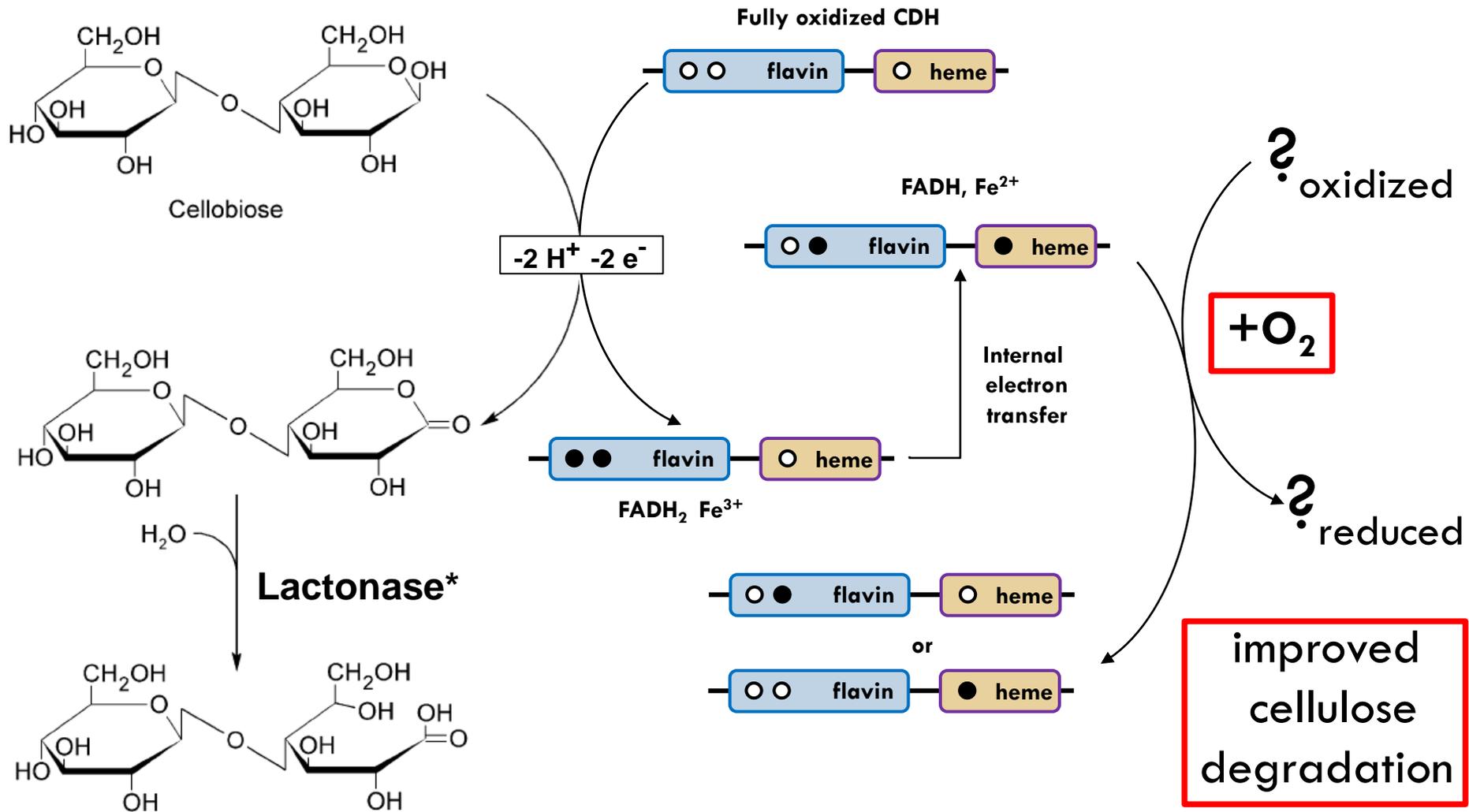
50 mM acetate pH 5.0, 40 °C

CDH effect requires molecular oxygen



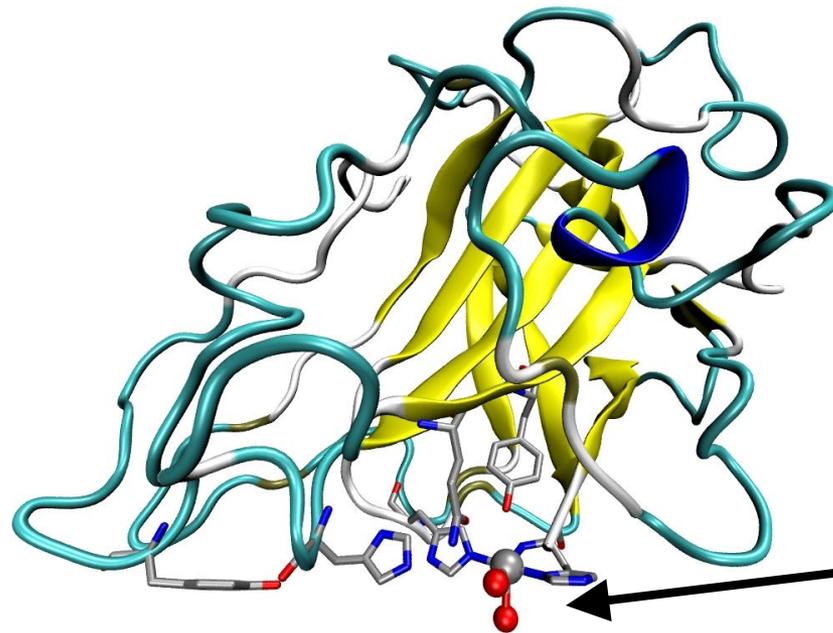
CDH stimulation is dependent upon molecular oxygen.

Redox chemistry enhances cellulose degradation



*Beeson, WT *et al.* (2011) *Appl. Env. Micro.* **77**, 650-ff.

N. crassa GH61s secreted during growth on cellulose



Metal binding site
Flat surface

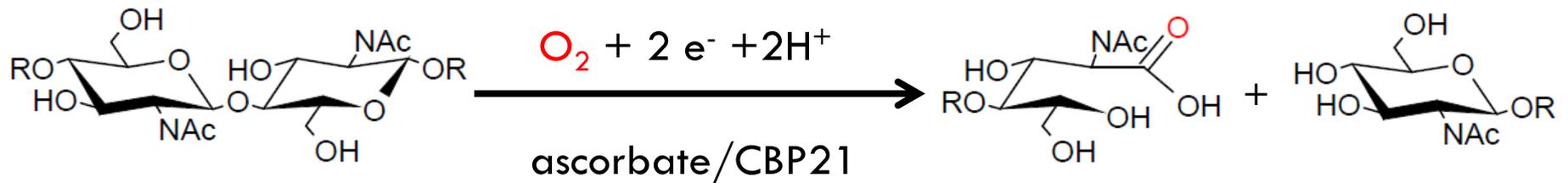
Harris P.V., *et al.* Biochemistry (2010)

| NCU Number | Protein Name | Predicted Function | Weight percent of supernatant |
|-------------------|---------------------|-----------------------------|--------------------------------------|
| NCU02240 | GH61-1 | cellulase enhancing protein | 3.4% |
| NCU07898 | GH61-2 | cellulase enhancing protein | 6.6% |
| NCU01050 | GH61-4 | cellulase enhancing protein | 1.0% |
| NCU08760 | GH61-5 | cellulase enhancing protein | 4.6% |

Phillips C.M., *et al.* J Prot Res (2011)

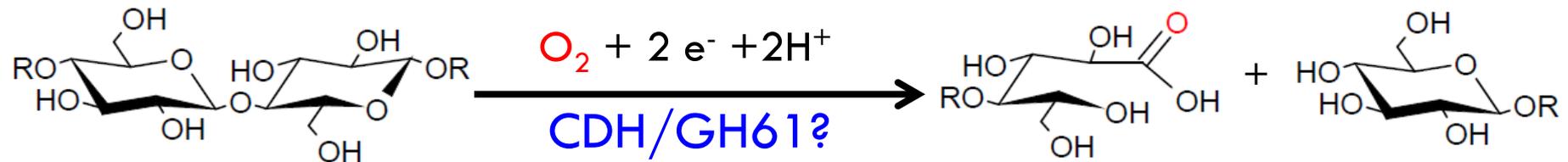
Lessons from chitin degradation

Chitin degrading bacteria



Vaae-Kolstad G., *et al.* Science (2010).

Cellulose degrading fungi?



Pure *N. crassa* GH61 proteins

MW

70

60

50

40

30

25

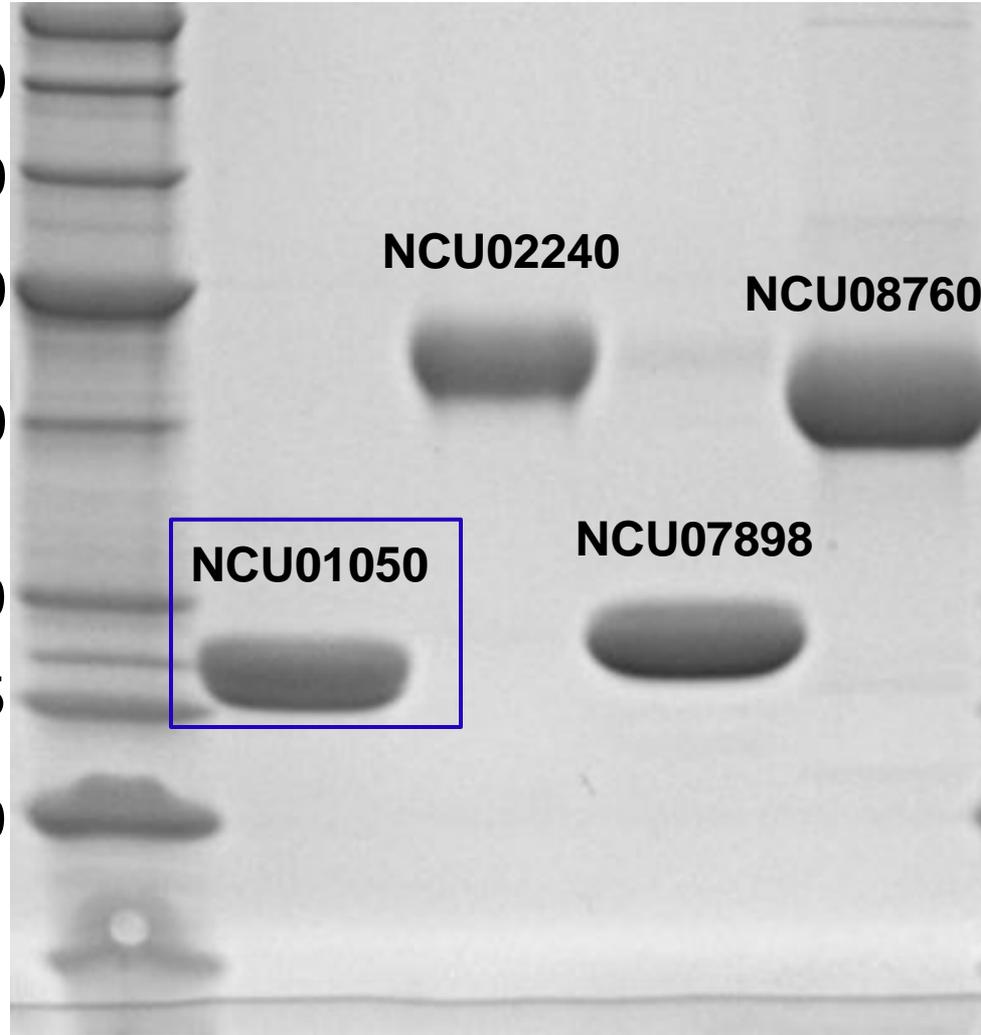
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NCU02240

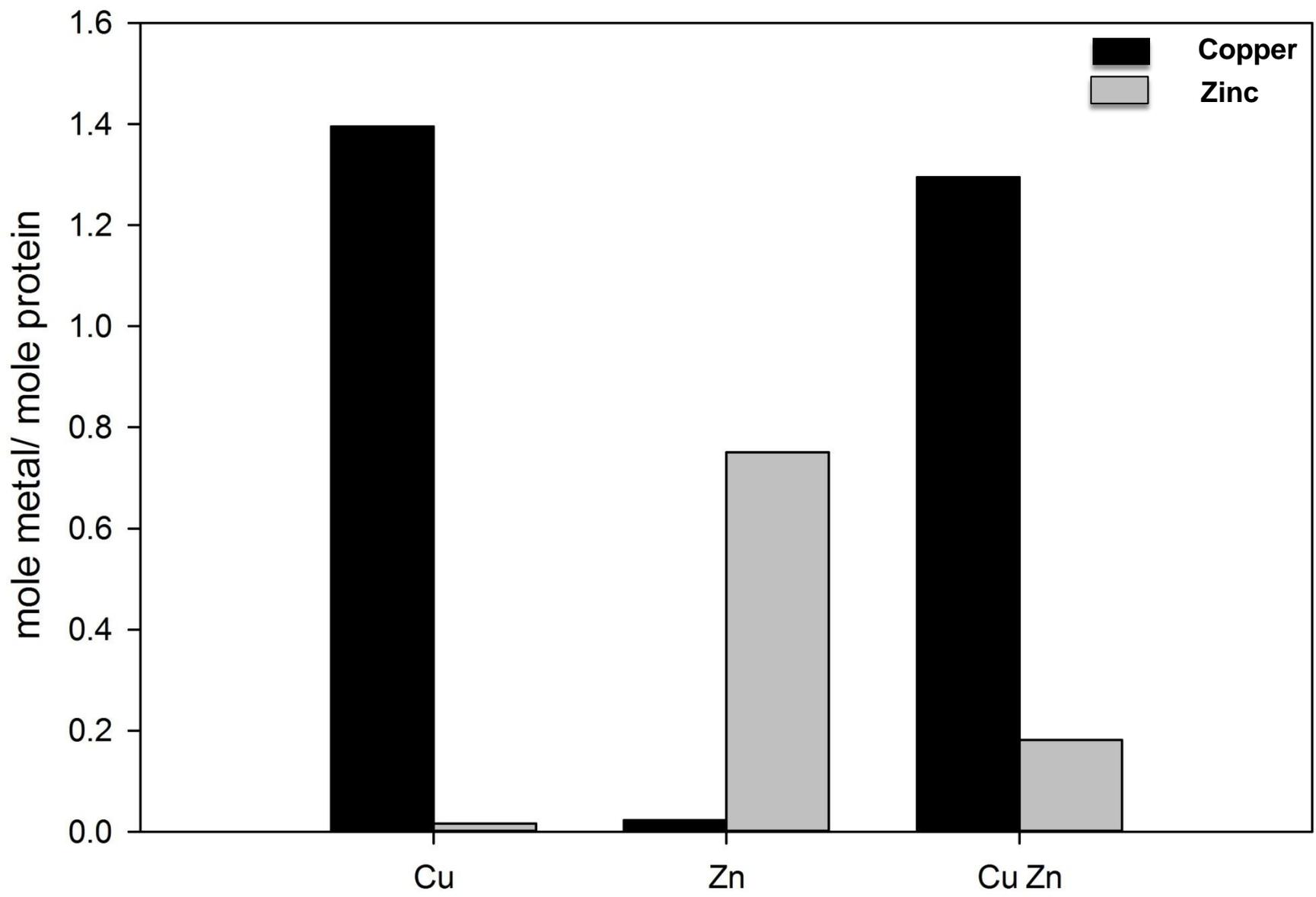
NCU08760

NCU01050

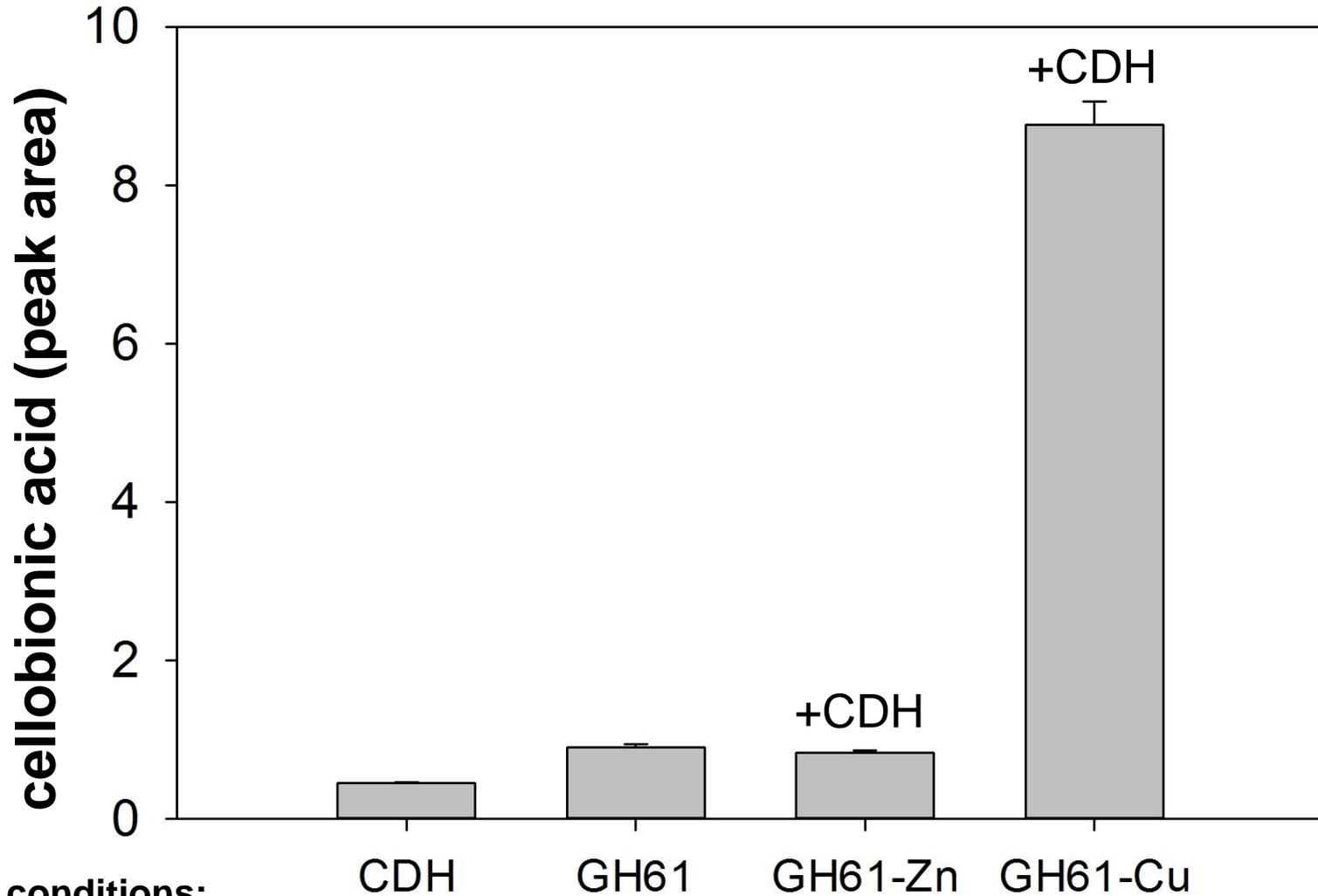
NCU07898



Purified GH61 NCU01050 prefers Cu to Zn



Copper-bound GH61 increases CDH activity



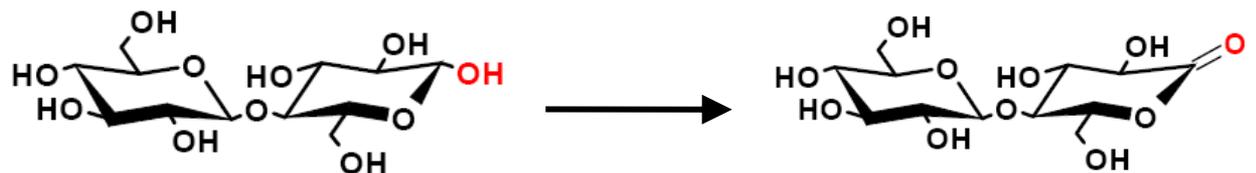
Assay conditions:

1 mM Cellobiose

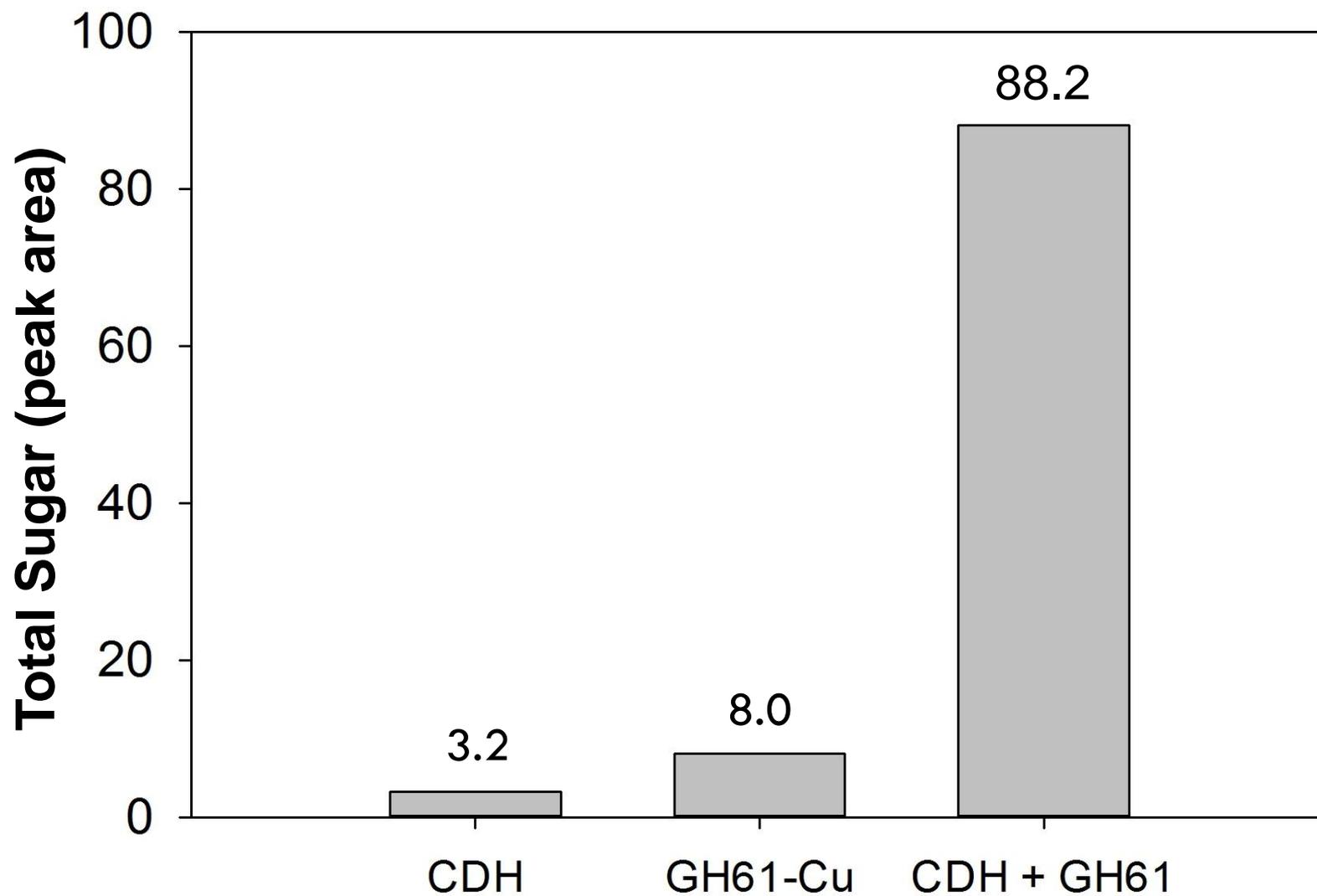
0.1 mg/mL CDH-2

50 mM acetate pH 5.0, 25 °C

30 minutes, triplicate

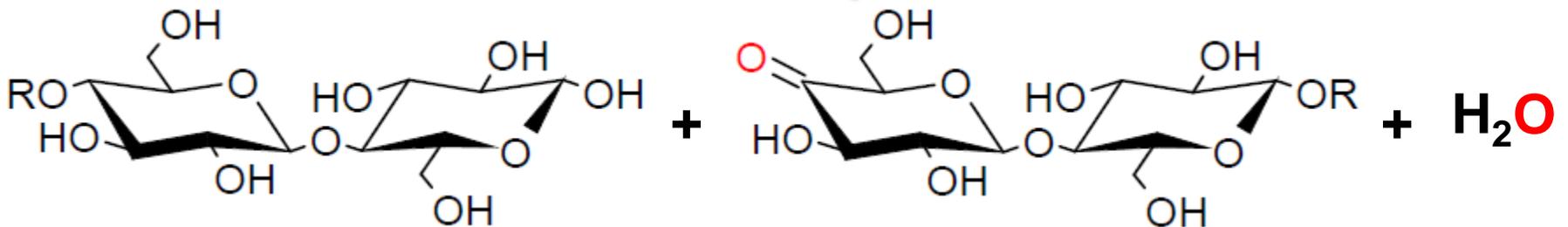
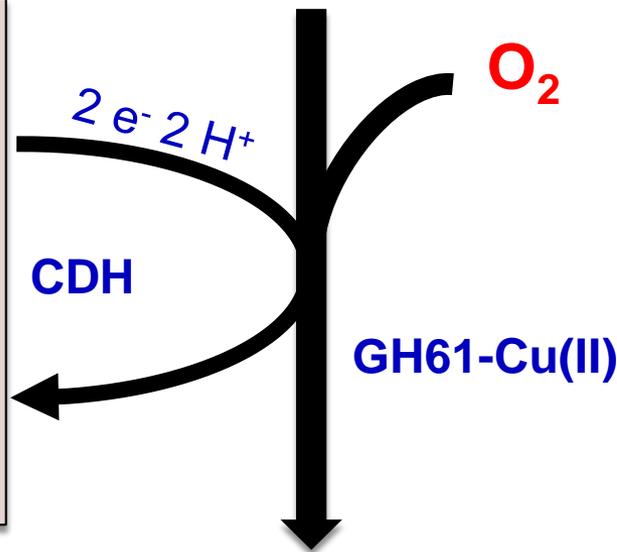
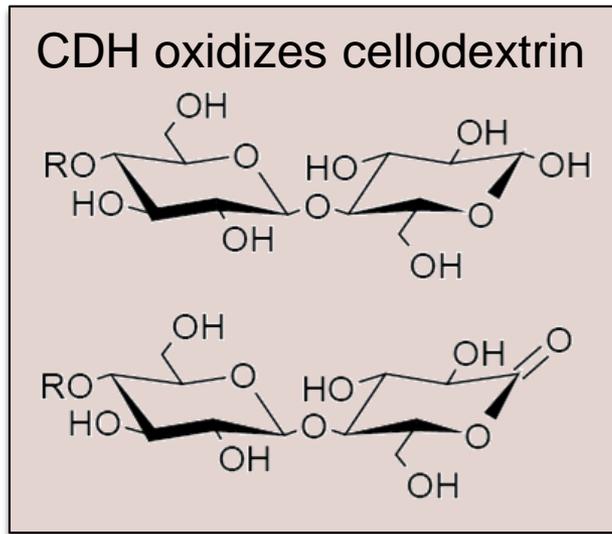
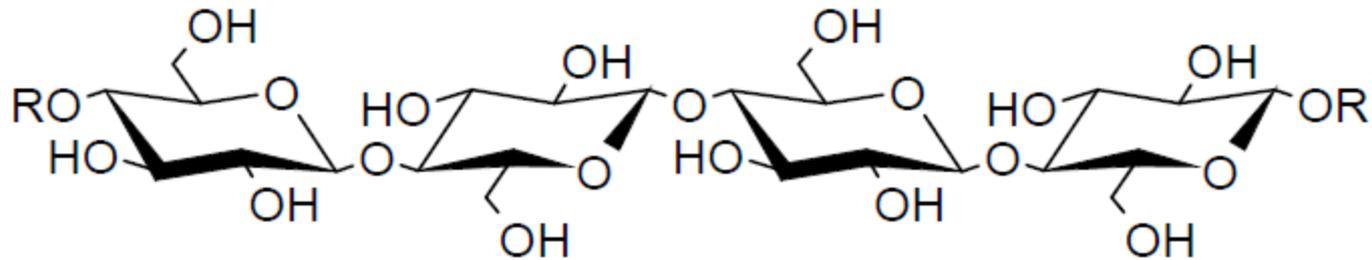


Degradation of cellulose* by GH61 and CDH

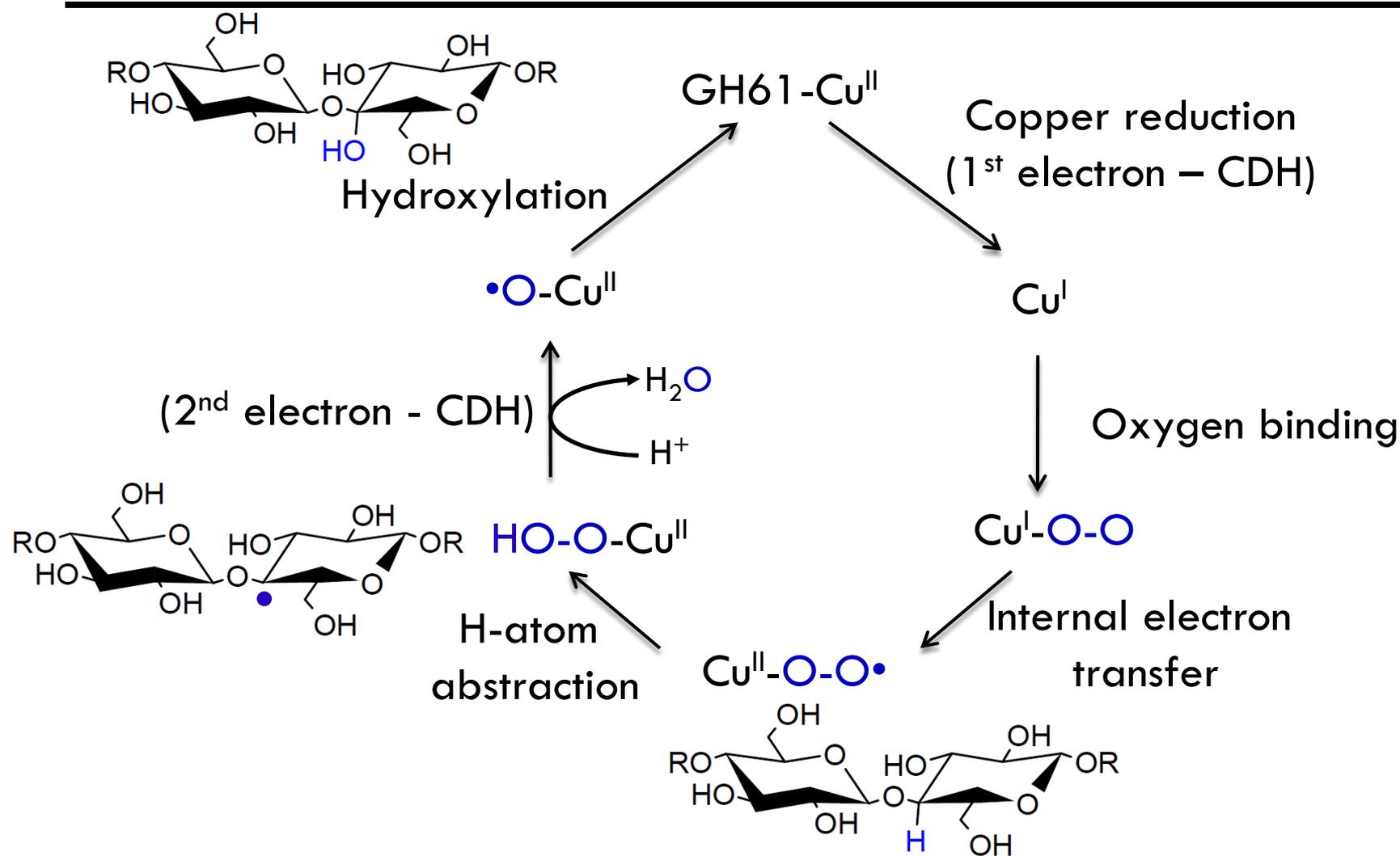
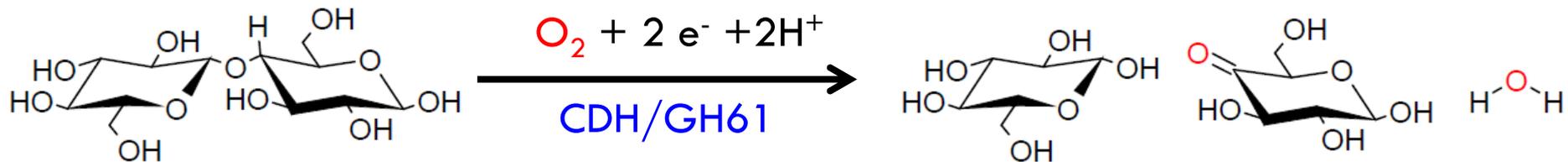


*Phosphoric acid swollen cellulose, PASC

Hypothesis for oxidative cleavage of cellulose



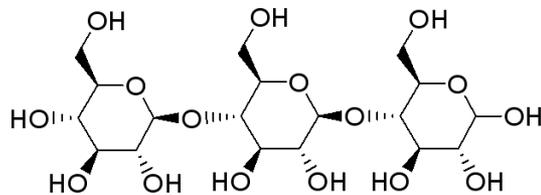
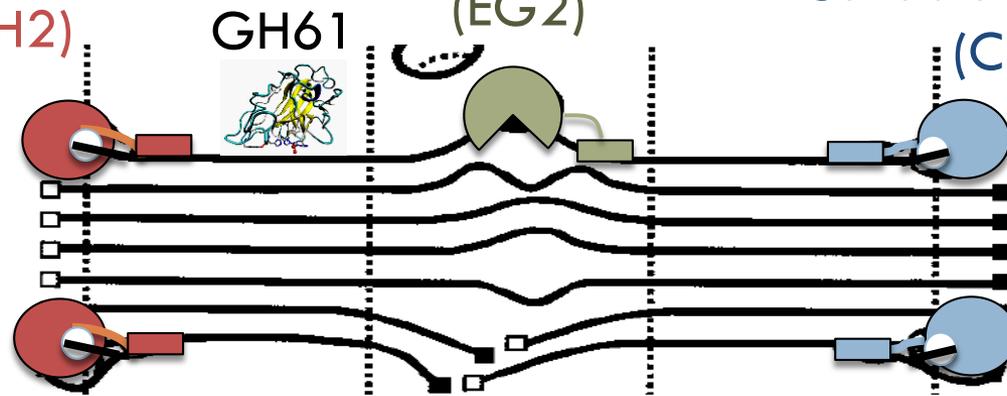
Possible mechanism of GH61 proteins



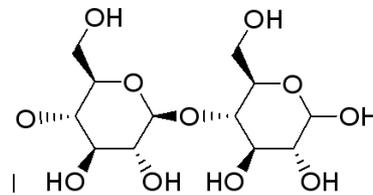
The core cellulases require exposed glucan chains, but GH61s would not

Neurospora crassa cellulase system

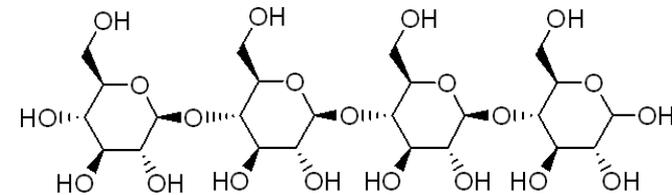
Cellobiohydrolase 2 (CBH2) Endoglucanase 2 (EG2) Cellobiohydrolase 1 (CBH1)



Cellotriose



Cellobiose

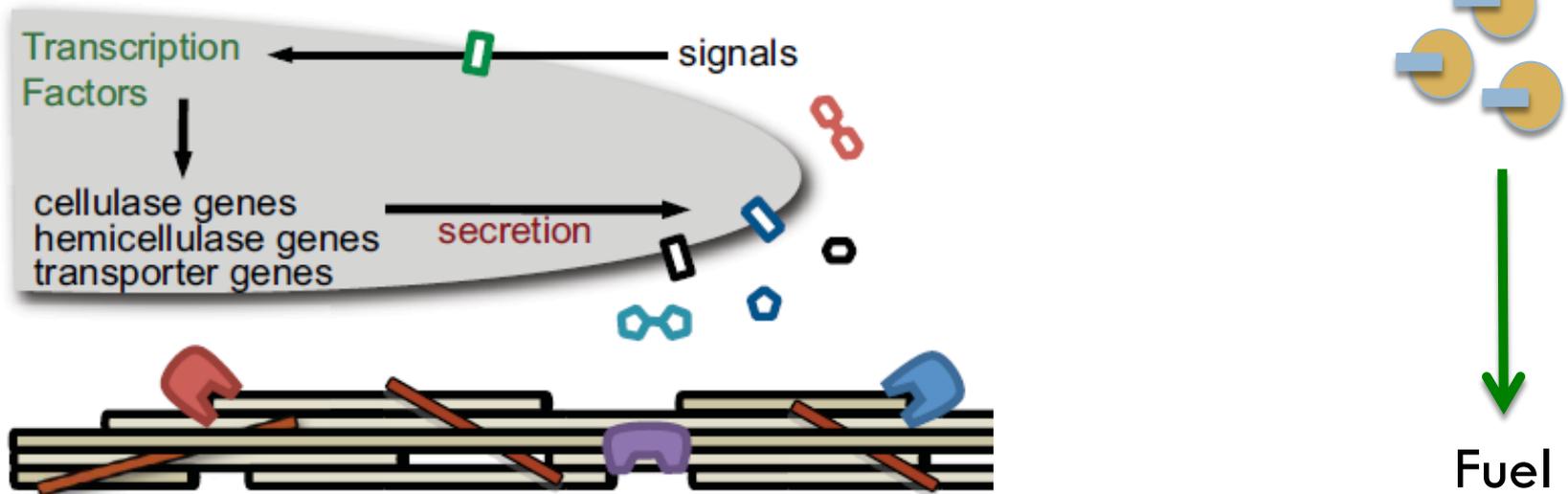


Cellotetraose

Summary: From model organisms to practical applications

- Cellodextrin transport plays a key role in fungal degradation of biomass.
- Cellodextrin transport can be integrated into yeast for biofuel production. Particularly useful for co-fermentation.
- Fungi use CDH/GH61 pairs to oxidatively cleave glucan chains in cellulose to enhance cellulase activity.

Induction



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Energy
Biosciences
Institute

Acknowledgements



Will Beeson on top of some highly recalcitrant biomass



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Cellodextrin Transport Improves Co-Fermentation

Table S4. Cofermentation of glucose, cellobiose, and xylose (10 g/L, 80 g/L, and 40 g/L, respectively) using bioreactor by DA24-16BT3 strain using different inoculums

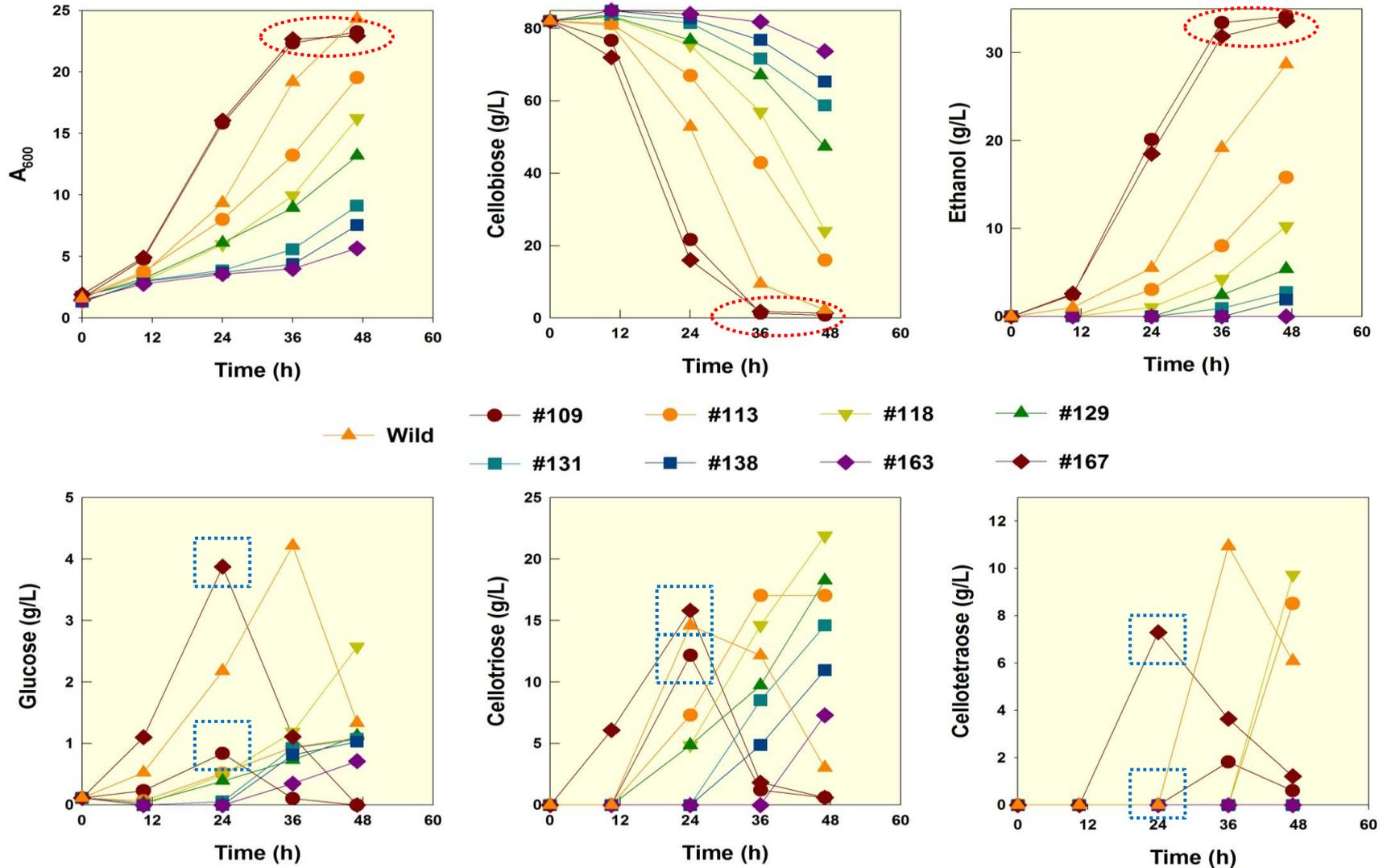
| Target initial OD (A_{600}) | Produced ethanol (g/L) | Sugar consumption rate (g/L·h) | Ethanol yield (g/g) | Productivity (g/L·h) |
|---------------------------------|------------------------|--------------------------------|---------------------|----------------------|
| ~1 (1.2) | 47.9 | 1.93 | 0.37 | 0.71 |
| ~10 (10.2) | 48.1 | 2.18 | 0.37 | 0.82 |

Alanine mutated CDT-1 fermentation properties

- In order to evaluate each ALA mutated CDT-1, 2 of fast growers, 2 of middle growers, 2 of slow growers, and 2 of no growers were selected.
- They were introduced into BY4742 with gh1-1.
- After pre-culture in YPC20 medium, fermentations were started in YPC80 medium.

| | Well number | Well number |
|-----------|-------------|-------------|
| Fast | 109 (G91A) | 167 (F335A) |
| Middle | 113 (Q104A) | 129 (F170A) |
| Slow | 131 (R174A) | 138 (E194A) |
| No growth | 118 (G126A) | 163 (Y313A) |

Alanine mutated CDT-1 fermentation properties



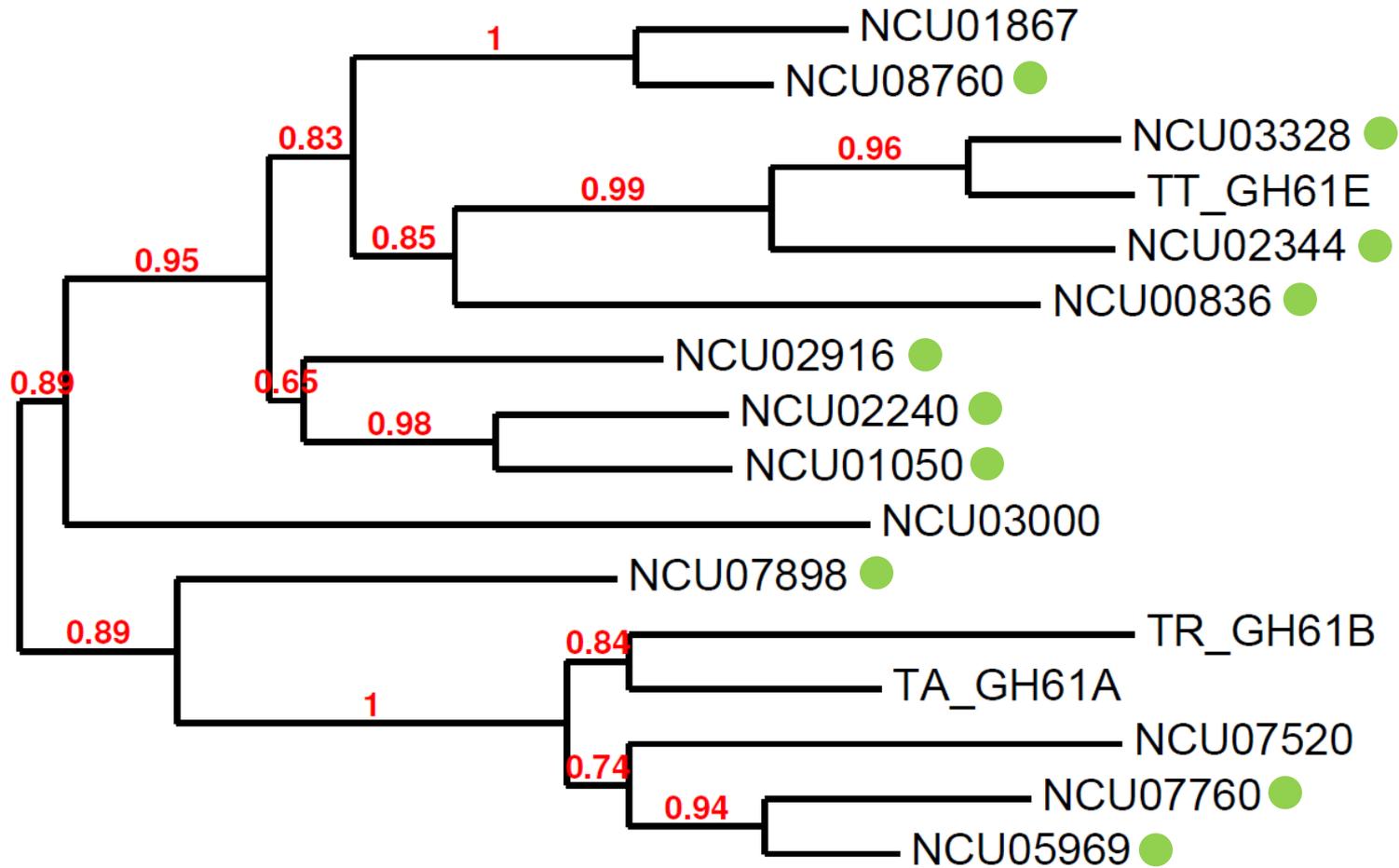
CDT-1 kinetic parameters (I will convert into bar graph)

| | K_M (μM) | V_{max} (pmol/s, norm. to 2×10^8 cells) | V_{max} (pmol/s, norm. for GFP fluo.) |
|-------|-------------------------|---|--|
| WT | 7.6 ± 1.5 | 0.60 ± 0.03 | 0.86 ± 0.05 |
| F335A | 114.8 ± 46.4 | 1.64 ± 0.24 | 1.75 ± 0.26 |
| G91A | 43.5 ± 10.1 | 1.51 ± 0.10 | 1.87 ± 0.12 |

The perennial grass, *Miscanthus*



GH61s upregulated during growth on cellulose



● >2 fold upregulated