Summer 6-6-2012

Pathway Engineering via Synthetic Biology

Huimin Zhao
Energy Biosciences Institute

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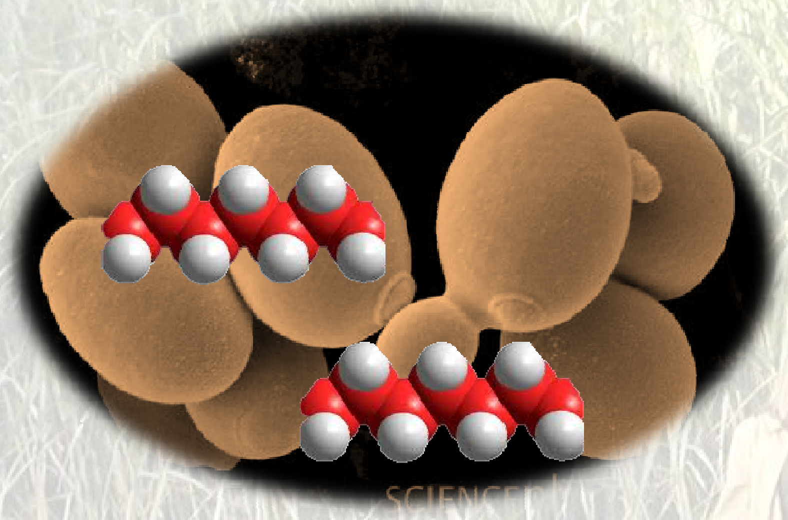


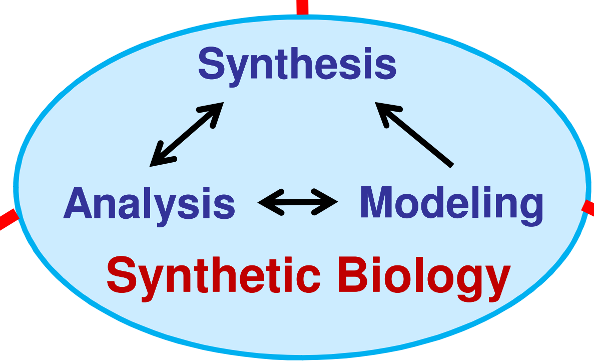
Energy Biosciences Institute

1867

ECI Metabolic Engineering Conference IX, Biarritz, France, June 6, 2012

Research Interests in Zhao Group

Pathways and Genomes
- Phloroglucinol biosynthesis
- FR900098 biosynthesis
- Xylitol biosynthesis
- Advanced biofuels
- Alkaloid biosynthesis



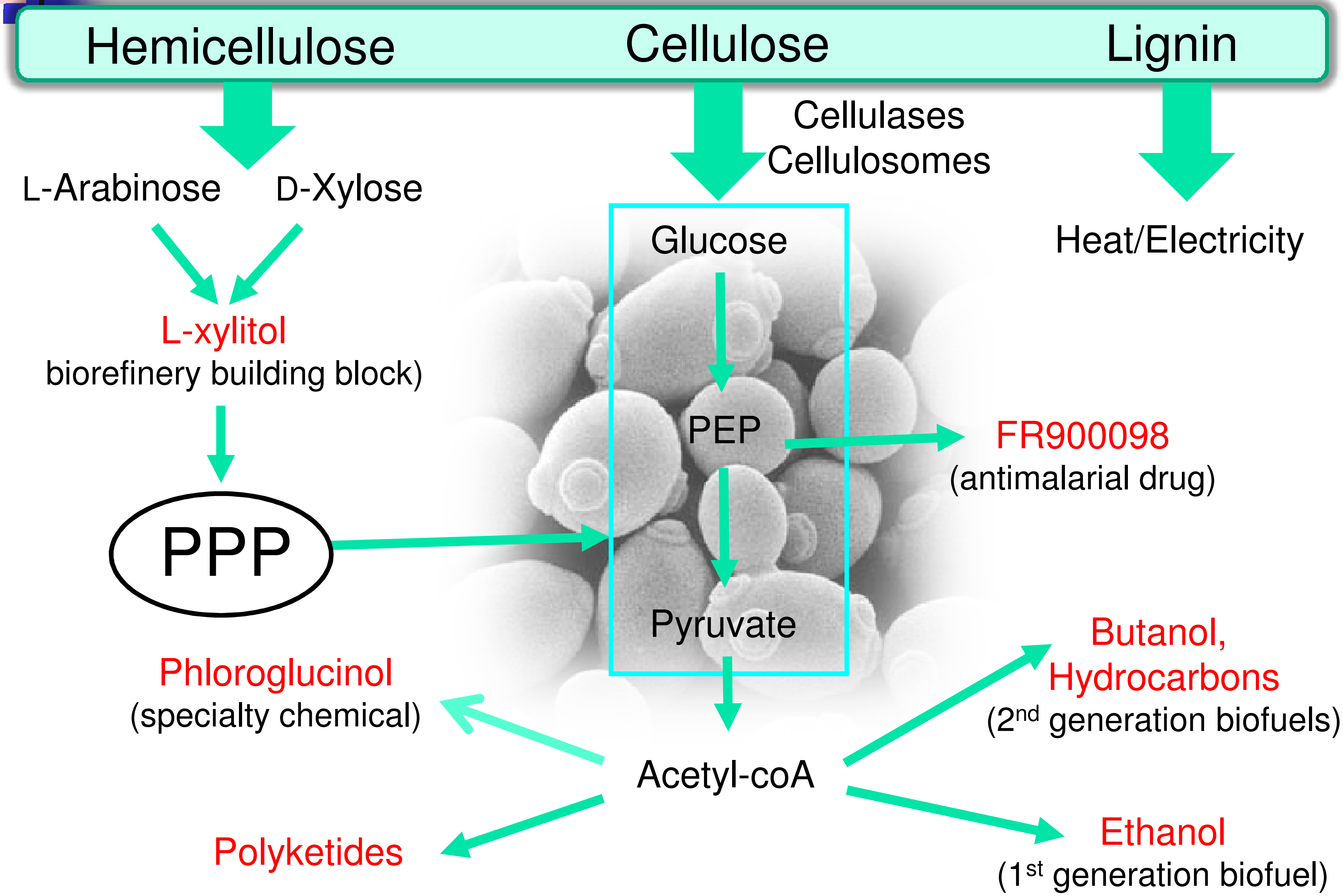
Proteins
- Gene switches/circuits
- Artificial nucleases
- P450 enzymes
- Type III PKS
- N-Oxygenases

Tool Development
- Directed evolution
- Rational design
- High throughput methods
- Biosynthesis
- Genome engineering

Grand Challenge #1 (Energy & Sustainability): *Urgent need for oil replacement*
→ Use renewable feedstocks to produce fuels, chemicals, and drugs

Grand Challenge #2 (Health): *Need for new therapeutics*

#1. Engineering Microbial Factories (Fuels and Natural Products @ UIUC)



Metabolic Engineering Research Lab (MERL) @ Singapore

Biomass

2 → Sugars → 1

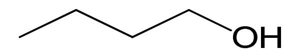
Biomass utilization

Biochemical production

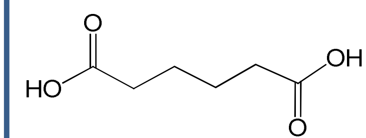
Metabolic Engineering

↑ 3

Tool Development
- Computational
- Genetic manipulation



n-butanol



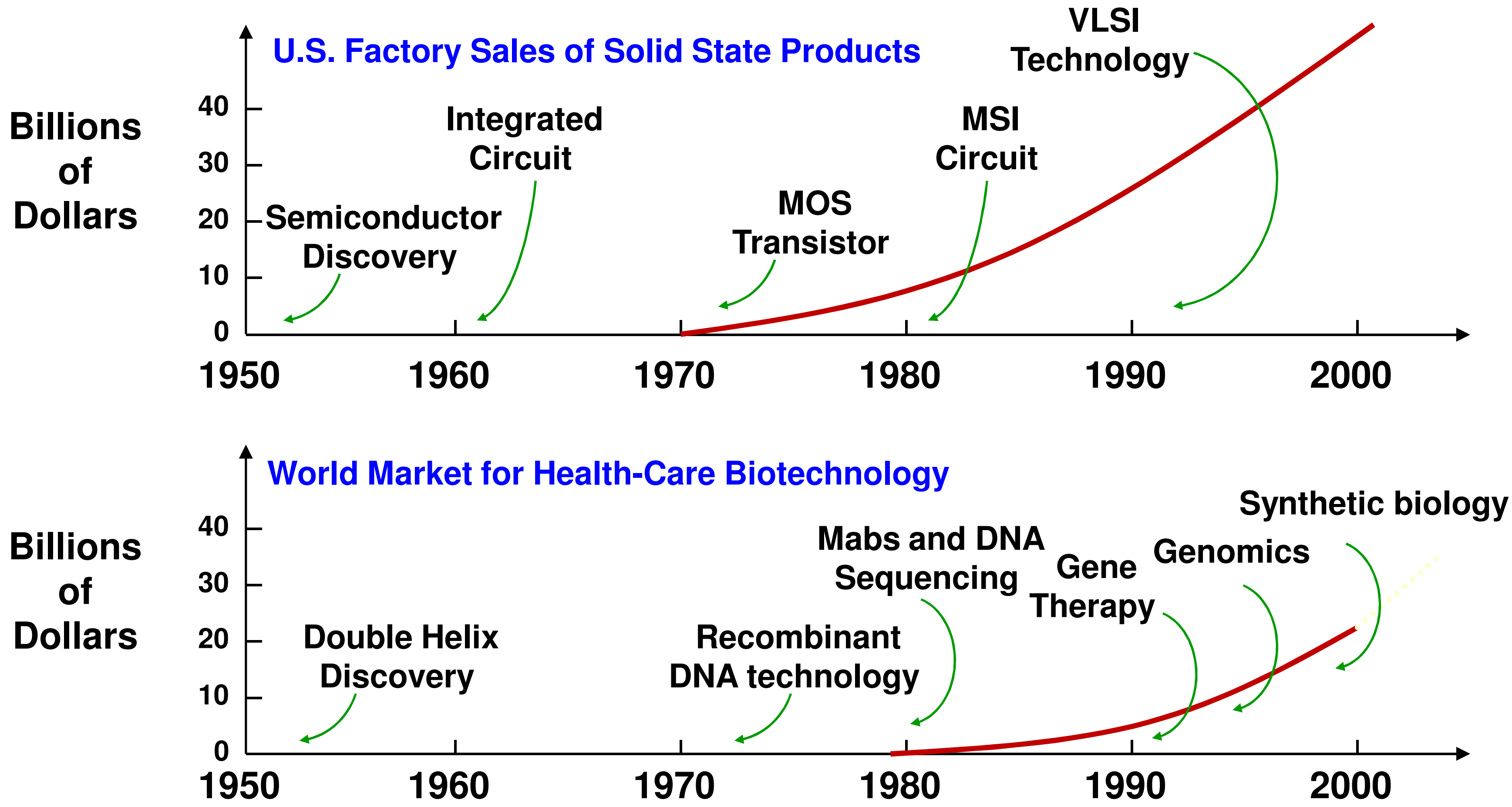
Adipic acid

&

Others

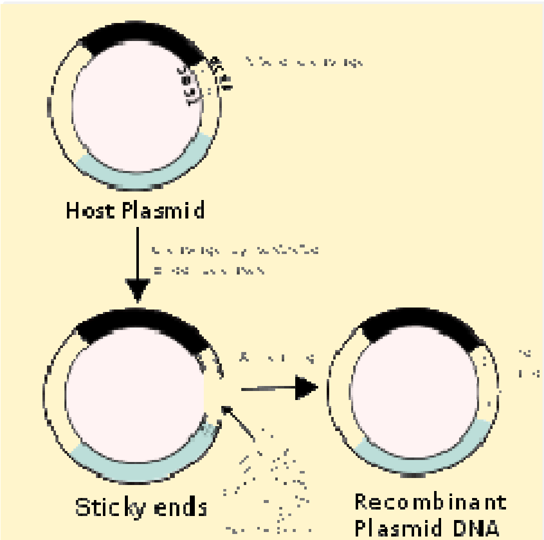
Overall Goal: Develop and apply systems and synthetic biology approaches to engineer microorganisms capable of cost-effectively producing industrial chemicals from renewable feedstocks.

Microelectronics and Biotechnology

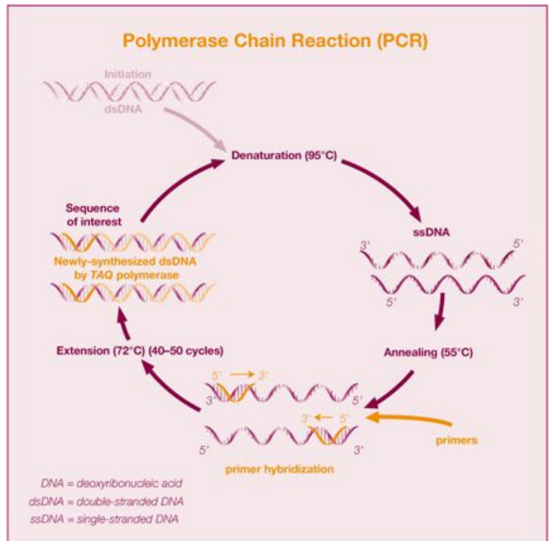


Tools Driving Biotechnology

First Generation Biotech

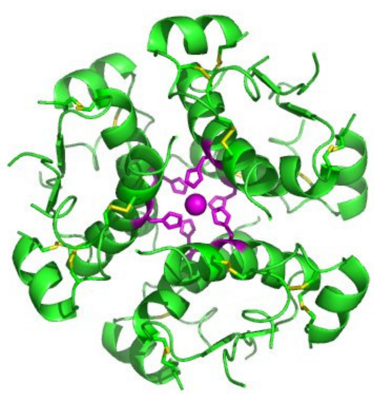


Recombinant DNA Technology
"Cut and paste"



PCR
"DNA Amplification"

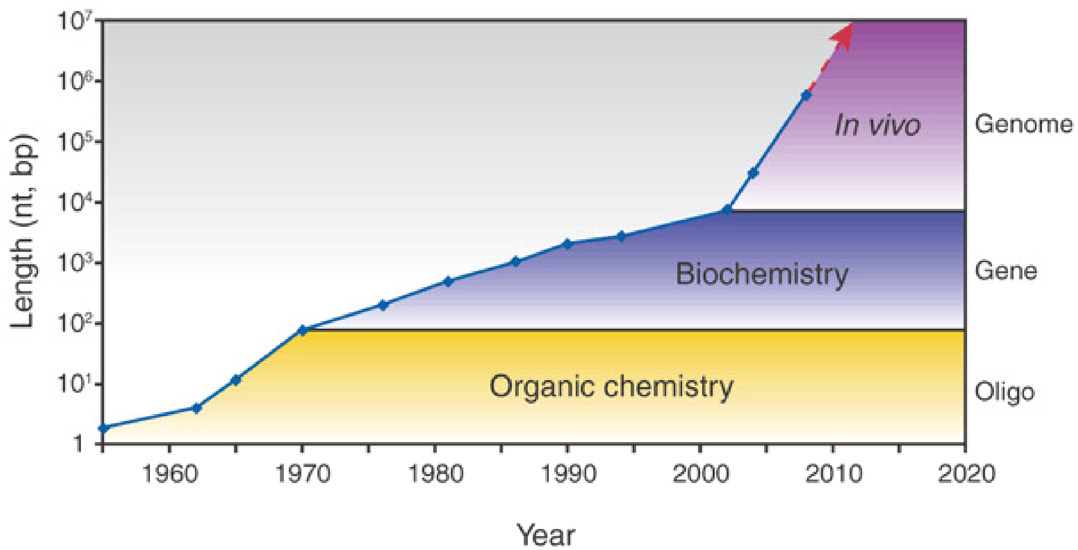

DNA Sequencing
"Read out the genetic code"

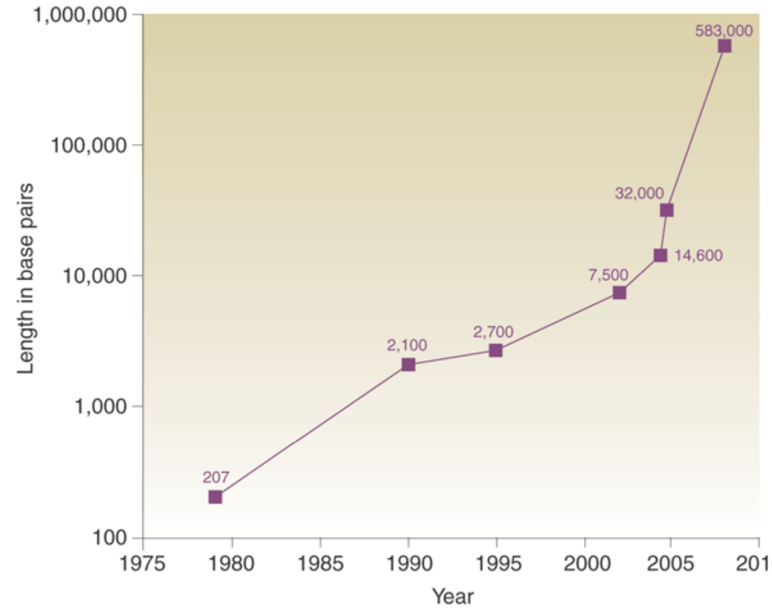

human insulin

First product: human insulin, produced in *E. coli* in 1978.
- Recombinant human growth hormone
- Recombinant blood clotting factor VIII
- ……

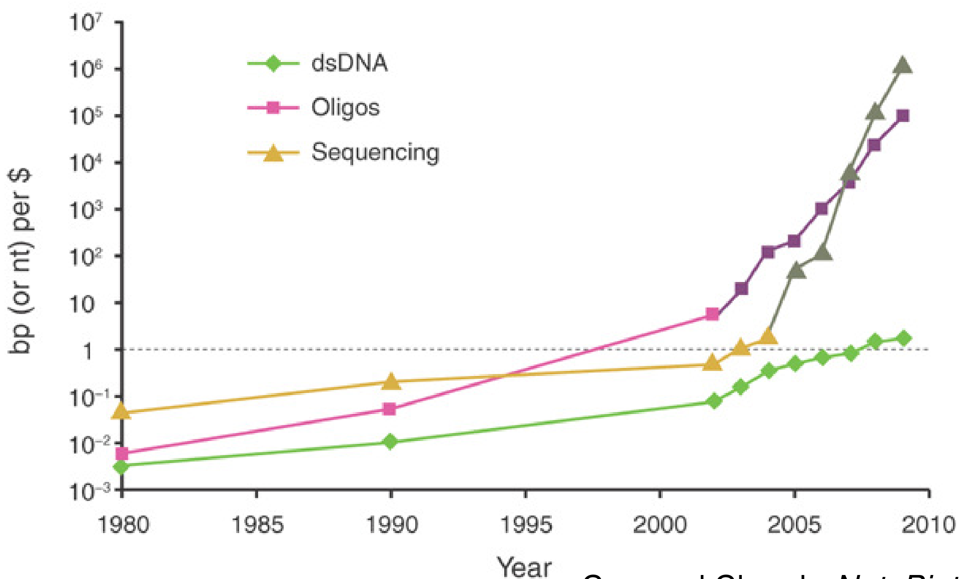
Global market size for recombinant proteins: ~$60B in 2009

Transformative Advances in DNA Sequencing and Synthesis





Carlson, *Nat. Biotech.* 27, 1091 (2009)



Carr and Church, *Nat. Biotech.* 27, 1151 (2009)

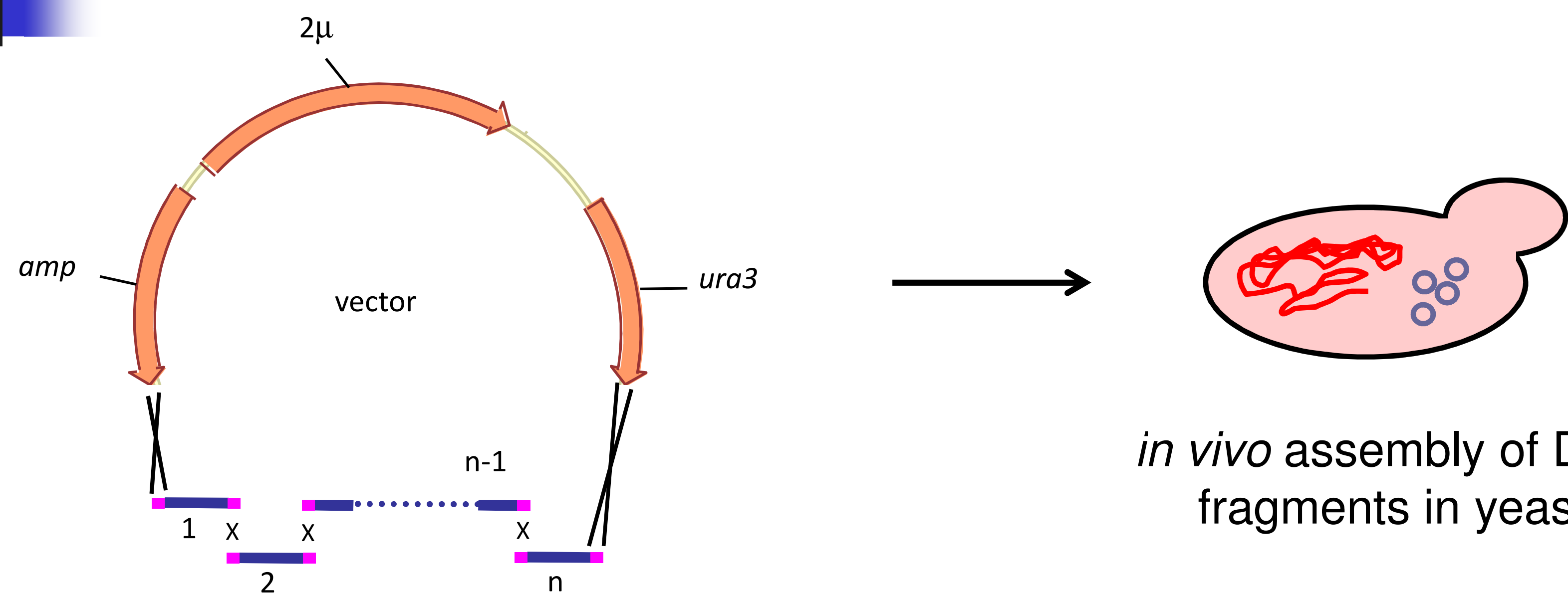
10-1000's genes

complex chemicals and materials

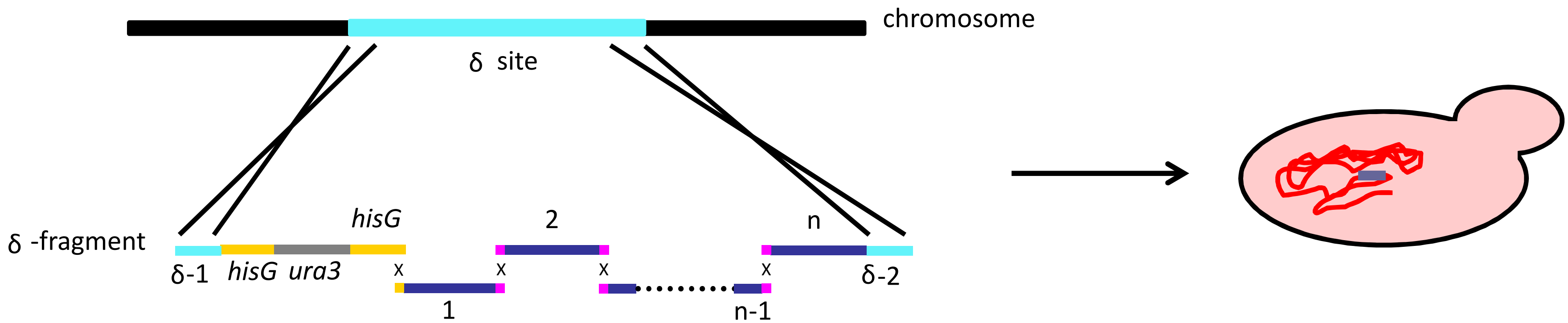
organisms as products

...

Building Large DNA Molecules via One-step DNA Assembler

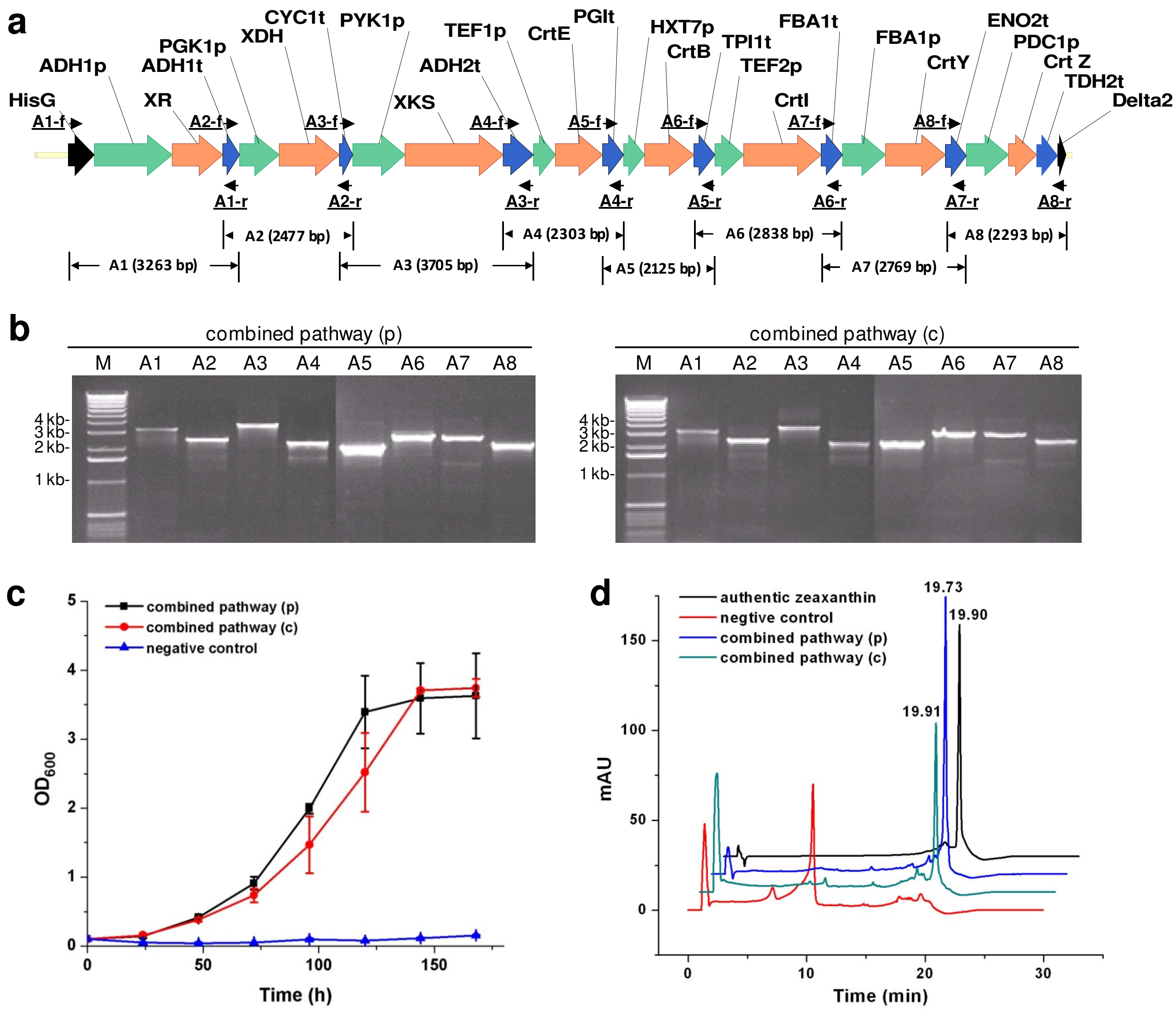


in vivo assembly of DNA fragments in yeast

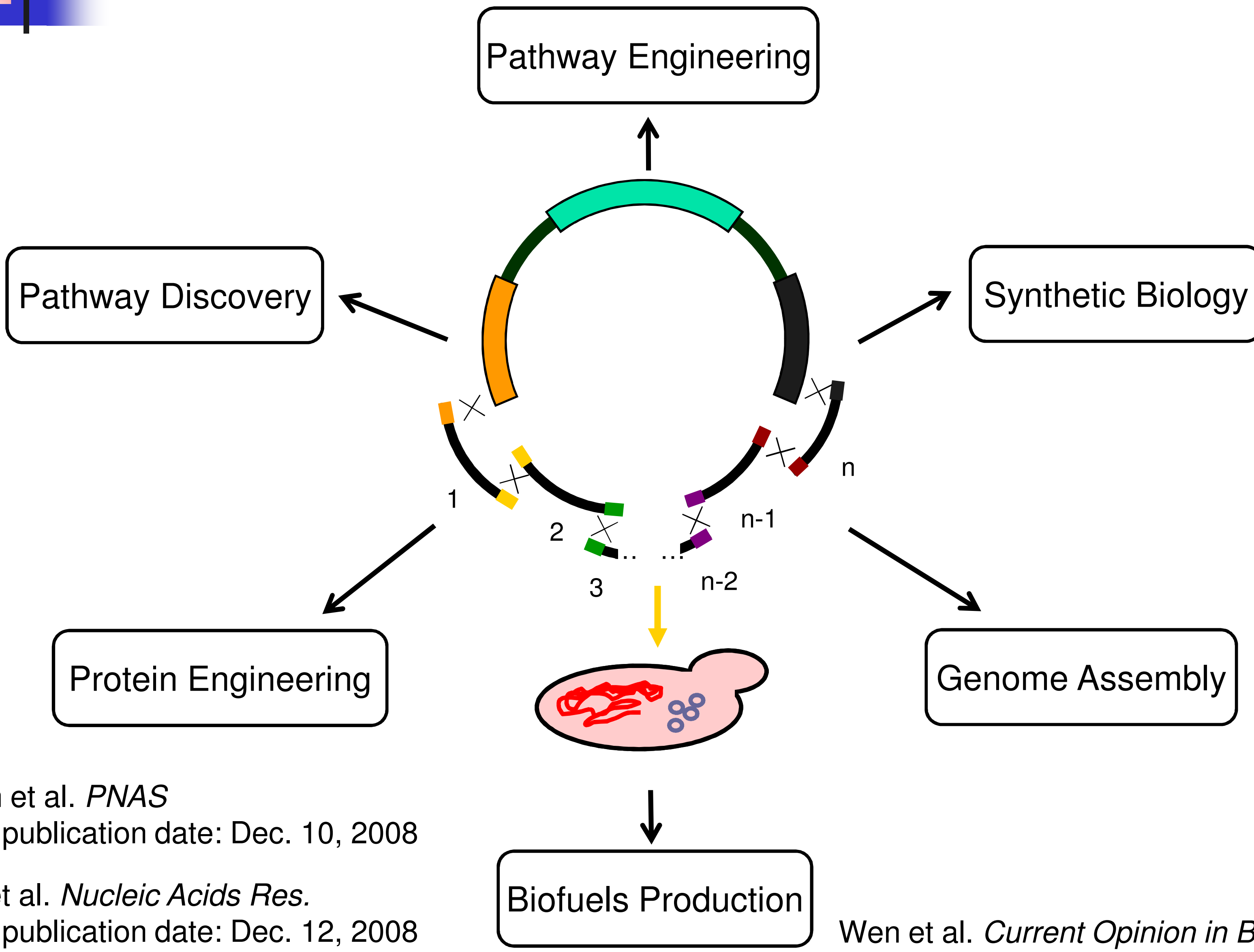


Shao et al. *Nucleic Acids Res.* (2009)

Eight-gene Pathway: A Combined Xylose and Zeaxanthin Pathway



Broad Application of DNA Assembler

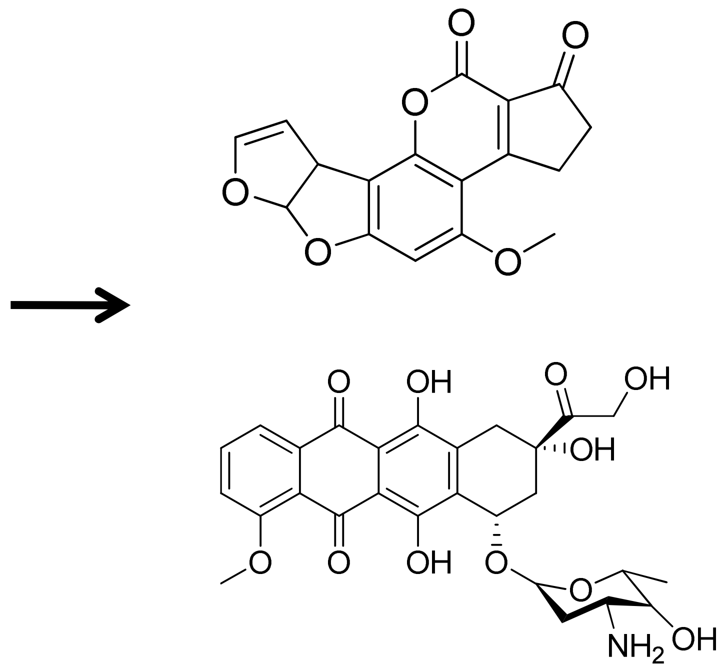


Gibson et al. *PNAS*
Online publication date: Dec. 10, 2008

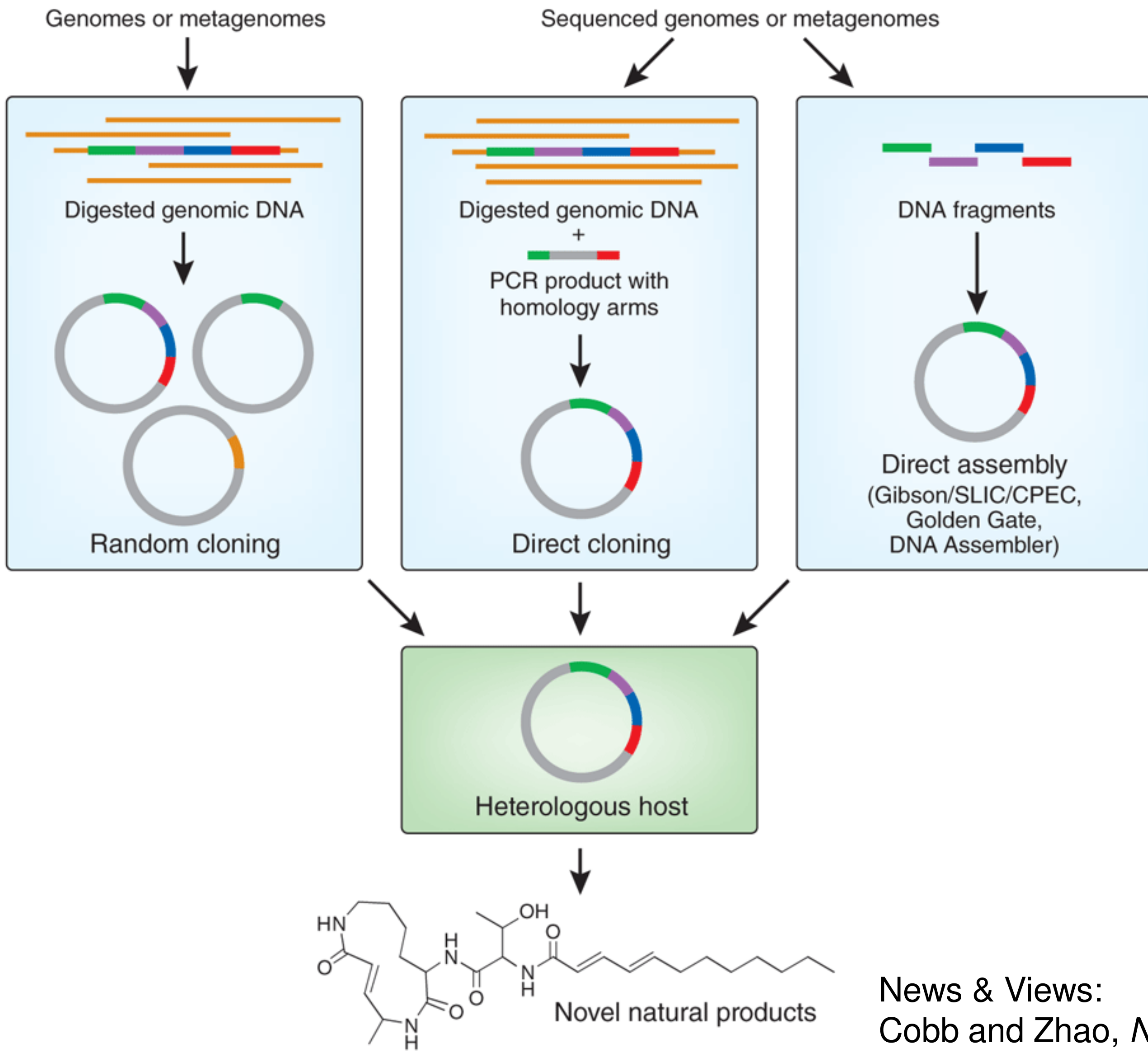
Shao et al. *Nucleic Acids Res.*
Online publication date: Dec. 12, 2008

Wen et al. *Current Opinion in Biotech*. (2009)

Discovering New Drugs



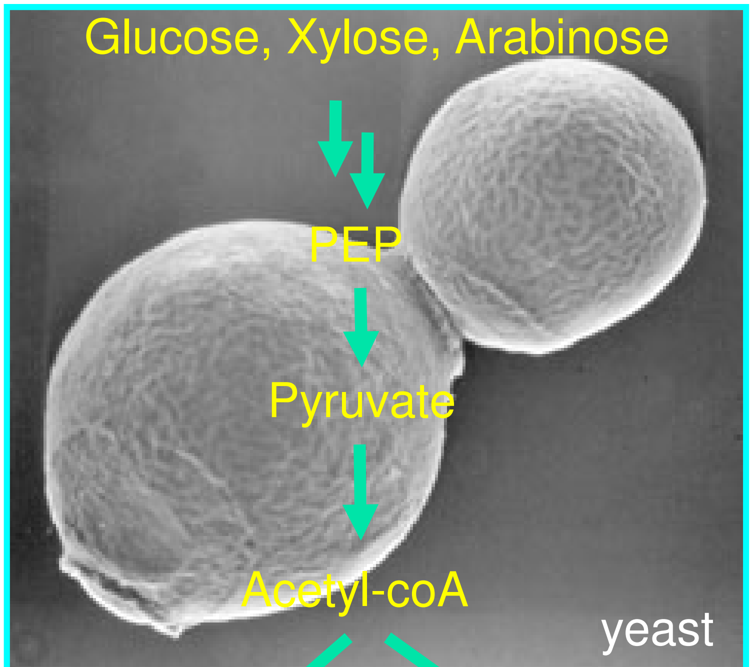
Activating Cryptic Pathways from Sequenced Genomes and Metagenomes



News & Views:
Cobb and Zhao, *Nature Biotech* 2012

Engineering a Microbial Factory for Advanced Biofuels Production

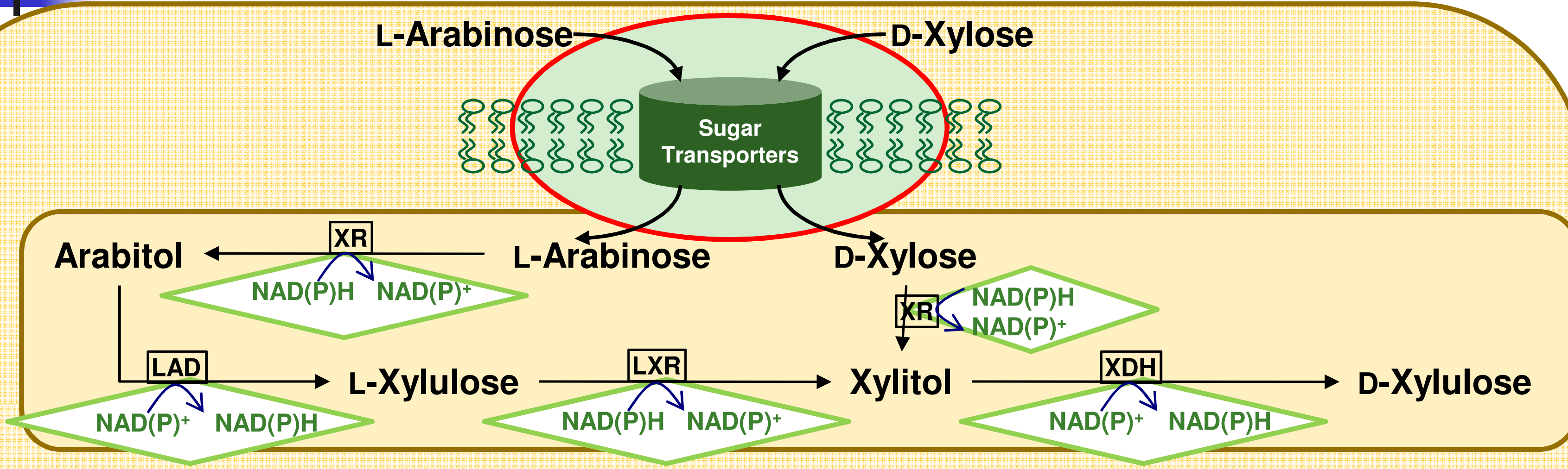
Hemicellulose/Cellulose

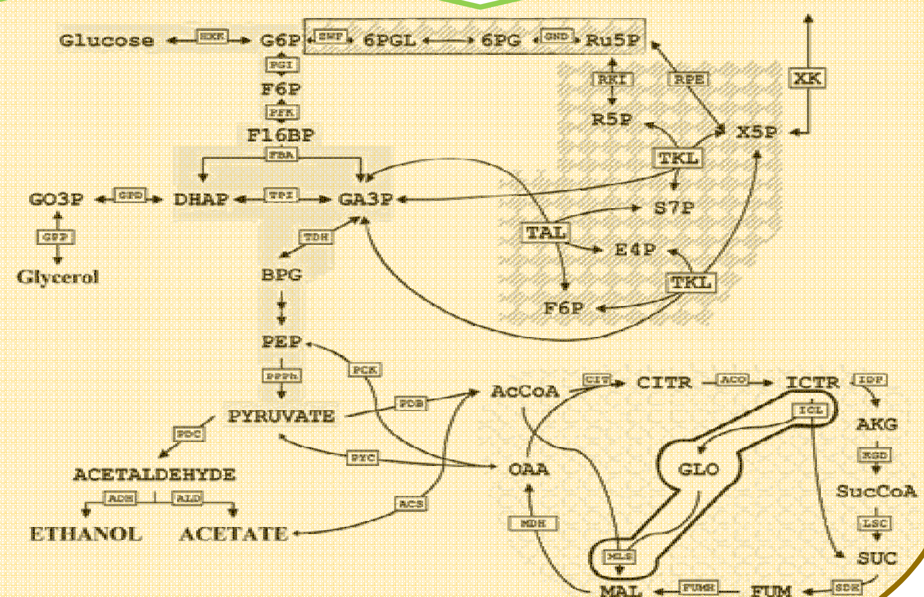


Ethanol

Advanced biofuels
(Butanol, Hydrocarbons)

Pentose Utilization in Yeast





Sugar Uptake

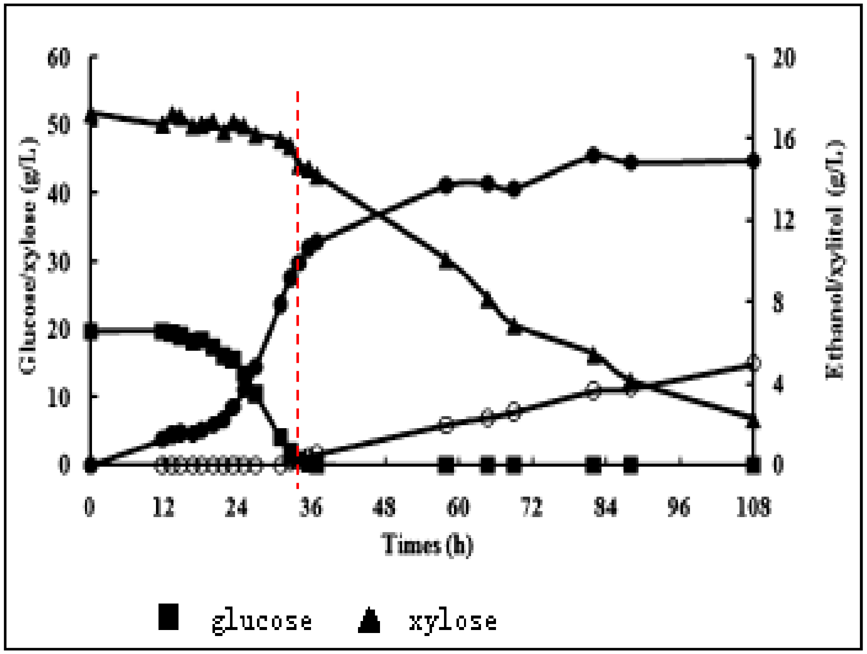
Heterologous Pathway

Redox Imbalance

Metabolic Flux

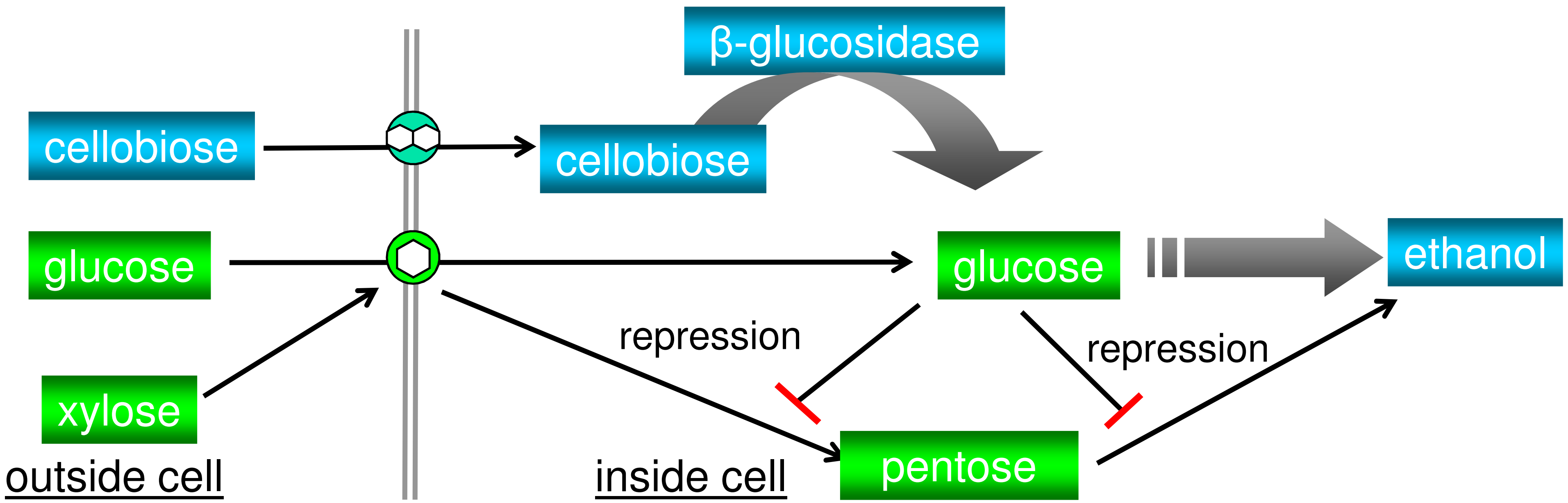
Glucose Repression in Mixed Sugar Fermentation

- Glucose repression occurs in *S. cerevisiae*
- Alternative carbon source fermentation is inhibited in the presence of glucose
- Lag time in xylose and arabinose consumption curve



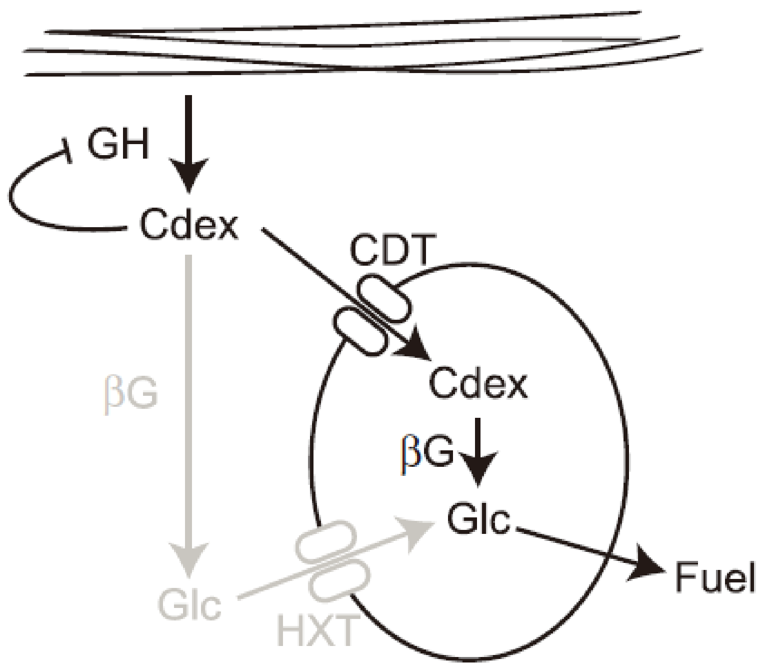
Olsson et al. Appl Microbiol Biotechnol (2009) 82:909–919

Coexpression of Cellobiose Transporter and β-Glucosidase



Coexpression of Cellobiose Transporter and β-Glucosidase

- Cellodextrin transport system from *Neurospora crassa*
  - Cellodextrin transporters: NCU00801 (*cdt1*), NCU00809, NCU08114(*cdt2*)
  - β-glucosidase: NCU00130 (*gh1-1*)

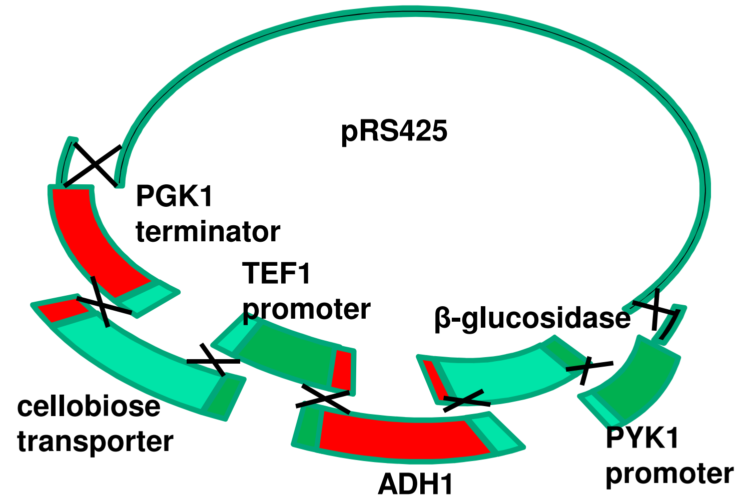


- *S. cerevisiae* with a heterologous cellodextrin transport system showed improved growth rate.

Galazka JM, *et al. Science* 330, 84 (2010)

Coexpression of Cellobiose Transporter and β-Glucosidase

- Genes
  - 3 transporters: *cdt-1*, *cdt-2*, *NCU00809*
  - 2 β-glucosidases:
    gh1-1 from *N. crassa*,
    b*gl1* from *A. aculeatus*



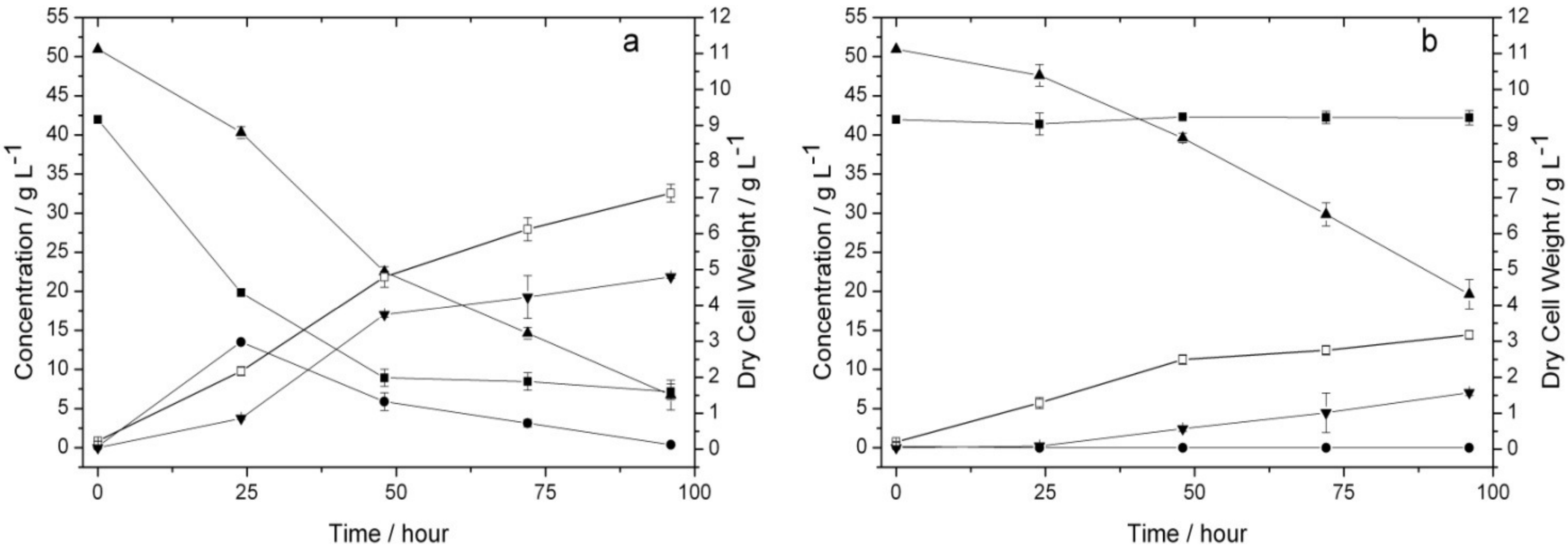
- Plasmids
  - Use DNA assembler method to integrate genes into pRS425 plasmid

- Strains
  - 6 plasmids constructed were transformed into *S. cerevisiae* strain with an integrated xylose utilization pathway

Strain	Transporter	β-glucosidase
SL01	cdt1	gt1-1
SL02	NCU00809	gt1-1
SL03	cdt2	gt1-1
SL04	cdt1	bgl1
SL05	NCU00809	bgl1
SL06	cdt2	bgl1
SL00	-	-

Mixed Sugar Cultivation in Shake-flask: Cellobiose+Xylose

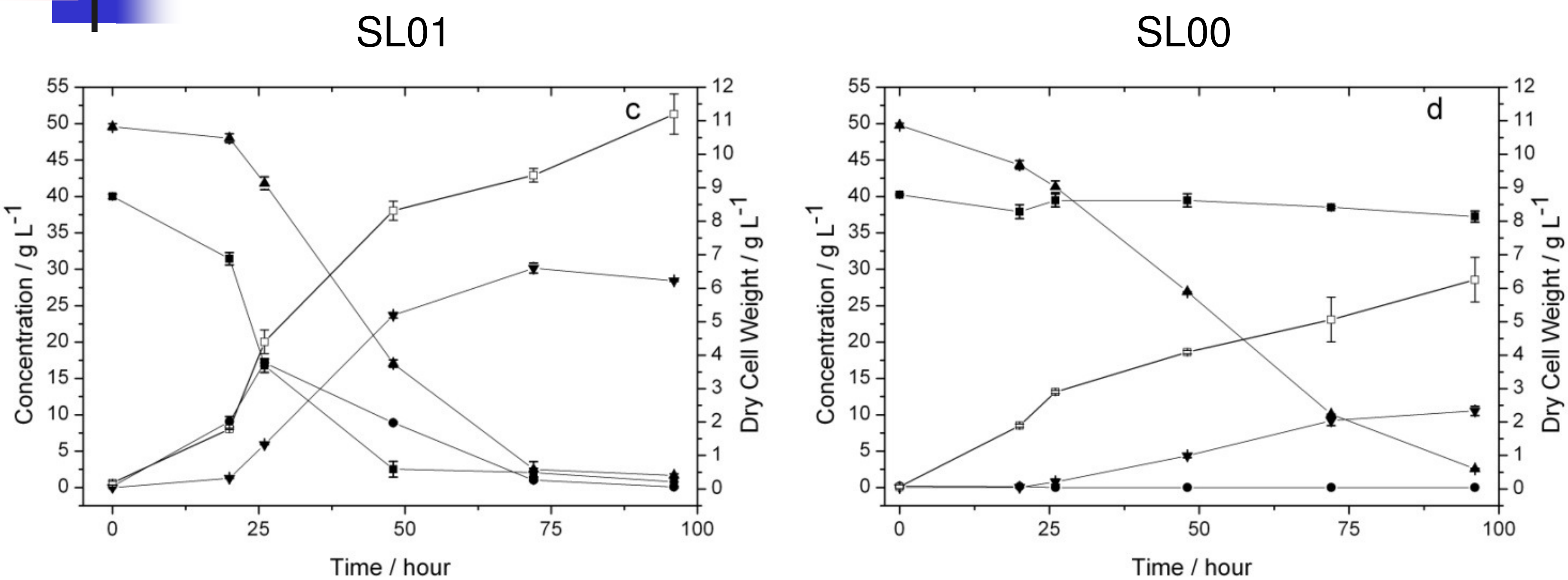
SL01 SL00



cellobiose (■) , xylose (▲), glucose(●), ethanol (▼) , Dry cell weight (□)

	SL01	SL00
Yield $_{\text{ethanol}}$	0.28	0.22
Productivity $_{\text{ethanol}}$ **(g/(L h))**	0.23	0.07

Mixed Sugar Cultivation in Bioreactor: Cellobiose+Xylose



cellobiose (■) , xylose (▲), glucose(●), ethanol (▼) , Dry cell weight (□)

	SL01	SL00
Yield $_{\text{ethanol}}$	0.39	0.24
Productivity $_{\text{ethanol}}$ **(g/(L h))**	0.49	0.09

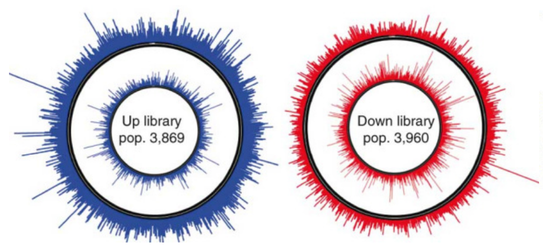
Li *et al. Mol Biosyst* 2010

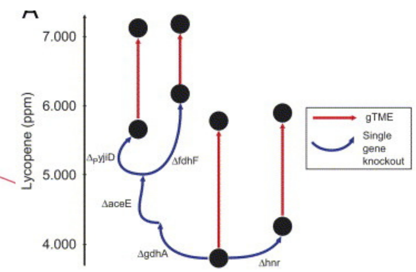
Balancing Metabolic Flux Remains a Big Challenge

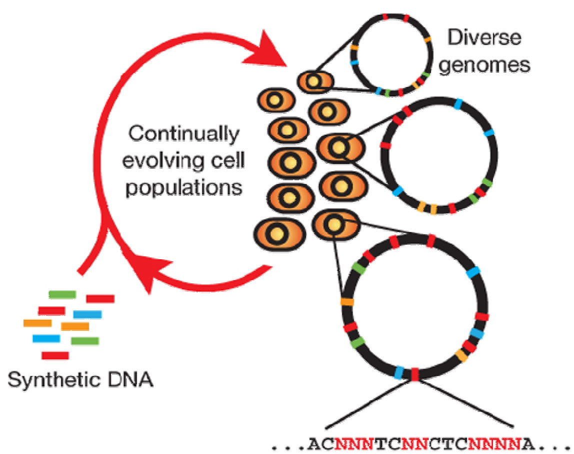
- Production of value-added compounds usually requires introduction of multi-step metabolic pathways
- Metabolic flux in multistep metabolic pathways need to be optimized to avoid metabolic burden
  - Overexpression of certain genes,
  - Redox imbalance from unmatched cofactor specificity
  - Accumulation of unstable or toxic intermediates
- Traditional approaches
  - Overexpression and deletion of certain genes in metabolic pathways
  - Modulating the expression levels of individual enzymes
  - Protein engineering to improve performance of rate limiting enzymes
  - Targeting a specific enzyme instead of the overall pathway
- Simultaneous optimization of multiple metabolic genes remains a big challenge

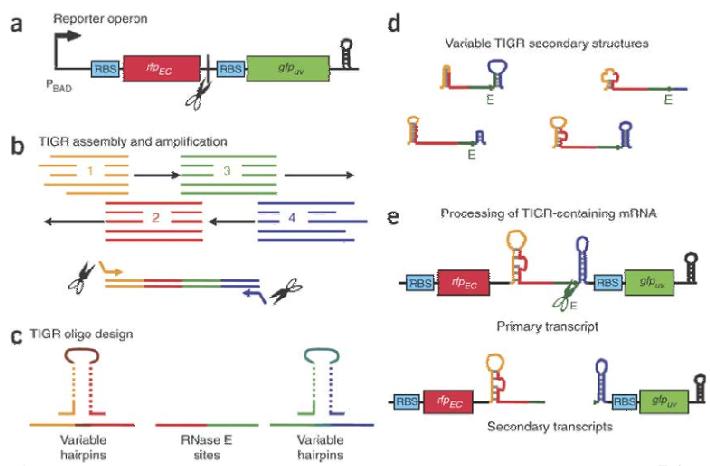
Balancing Metabolic Flux Remains a Big Challenge

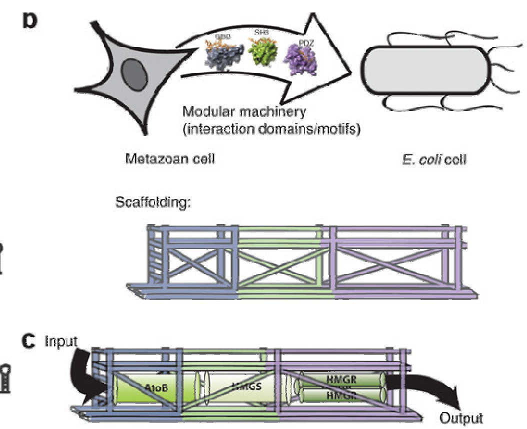
- Perturbation of global transcription machinery
- Genome-scale mapping of fitness altering genes
- Multiplex genome engineering
- Balance metabolic flux within the target pathway
  - Strengths of promoters
  - Ribosome binding sites
  - Intergenic regions
  - Synthetic scaffolds









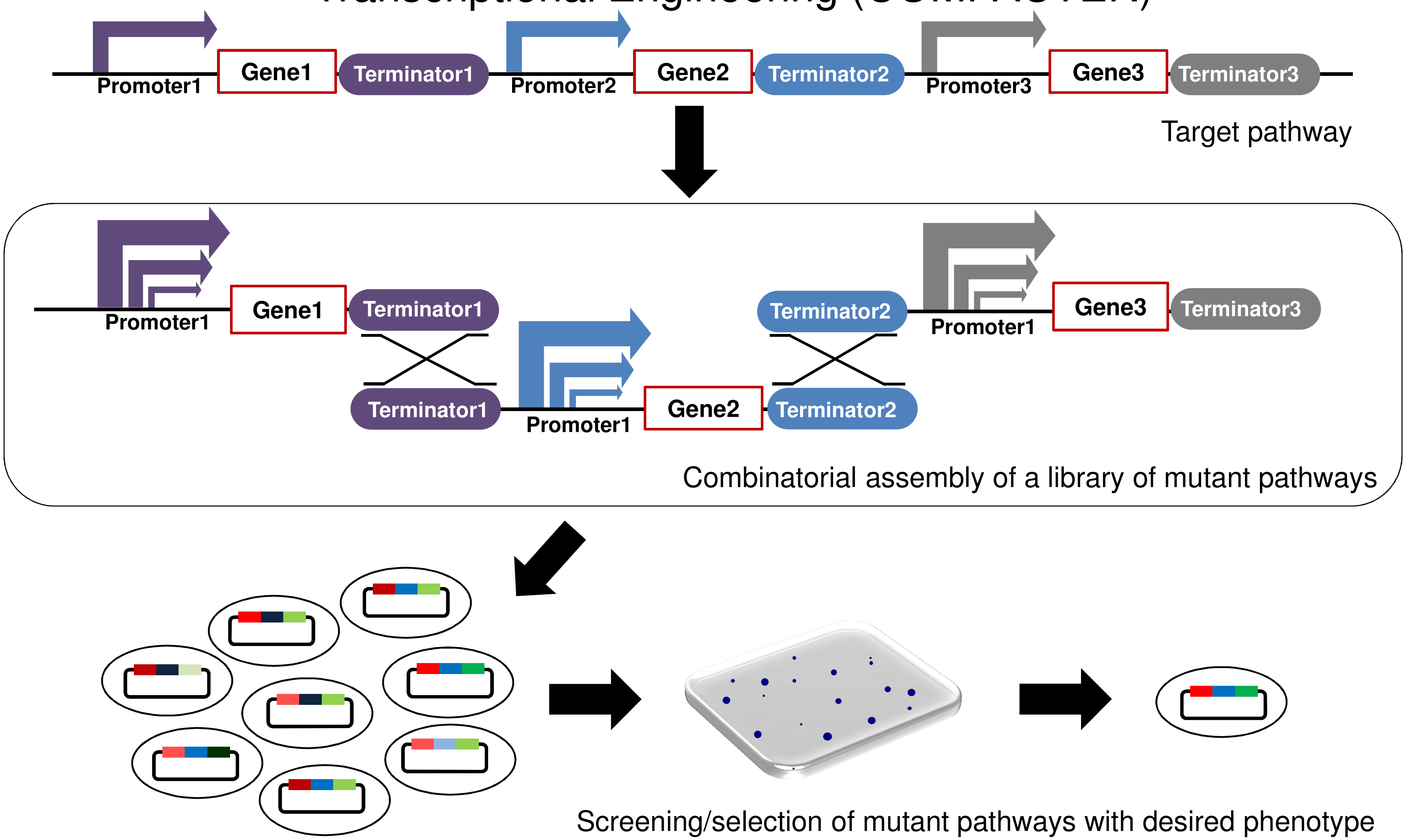


Warner et al., Nature Biotechnology 28, 856-U138 (2010)
Wang et al., Nature 460, 894-898 (2009)
Salis et al., Nat Biotechnol 27, 946-950 (2009)
Alper et al., Proc Natl Acad Sci U S A 102, 12678-12683 (2005)

Pfleger et al.,Nat Biotechnol 24, 1027-1032 (2006)
Dueber et al., Nat Biotechnol 27, 753-759 (2009)
Alper et al., Metab Eng 9, 258-267 (2007)
Warnecke et al., Metab Eng 12, 241-250 (2010)

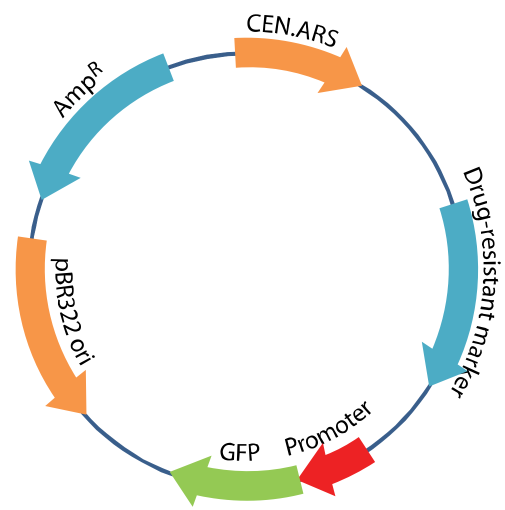
Pathway Optimization by COMPACTER

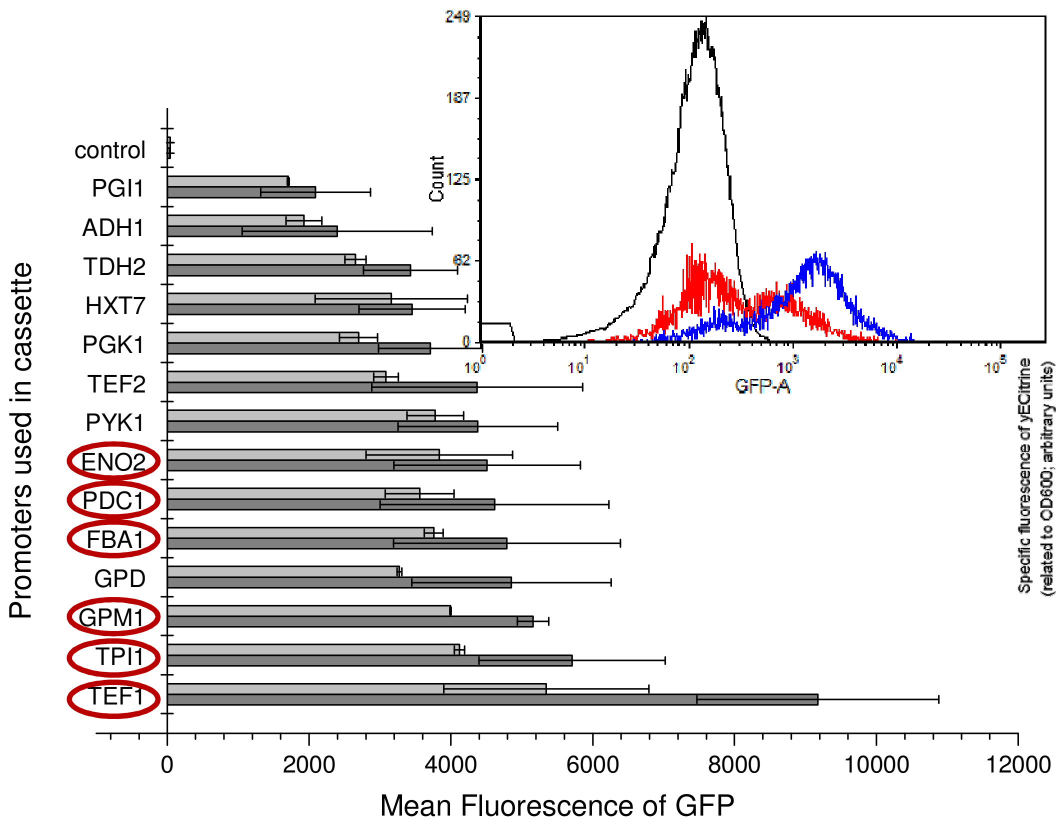
Customized Optimization of Metabolic Pathways by Combinatorial Transcriptional Engineering (COMPACTER)

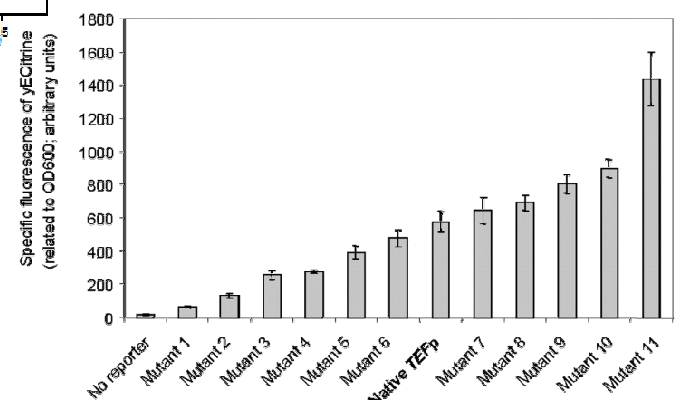


Promoter Mutants with Varying Strength

- Selected 6 yeast promoters
- Nucleotide analogue mutagenesis
- Isolating promoter mutants via FACS

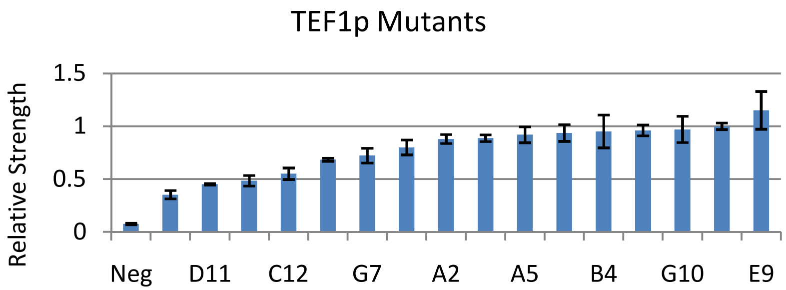


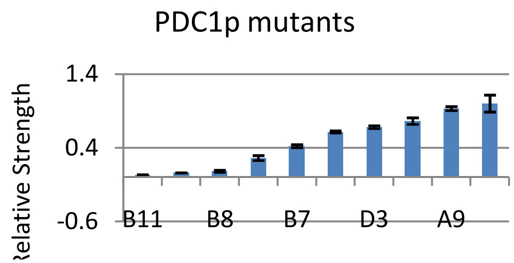


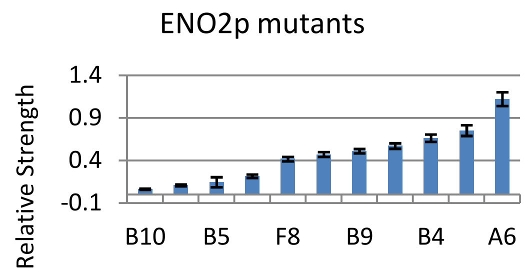


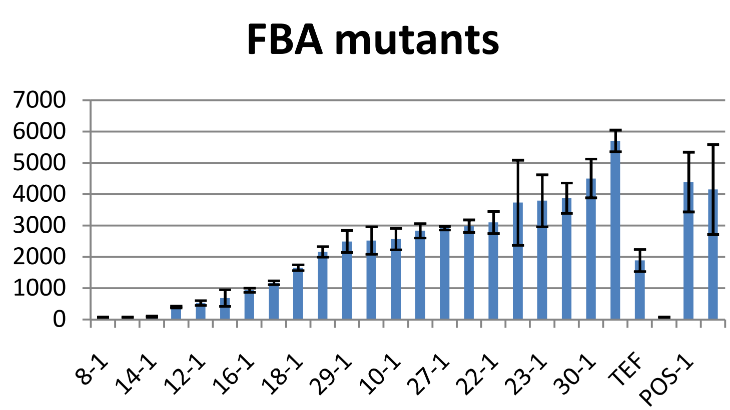
Alper et al. PNAS 102, 12678-12683 (2005)
Sun et al. B&B, 2012

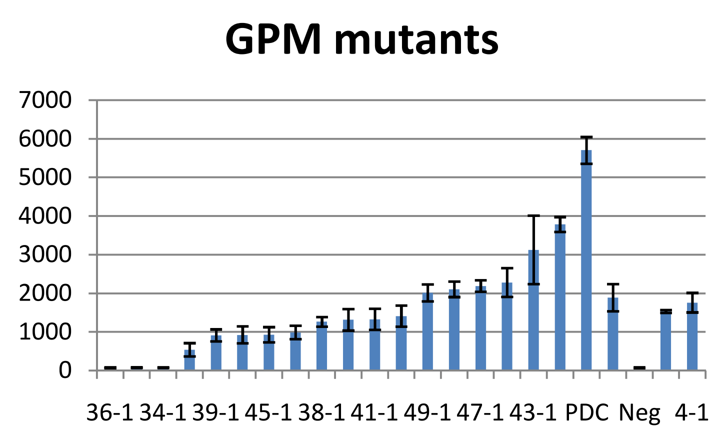
Promoter Mutants with Varying Strength



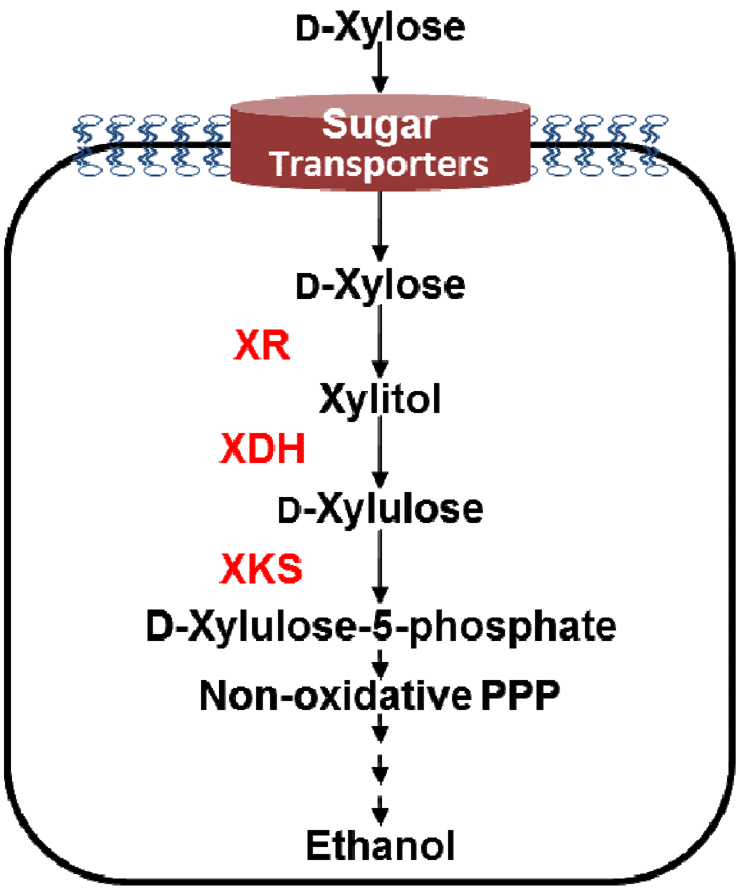




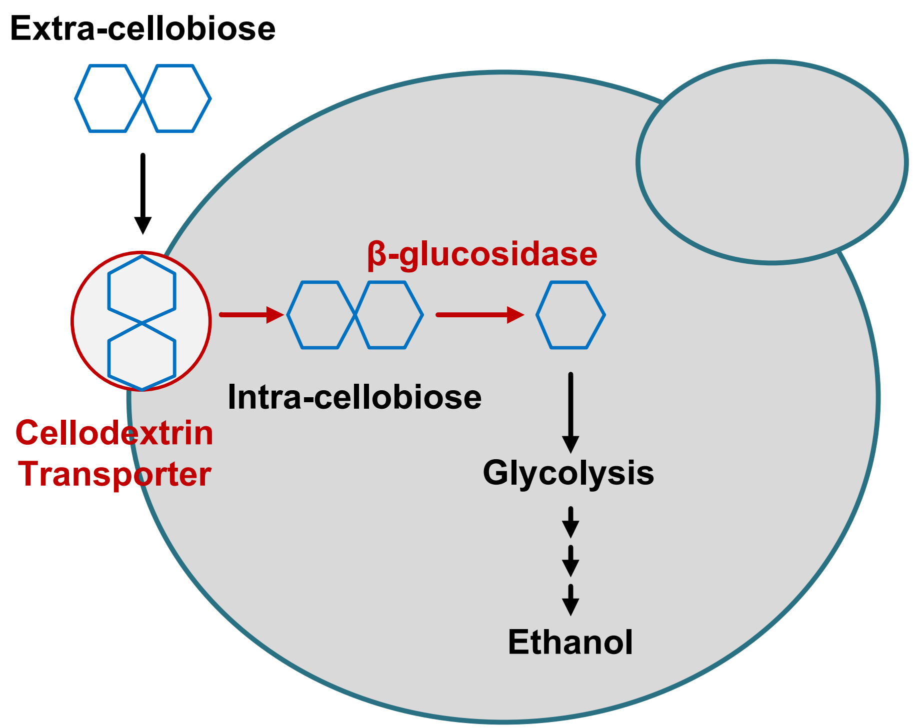




Pathway Optimization by COMPACTER



Xylose Utilizing Pathway

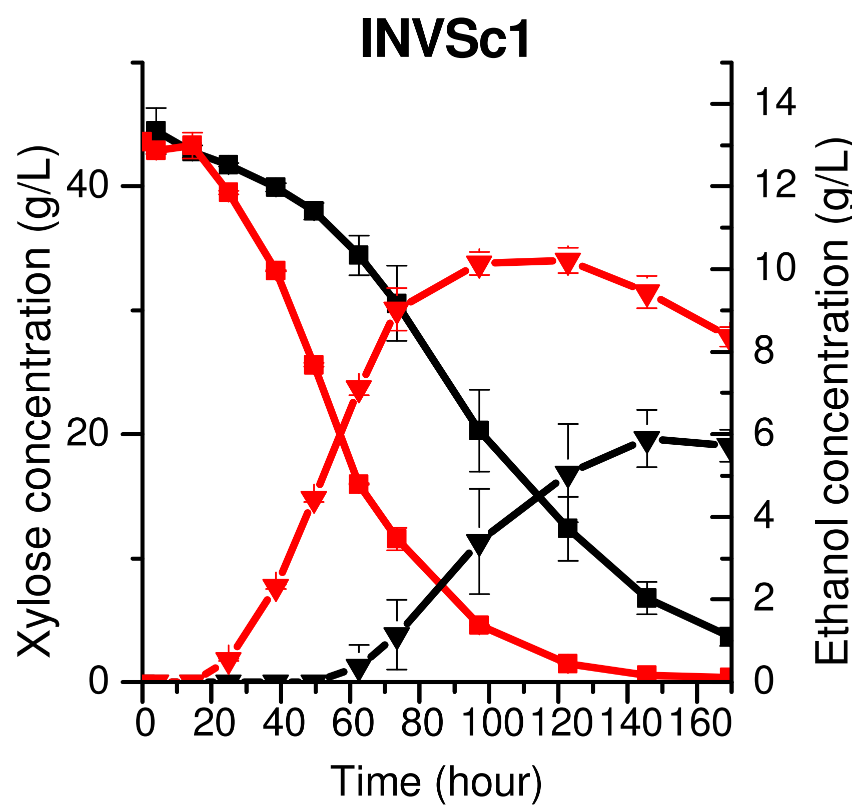


Cellobiose Utilizing Pathway

Optimization of the Xylose Utilizing Pathway in the INVSc1 Strain

- Host strain: INVSc1 (Invitrogen)
  - Diploid, auxotrophic mutation available
- Control
  - pRS416-PDC1p(WT)-csXR-TEF1p(WT)-ctXDH-ENO2p(WT)-ppXKS
- Backbone: pRS416
  - Single copy shuttle vector
- Library size: 10^4~10^5
- Fermentation:
  - Initial OD~1
  - Oxygen limited condition
  - YP media

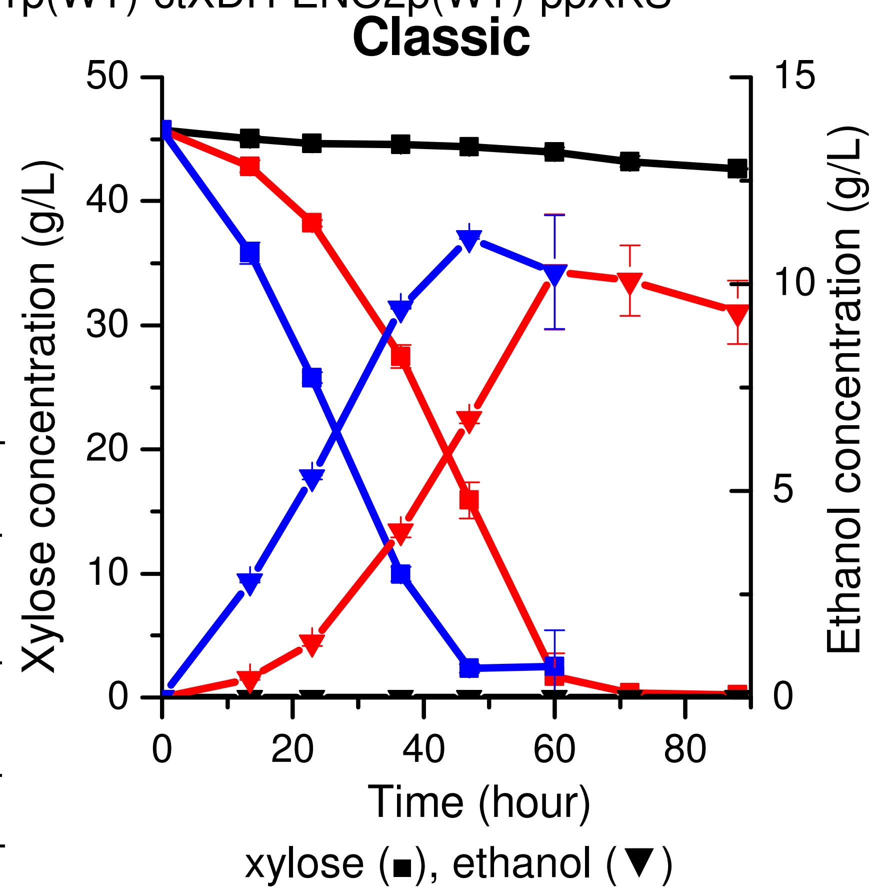
	WT	S3	Unit
Xylose consumption rate	0.24	0.40	g/L/hr
Ethanol production rate	0.04	0.10	g/L/hr
Ethanol yield	0.16	0.25	g/g xylose



Optimization of the Xylose Utilizing Pathway in an Industrial Strain

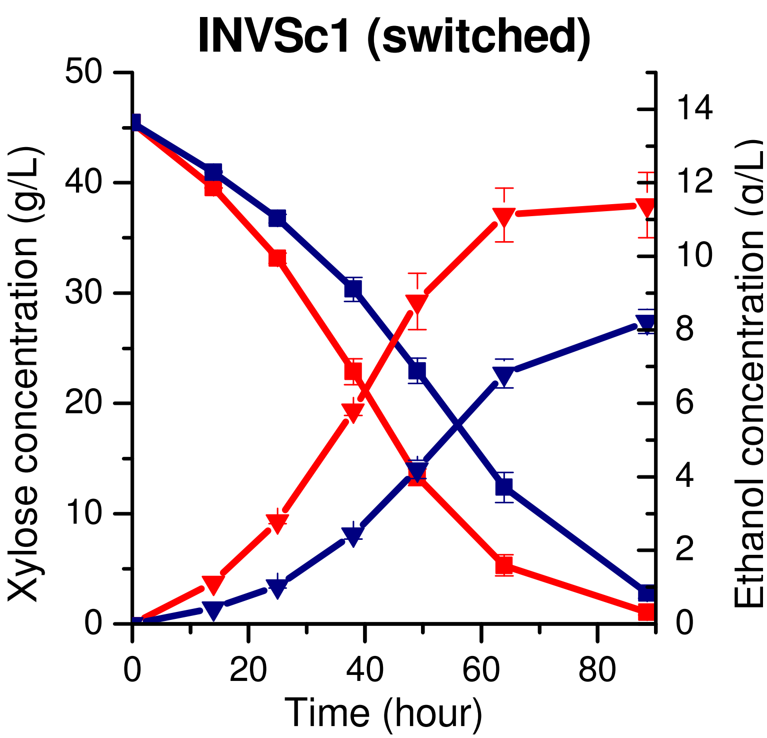
- Host Strain
  - Still Spirits (Classic) Turbo Distiller's Yeast
- Control
  - pRS-KanMX-PDC1p(WT)-csXR-TEF1p(WT)-ctXDH-ENO2p(WT)-ppXKS
- Backbone:pRS-KanMX
  - Single copy shuttle vector
- Library size: 10^3~10^4
- Fermentation:
  - Initial OD~10
  - Oxygen limited condition
  - YP media

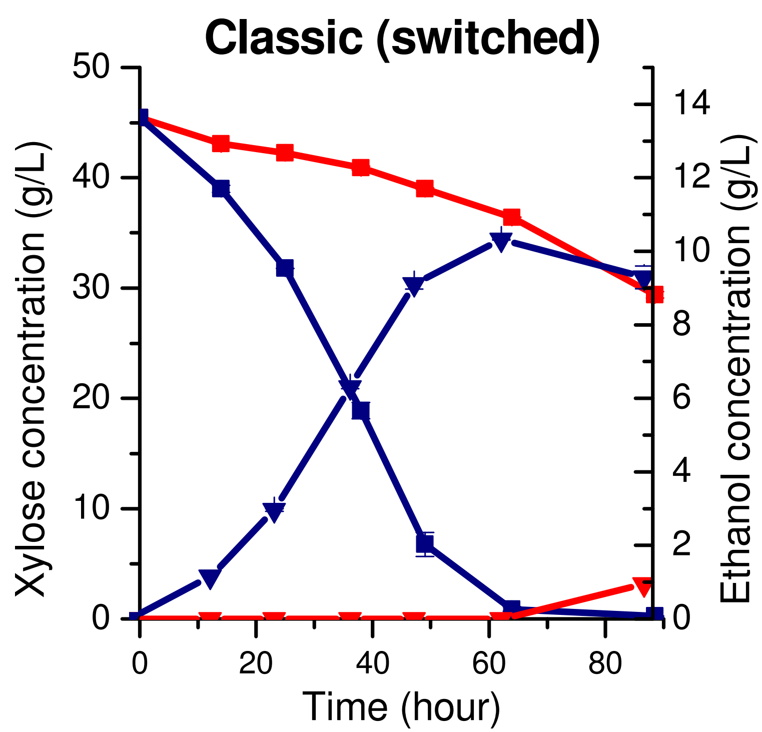
	YPD seed		YPX seed	Unit
	Classic WT	Classic S7	Classic S7	
Xylose consumption rate	0.06	0.74	0.92	g/L/hr
Ethanol production rate	0	0.17	0.24	g/L/hr
Ethanol yield	0	0.24	0.26	g/g xylose



Host-specific Pathway Optimization

- Switching optimized xylose utilizing pathways between laboratory and industrial strains

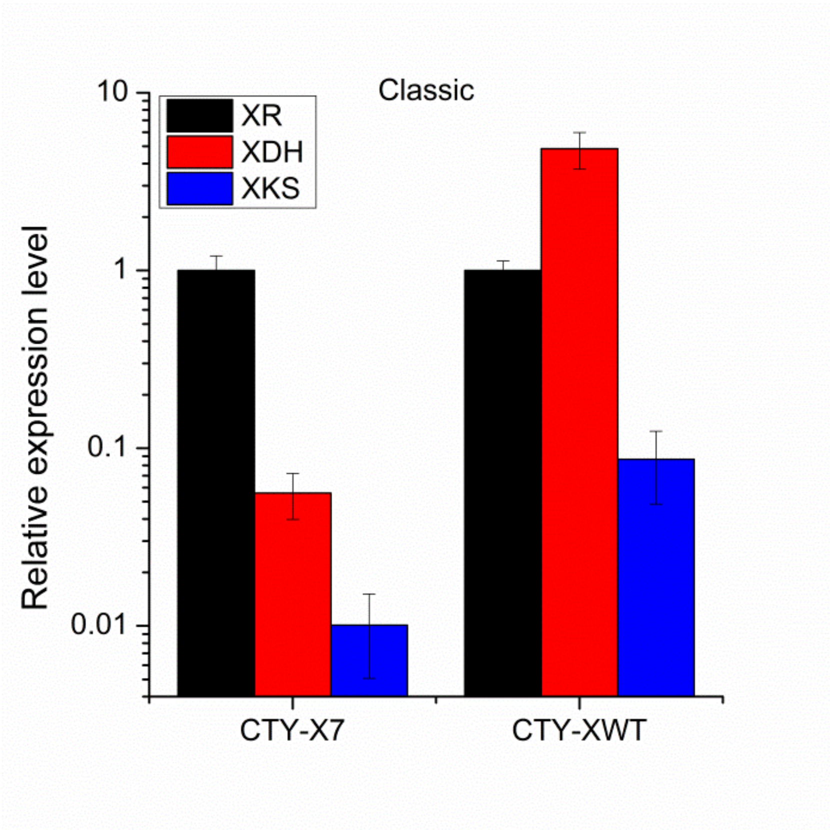


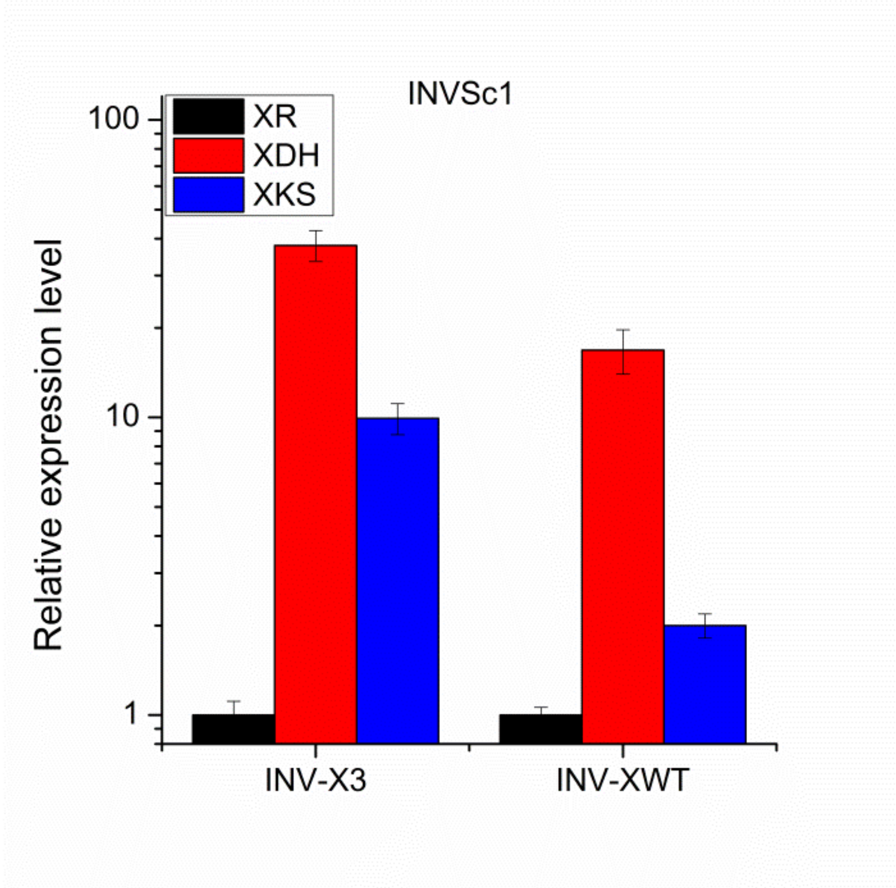


xylose (■), ethanol (▼)

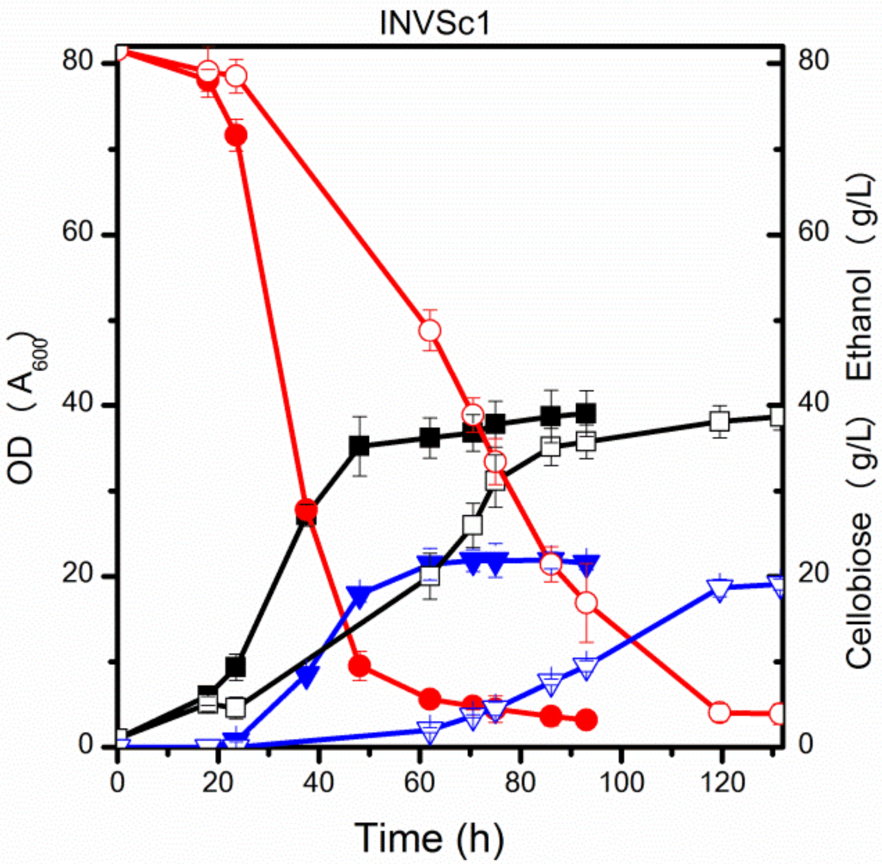
Host-specific Pathway Optimization

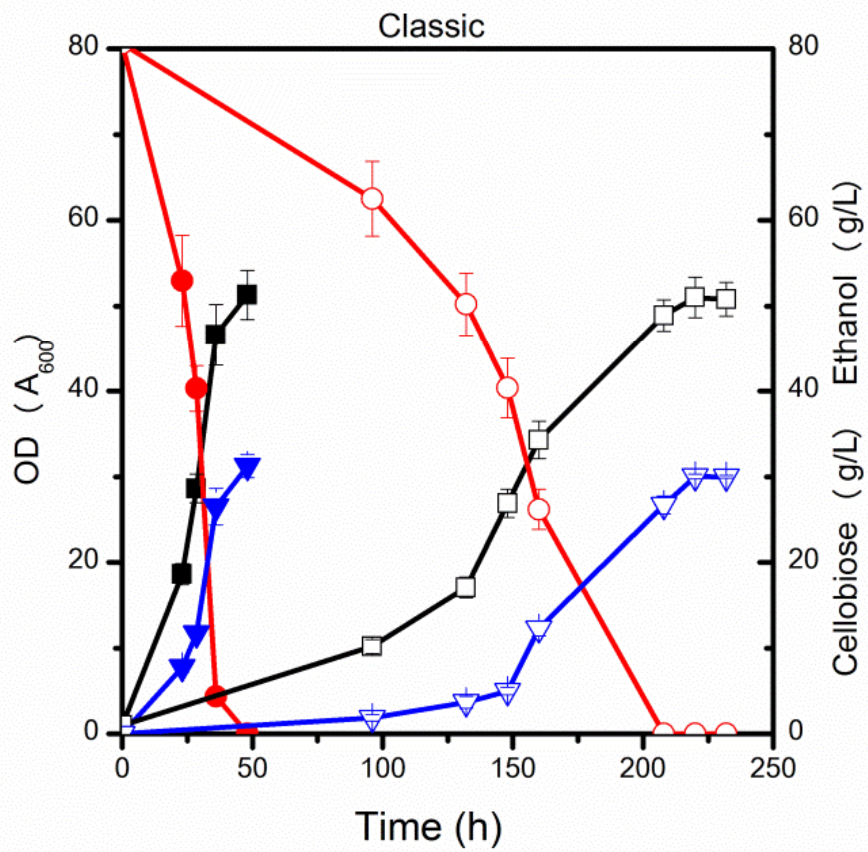
- qPCR analysis of the optimized xylose utilizing pathways between laboratory and industrial strains



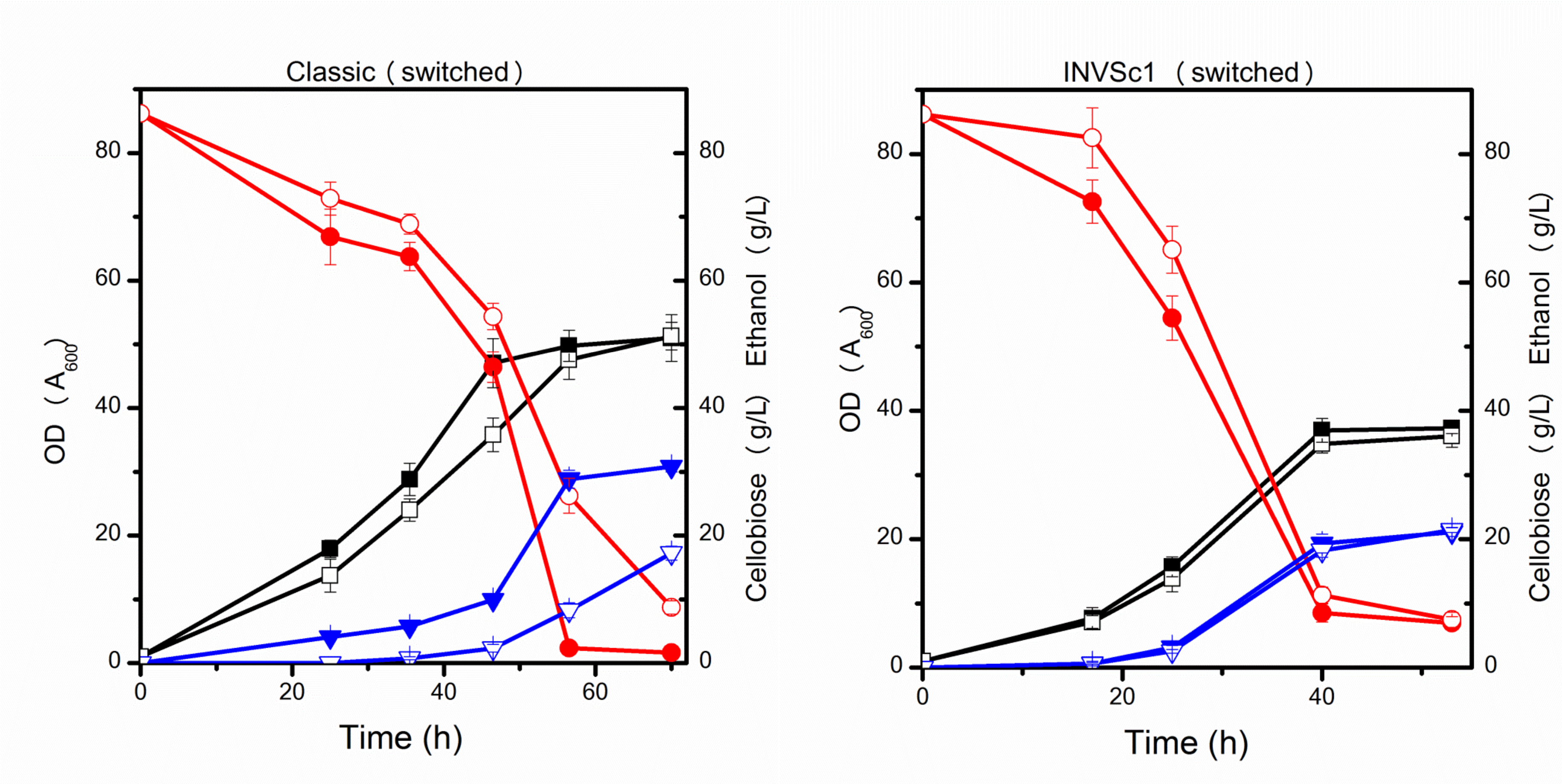


Optimization of the Cellobiose Utilizing Pathway





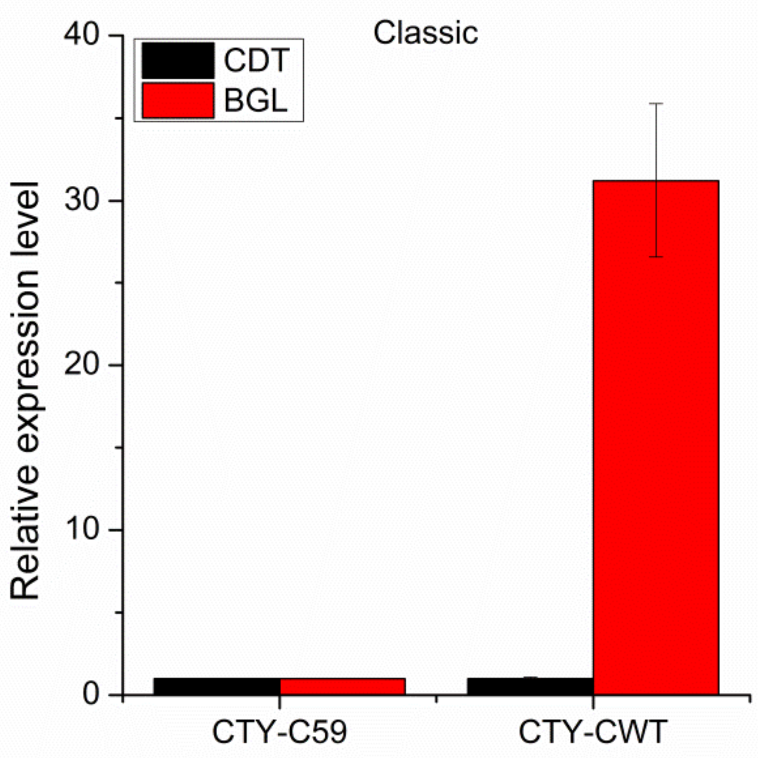
Optimized Xylose Utilizing Pathways are Strain Specific

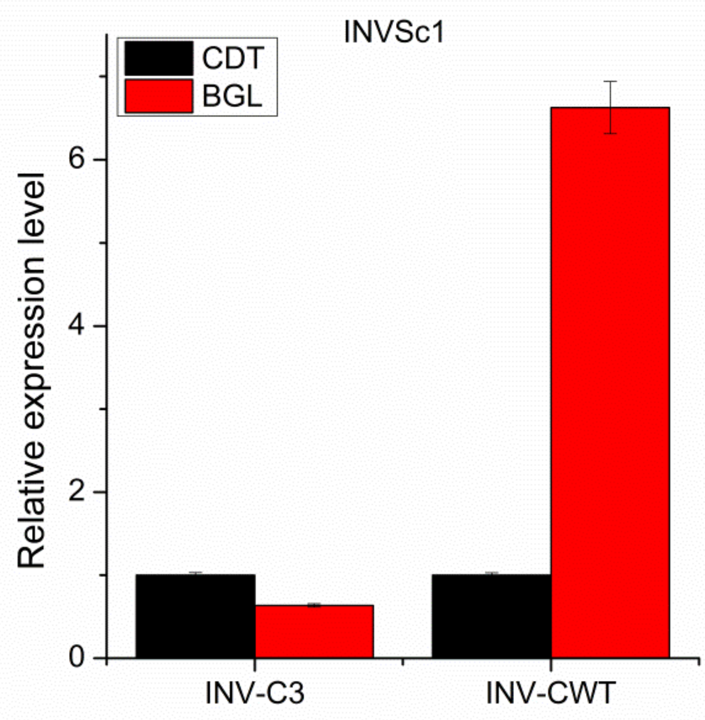


Open symbol: pathway optimized in INVSc1 strain, Solid symbol: pathway optimized in Classic strain, Red circle: cellobiose, Black square: OD (A_{600}), Blue down triangle: ethanol.

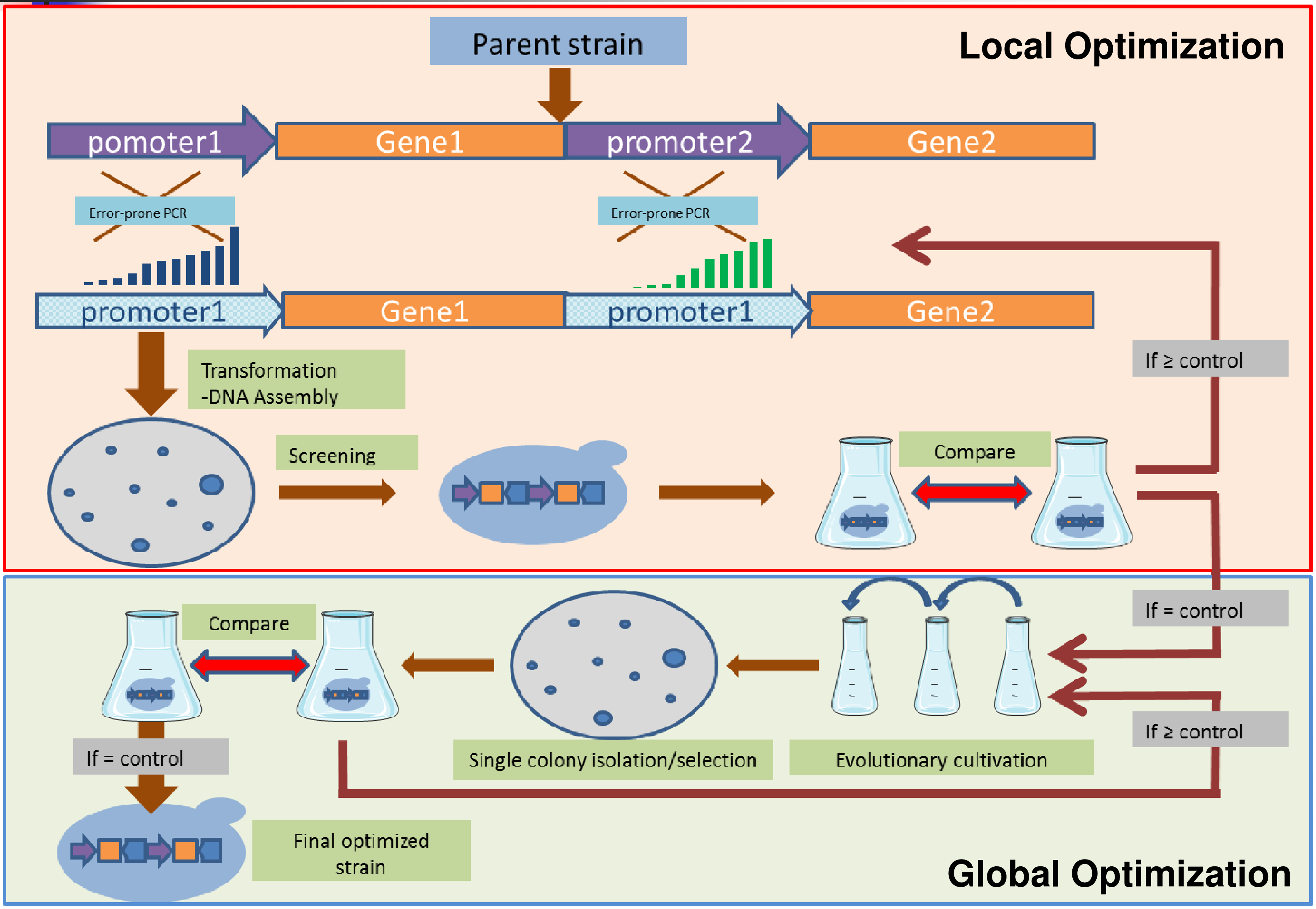
Optimized Cellobiose Utilizing Pathways are Strain Specific

- qPCR analysis of the optimized cellobiose utilizing pathways between laboratory and industrial strains

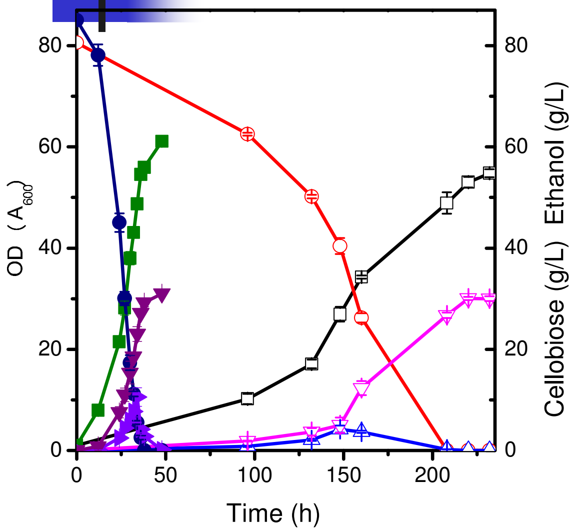


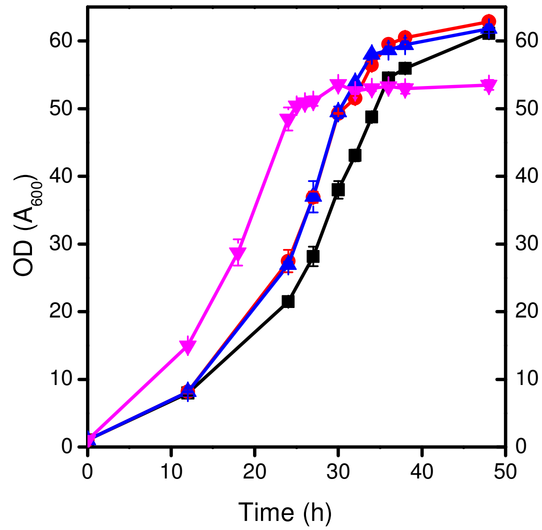


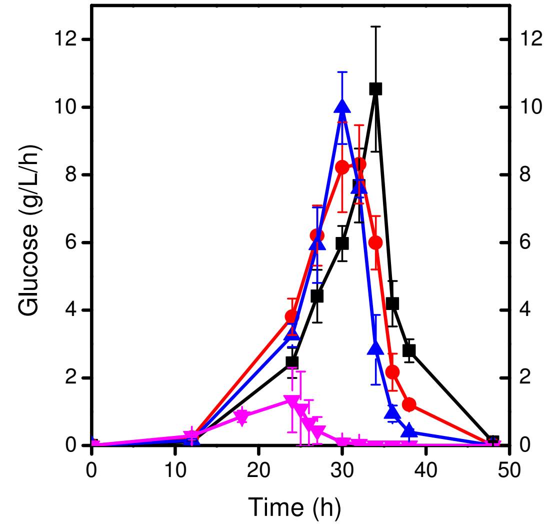
Directed Evolution for Strain Development



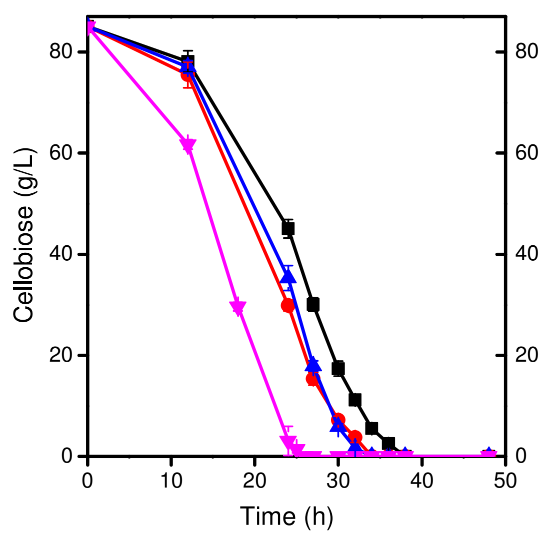
Directed Evolution for Strain Development

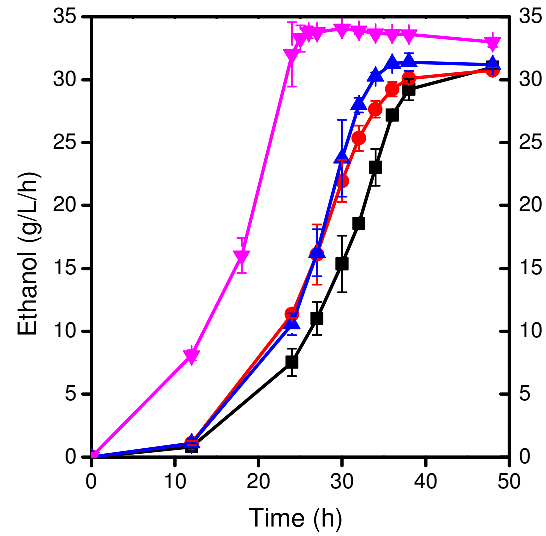






- ❑ #9,#91 and #9118 have same final OD, ethanol concentration and glucose accumulation
- ❑ A#9118 has lower OD and higher ethanol
- ❑ A#9118 has much lower glucose accumulation
- ❑ No mutations were found in promoter regions in A#9118



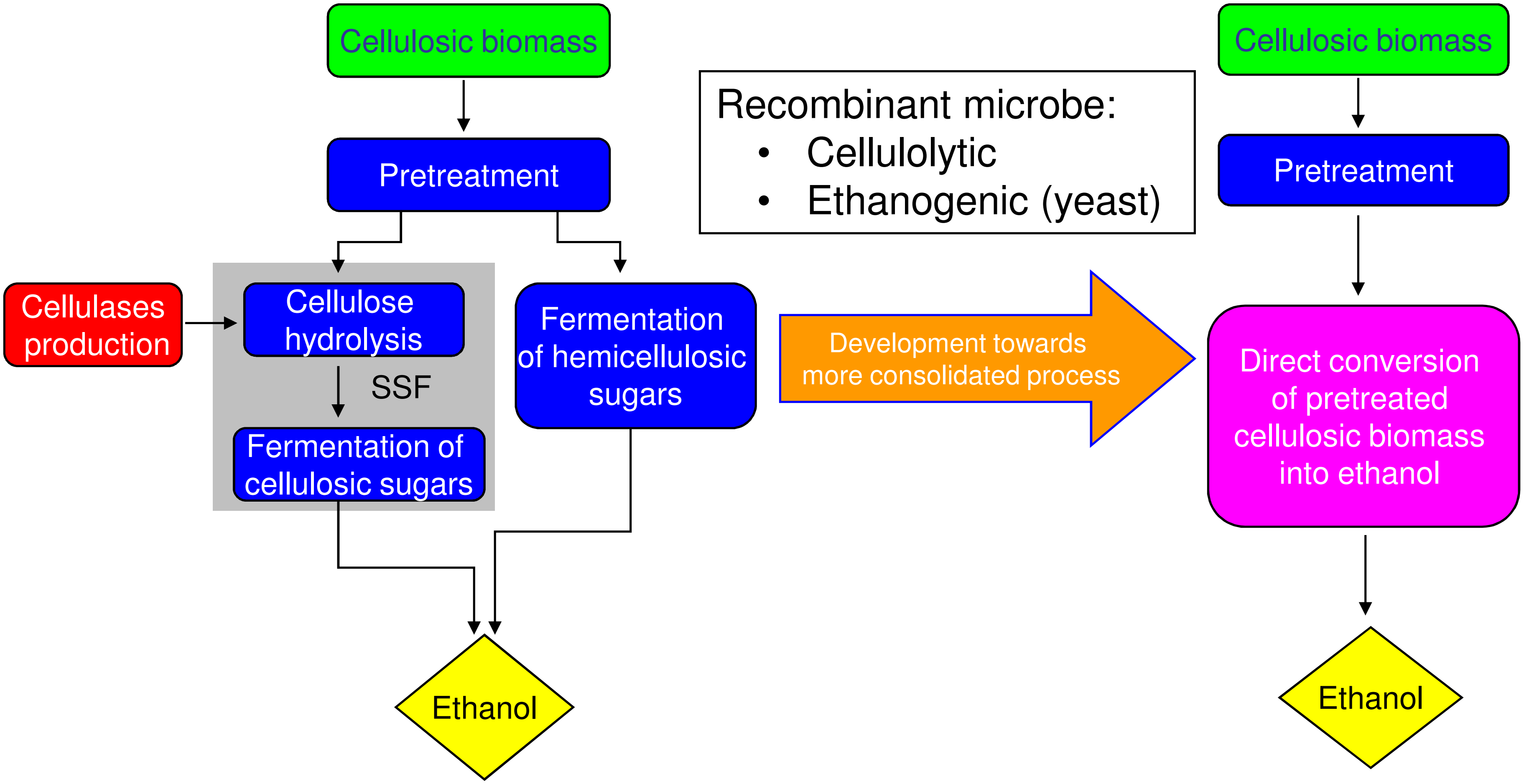


Directed Evolution for Strain Development

Cellobiose fermentation performance of evolved yeast strains #9, #9-1, #9-1-18 and A#9-1-18

	WT	#9	#9-1	#9-1-18	A#9-1-18
Cellobiose consumption (g cellobiose/L/h)	0.388	2.24	2.5	2.5	3.27
Ethanol productivity (g ethanol/L/h)	0.137	0.77	0.81	0.89	1.30
Yield (g ethanol/g cellobiose)	0.373	0.36	0.36	0.37	0.40

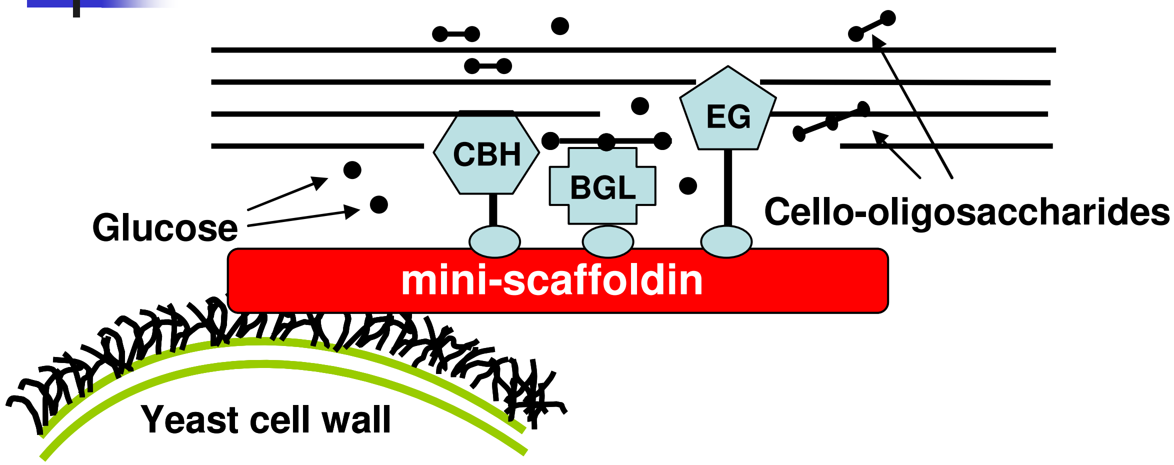
Consolidated Bioprocessing (CBP)



- Consolidated bioprocessing (CBP): save ~10-20 cents/gallon of ethanol

L. Lynd *et al.* Curr Opin Biotechnol 577 (2005)

Direct Conversion of Cellulose to Ethanol by Engineered Mini-cellulosomes

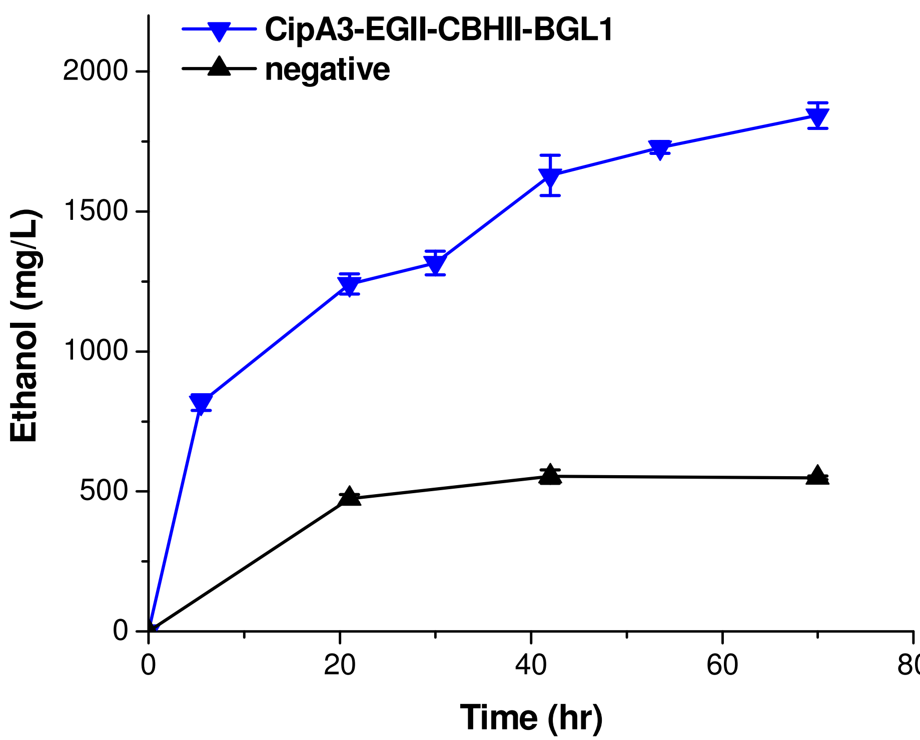


- EG: Endogluconase
- CBH: Exogluconase
- BG: β-glucosidase

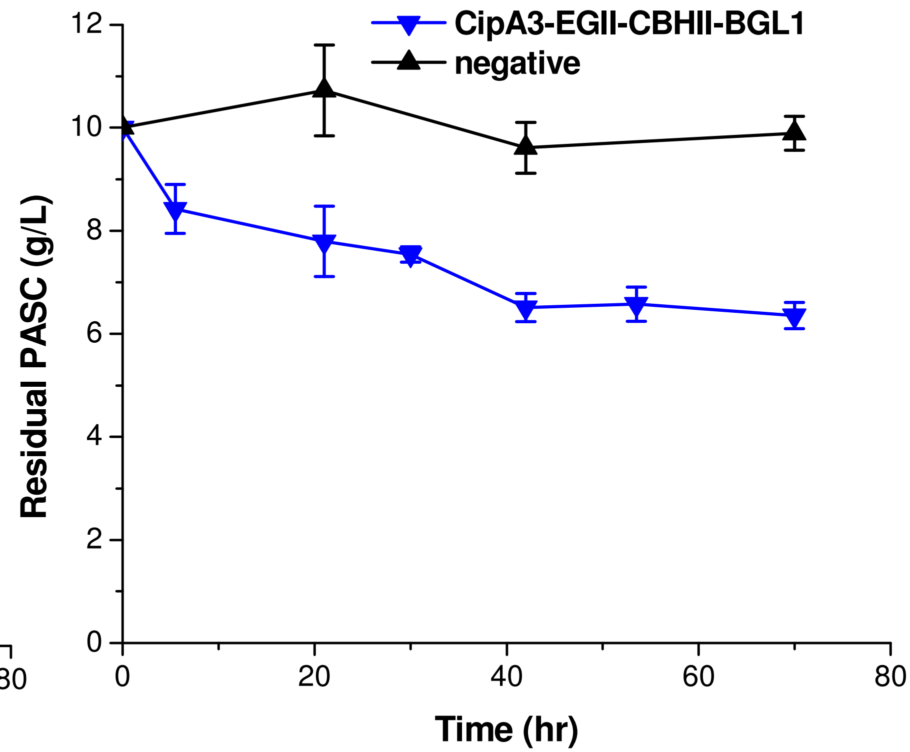
- Yeast surface display of functional minicellulosomes
  - Functional display of a mini-scaffoldin
  - Successful assembly of minicellulosomes through cohesin-dockerin interaction
  - Synergistic hydrolysis of cellulose
  - Direct fermentation of hydrolysate (glucose) to ethanol

Direct Ethanol Production from PASC

Ethanol production



Residual PASC

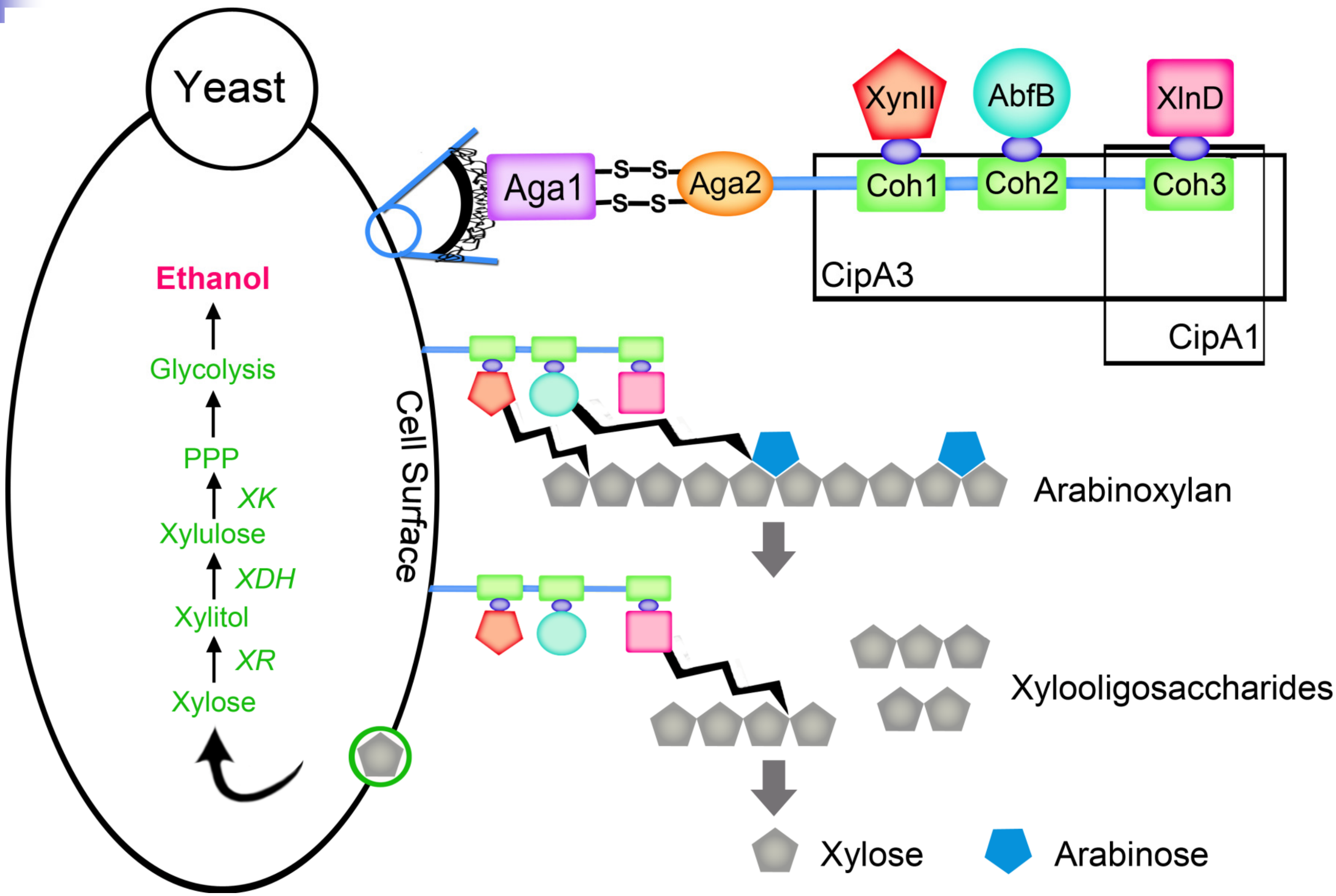


Yield: 0.31 grams of ethanol per gram of PASC

62% of theoretical yield

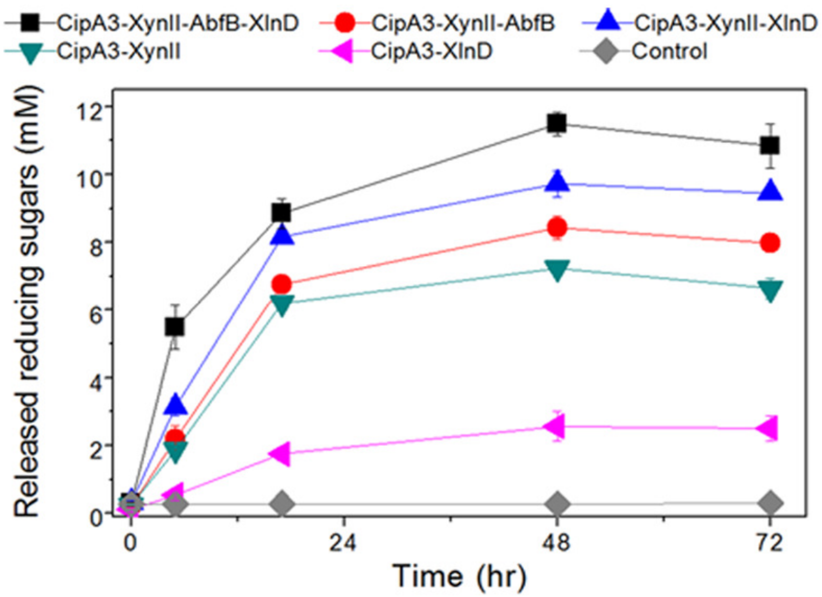
Wen, F., Sun, J. and Zhao, H. *AEM* (2010)

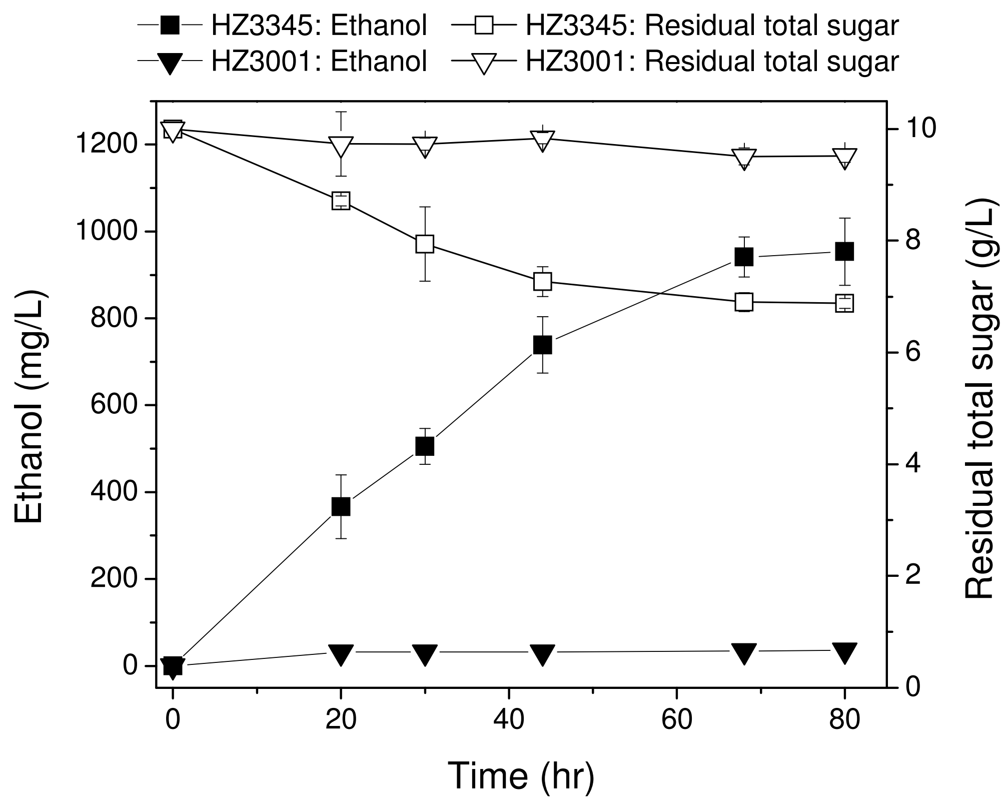
Direct Conversion of Xylan to Ethanol by Engineered Hemicellulosomes



Sun, J. et al. *AEM* (2012)

Direct Conversion of Xylan to Ethanol by Engineered Hemicellulosomes





Yield: 0.31 grams of ethanol per gram of birchwood xylan

Sun, J. et al. *AEM* (2012)

Summary

- Developed a DNA assembler method for constructing large DNA molecules such as pathways, plasmids, and genomes.
- Developed a DNA assembler based synthetic biology method (COMPACTER) for optimizing the metabolic flux in a heterologous pathway
- Engineered a yeast strain capable of simultaneously and efficiently utilizing C5/C6 sugars
- Engineered yeast strains for consolidated bioprocessing of cellulose and xylan respectively.

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