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Consolidated bioprocessing – the realities

Synthesis of three advanced biofuels from ionic liquid-pretreated switchgrass using engineered *Escherichia coli*

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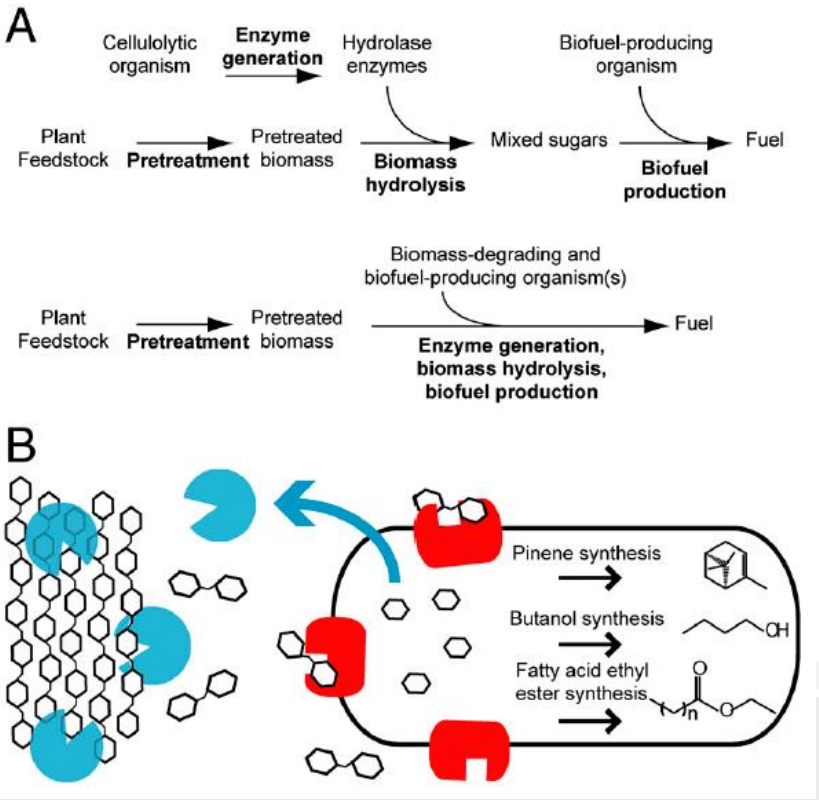
- Jay Keasling is the golden child of US industrial biotechnology and biofuels.
- Made his name by getting large Gates grants on making artemisinin in yeast (which we covered in Mol Biotech)
- He is at UC Berkeley.

The pitch…

- Starts off selling *E. coli* as a host, the high costs of enzymes in using cellulosic feedstocks, and his vision of consolidated bioprocessing.



- A cellulolytic strain of *E. coli* would be desirable, but doesn't exist, partly due to poor protein secretion.
- Need to secreted about 1000-fold better to get enough enzyme out there.

Fig. 1. Consolidated bioprocessing of plant biomass into biofuels by *E. coli*. (*A*) Two processes for biofuel production. Typically, cellulase and hemicellulase enzymes are produced in a process step separate from biomass hydrolysis and biofuel production (top). Consolidated bioprocessing (bottom) combines enzyme generation, biomass hydrolysis, and biofuel production into a single stage. (*B*) Engineering *E. coli* for use in consolidated bioprocessing. Cellulose and hemicellulose are hydrolyzed by secreted cellulase and hemicellulose enzymes (cyan) into soluble oligosaccharides. β-glucosidase enzymes (red) further hydrolyze the oligosaccharides into monosaccharides, which are metabolized into biofuels via heterologous pathways.

Getting secreted cellulases & hemicellulases

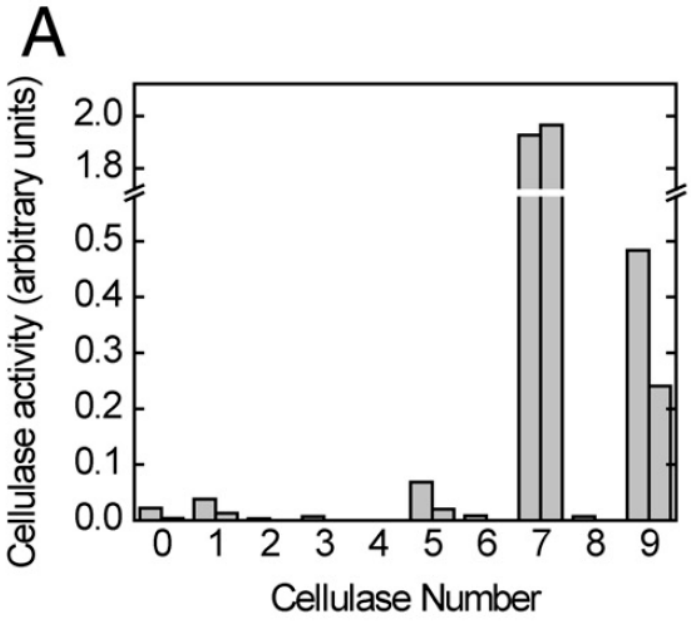
They has previous shown that the *Clostridium stercorarium* endoxylanase Xyn10B can be produced extracellularly by *E. coli* when fused with the protein OsmY.

- Screened 10 GH9-family cellulases with OsmY fusions and measured the amount of activity in the supernatent.

- Used a spectrophotometric assay with azo-CMC assay.

- Enzyme chops the CMC into small pieces, which stay in solution after a chemical precipitation step at the end to remove remaining substrate.

- The small pieces with the azo-dye can be detected by measuring A590.



The Cel enzyme from Bacillus sp. D04 (cellulase #7)was the best.

Short oligosaccharides are made

- Using their purified enzymes, Cel and Xyn10B, they demonstrate that IL-treated switchgrass can be partially degraded.
- Took a small amount of cell extract from cells making either enzyme.
- "OsmYCel released glucose equivalent to 5% of the cellulose, producing cellotriose and cellobiose, while OsmY-Xyn10B hydrolyzed 11% of the xylan, mostly into xylotriose and xylobiose (Fig. S2)."
- Only released 8% of the total sugars available.

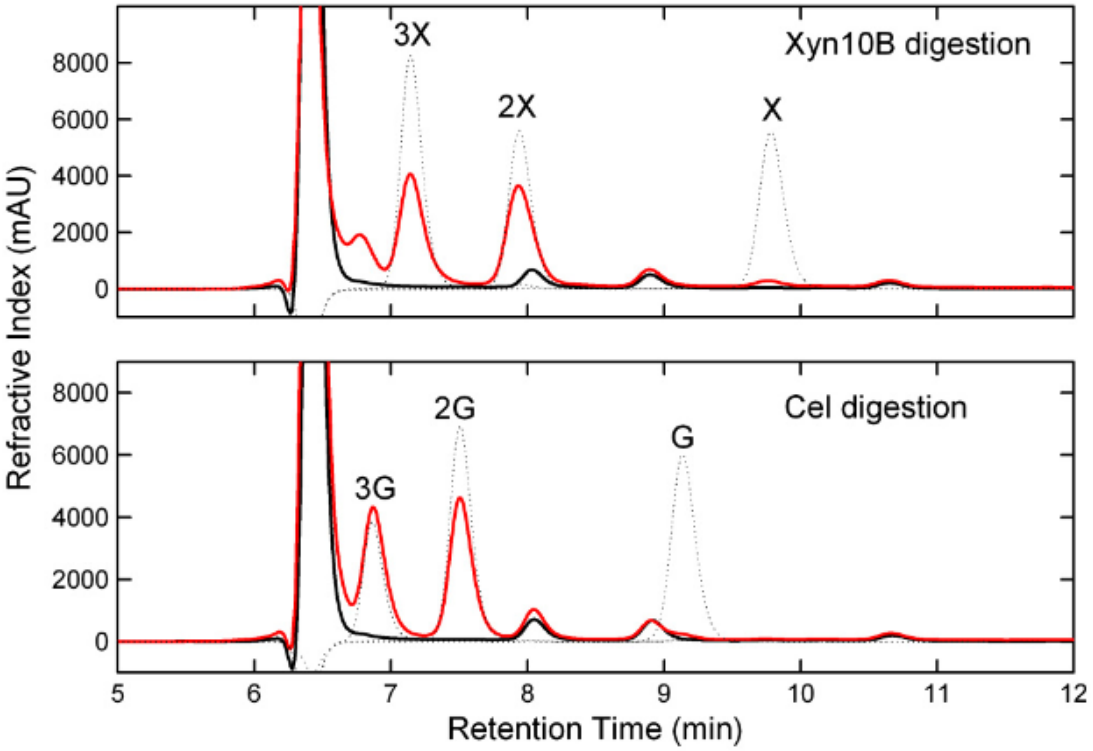
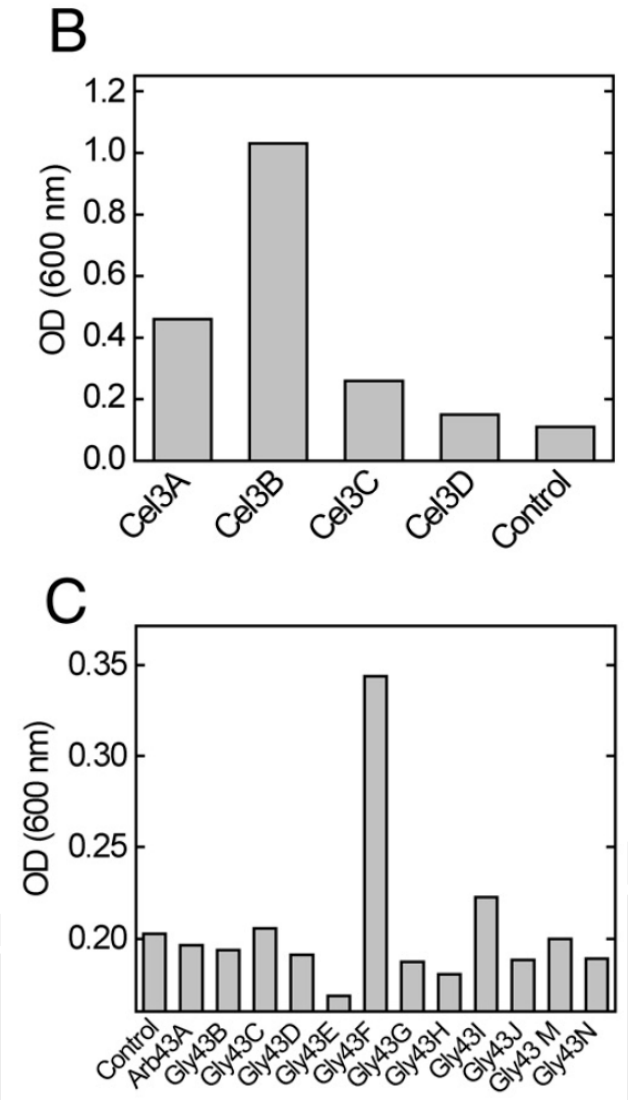


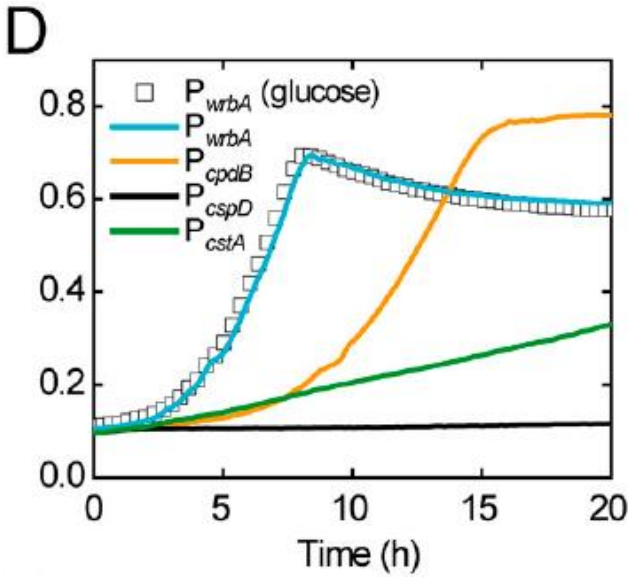
Fig. S2. The endocellulase Cel and the endoxylanase Xyn10B both hydrolyse IL-treated switchgrass. 10 mL MOPS-M9 with 3.4% IL-treated switchgrass was treated with either OsmY-Cel or OsmY-Xyn10B at 37 °C with agitation for 4 days. Soluble sugars released by hydrolysis were detected using HPLC. Chromatogram traces of the digestion reaction with OsmY-Xyn10B (top) or OsmY-Cel (bottom) with predigestion (black) and postdigestion (red) traces are shown. Peaks of standards are shown as dashed traces. Peaks indicated are xylotriose (3X), xylobiose (2X), and xylose (X), cellotriose (3G), cellobiose (2G), and glucose (G).

Getting *E. coli* to grow on oligosaccharides

- *E. coli* won't grow on these oligosaccharides, so need to break them down to monosaccharides.
- Screened four β-glucosidases cloned from *Cellvibrio japonicus*, a Gram-negative cellulolytic bacterium, and looked to see if this enabled growth on cellobiose. Cel3B was the best (Fig. 2B).
- Screened 12 xylobiosidase genes from C. japonicus and Gly43F enabled growth on enzymatically hydrolyzed beechwood xylan (Fig. 2C).
- **Fig. 1B is very poor** in showing where enzymes are located in the cell. If they are on the inside then how are the substrates getting in?

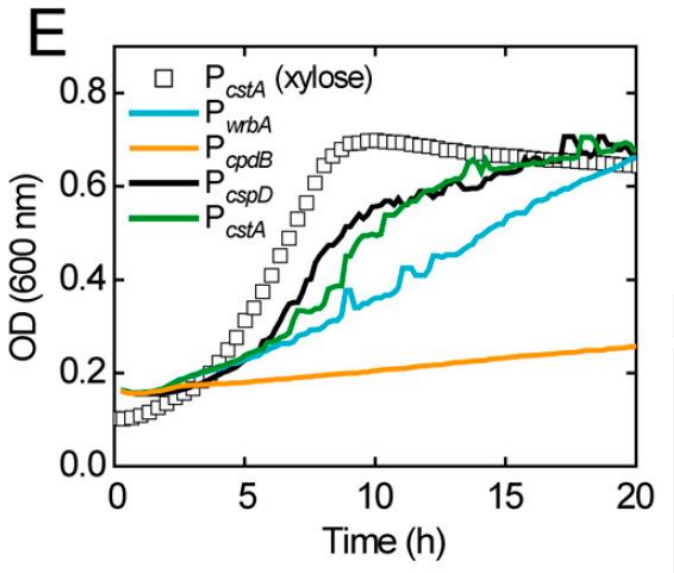


Switching to a native promoter



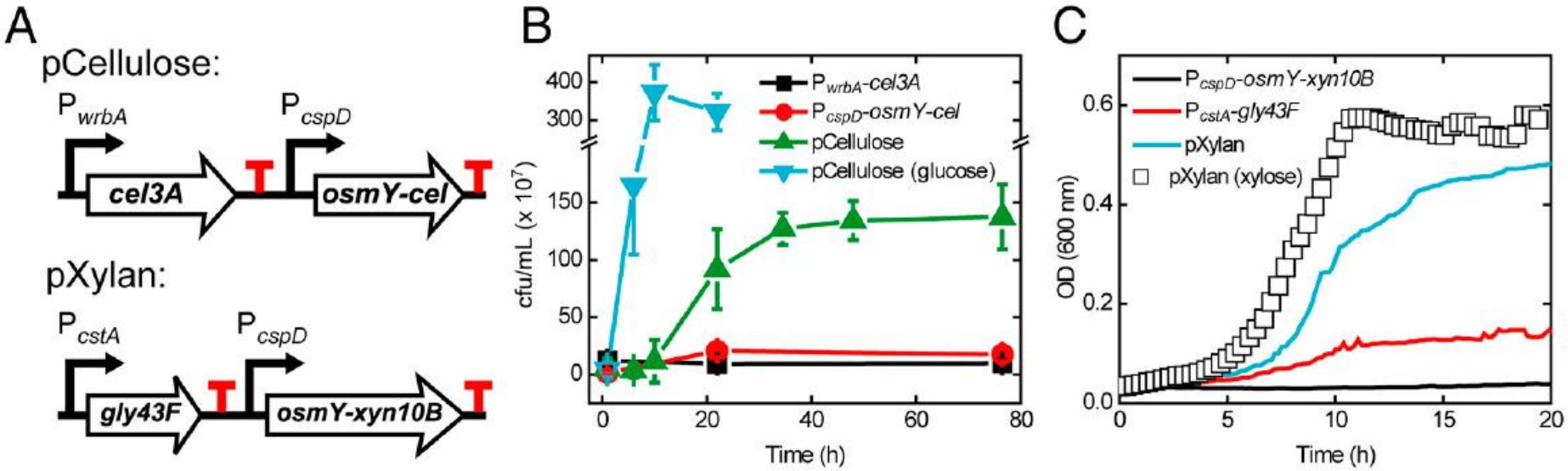
- Screened a number of *E. coli* promoters to work upstream of *cel3A*.
- One of these, *wrbA*, gives growth rates nearly the same on cellobiose as on glucose (and there is no added cellobiose transporter, so must be using a glucose transporter OK – he never considers transport!)

- Did the same with the xylosidase *gly43F*, but here other promoters were better, while none gave a good as growth as on free xylose.
- Although this is 'synthetic biology', it is all **highly empirical** and shows that we don't understand the relationship between promoter strength and final activity.



Synthetic clusters for cellulose and hemicellulose utilisation

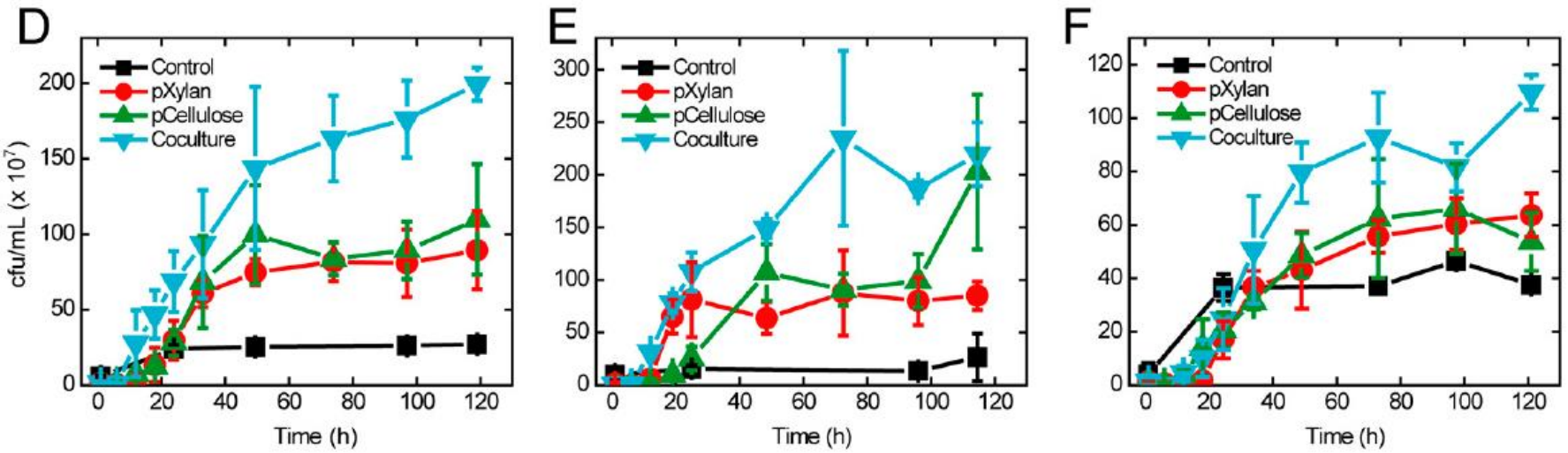
- Assembled their respective enzyme/promoter combination on a plasmid. Note that transcriptional terminators are added to insulate each gene (Fig. 3A).



- See growth on phosphoric acid swollen cellulose (PASC) with pCellulose (Fig. 3B). **Why do you think they have stopped measuring OD650?**
- See much faster growth with pXylan in beechwood xylan (Fig. 3C). **Why is growth better on xylan than cellulose?**

What's it like on a real substrate?

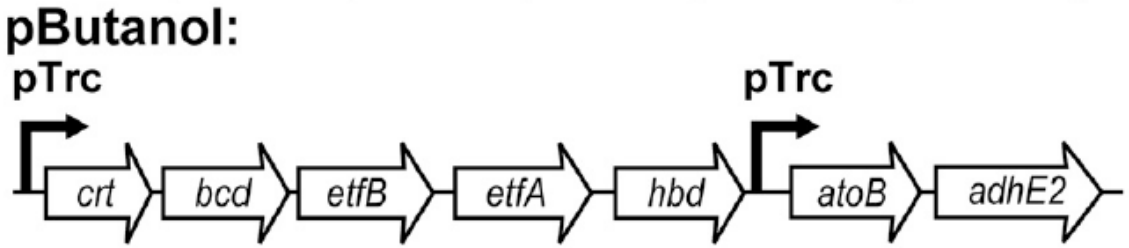
- Tried their bugs on some real substrates, the IL treated switchgrass, eucalyptus, and 'yard waste'.
- When they use co-culture they see twice the yield, suggesting both accessing different carbon sources (hexoses and pentoses).



- Is this really any good?
- Maybe they should have thought about transporters!

Connecting this to biobutanol

- Finally tried to show they could couple grow on a cellulosic feedstock to actually making a biofuel.
- They made a synthetic gene cluster for butanol production in *E. coli* Δ*adhE*.



"When bearing either pXylan or pCellulose, *E. coli* DH1 pButanol produced butanol from either xylan or cellobiose, respectively (Fig. S6B). A coculture of both strains yielded about 30 mg/L butanol from defined rich medium containing 3.3% w/vol IL-treated switchgrass as the main carbon source (Fig. 4C). "

Now remember that a wild-type *Clostridium* strain can easily get over 20 g/l, then this puts this into perspective!