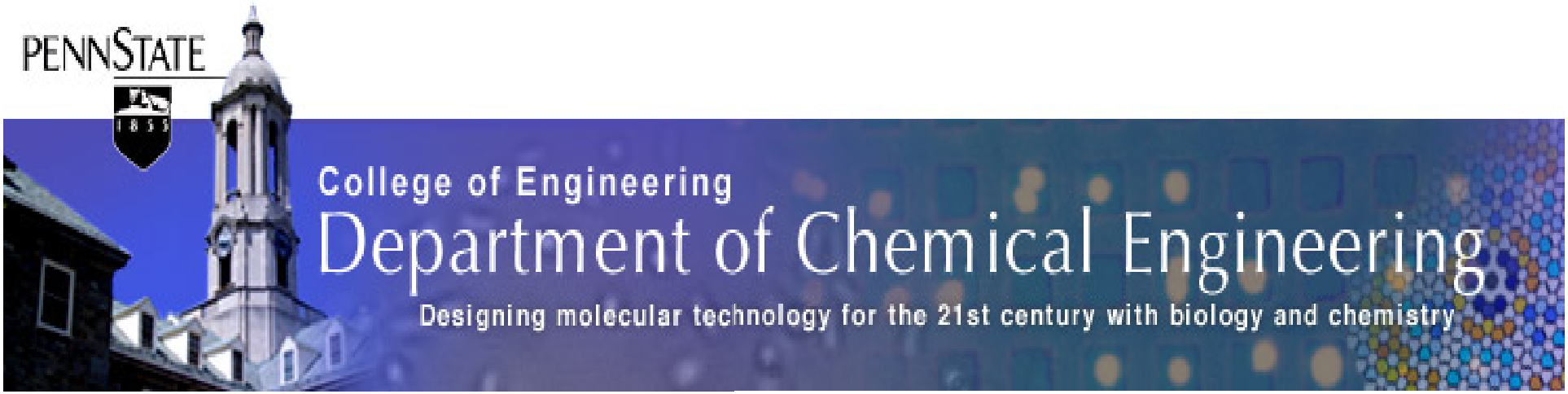


Engineering *E. coli* for xylitol production during growth on xylose

Olubolaji Akinterinwa

&

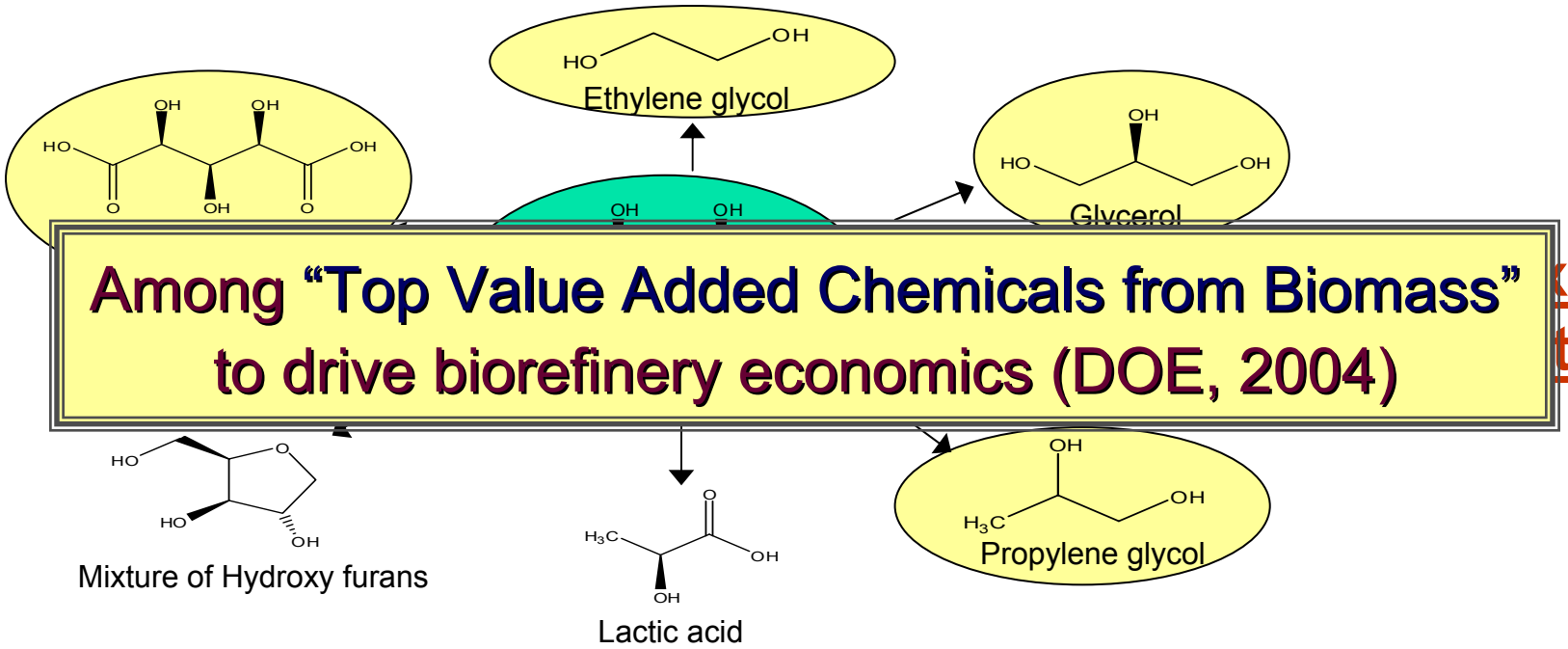
Patrick C. Cirino

IBE 2008 Annual Meeting, March 7th

008-17 Advances in Engineering Microbial Metabolism

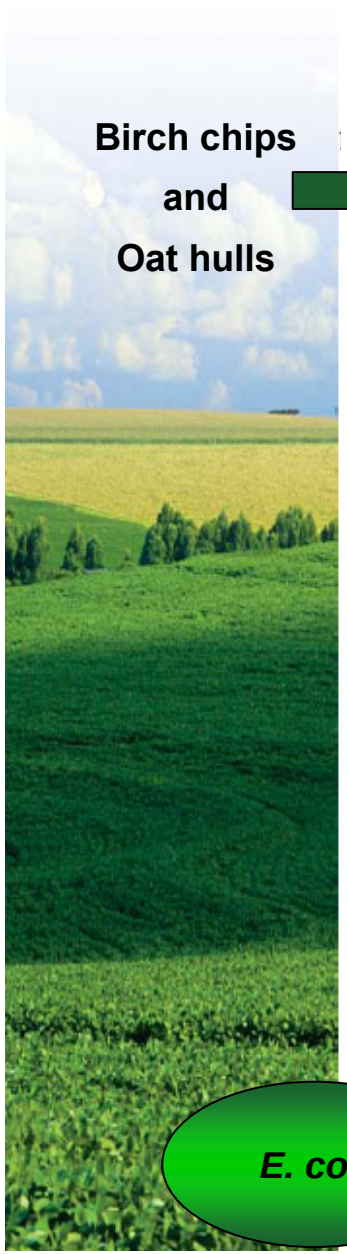
Xylitol

- Low calorie sweetener
- Promotes oral health
- Non-insulin mediated metabolism
- Obtained from reduction of xylose



xylitol
tives

Production of Xylitol



Birch chips
and
Oat hulls

WATER

PRETREATMENT

EXTRACTION

LIGNIN/CELLULOSE

CATTLEFEED

XYLANS

ENZYMATIC HYDROLYSIS

XYLOSE

BIOPRODUCTION
Environment - friendly
Potentially cheaper

CHROMATOGRAPHIC SEPARATION

PURIFICATION

XYLITOL

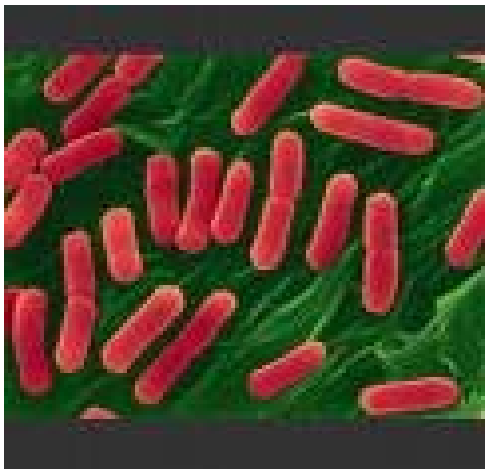
in vivo

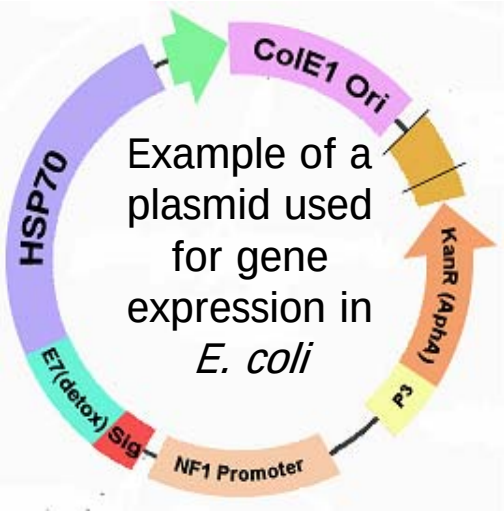
YEASTS BACTERIA

E. coli

Source: www.sunopta.com/bioprocess/picture_gallery.aspx

E. coli



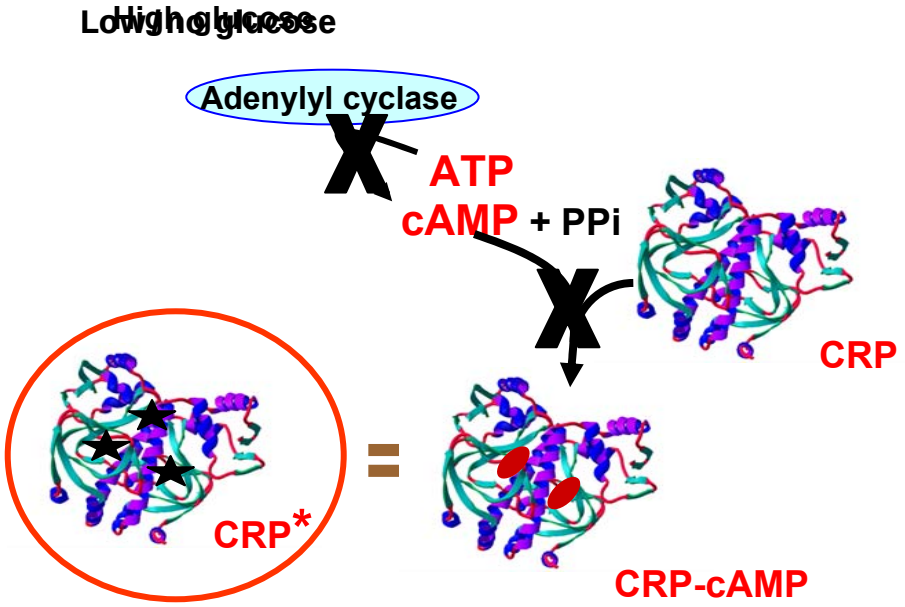


- Well-studied organism
  - Metabolic network models
- Easy to cultivate
- Easy to manipulate
  - Gene knock-in and knock-out
  - Gene over-expression using plasmids
- Able to utilize hexoses and pentoses

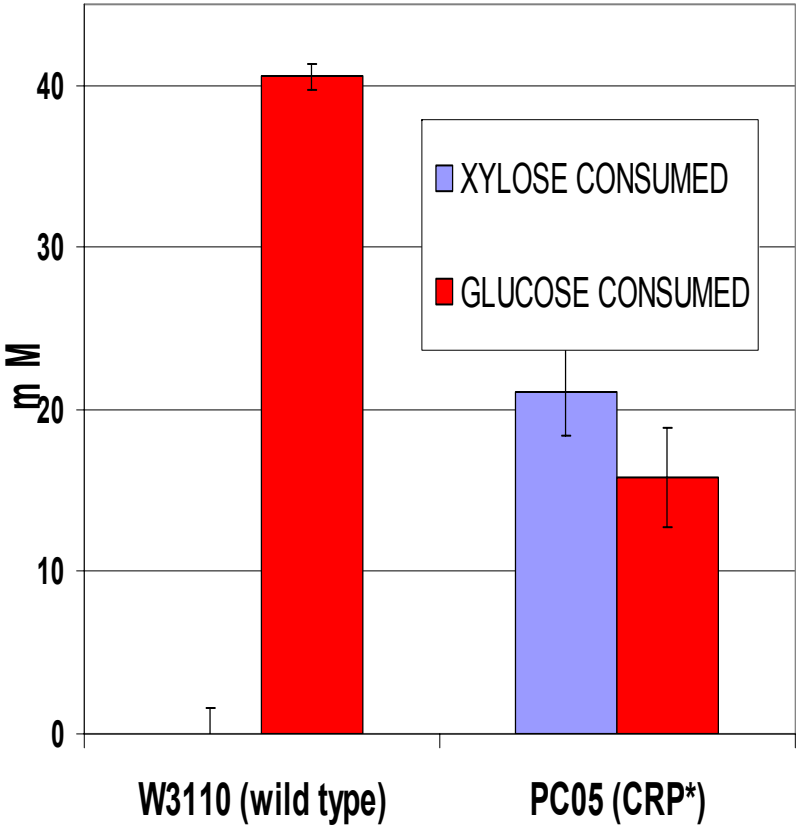
Diauxie in *E. coli*

Diauxie: preferential utilization of glucose in sugar mixtures

- Engineered strain for simultaneous uptake of sugars using **cAMP-independent CRP mutant**

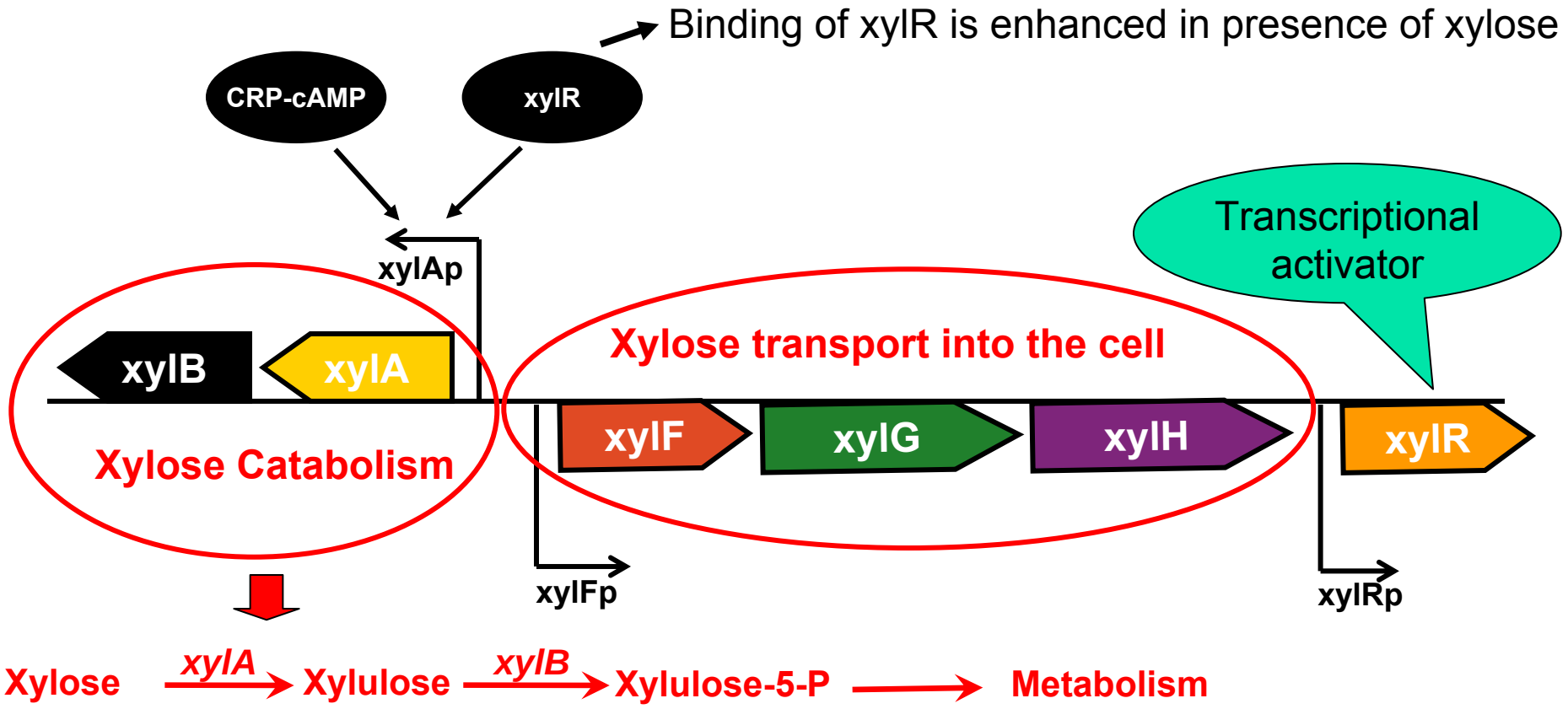


(transcriptional activator: controls about 200 genes)



[Marschall & Hengge-Aronis, Molecular Microbiology (1995) 18, 175-184]

E. coli's Xylose Catabolic Operon

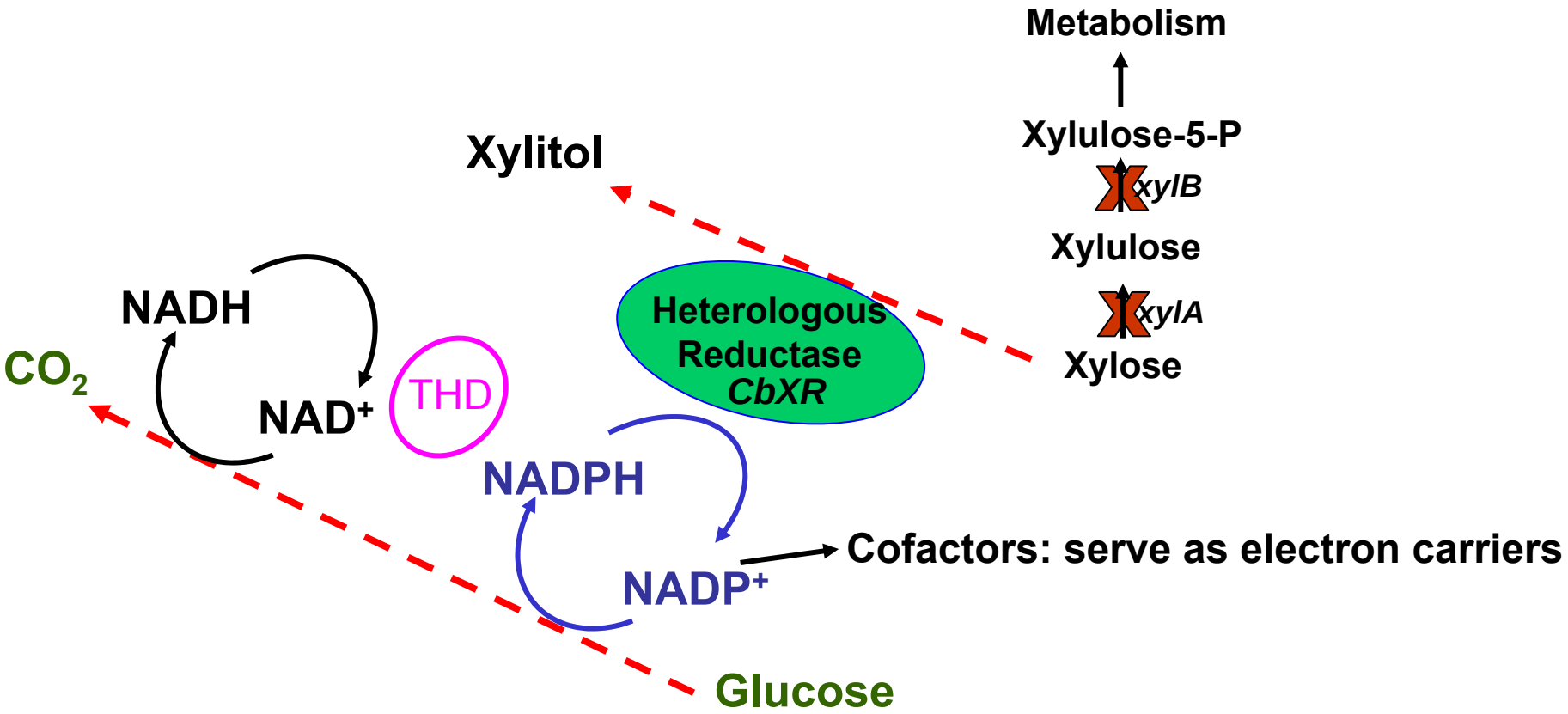


Note: XylB expression should not be affected in *xylA* deletion strains and vice versa

Platform for Xylitol Production in *E. coli*

Aim: Use glucose-xylose mixtures

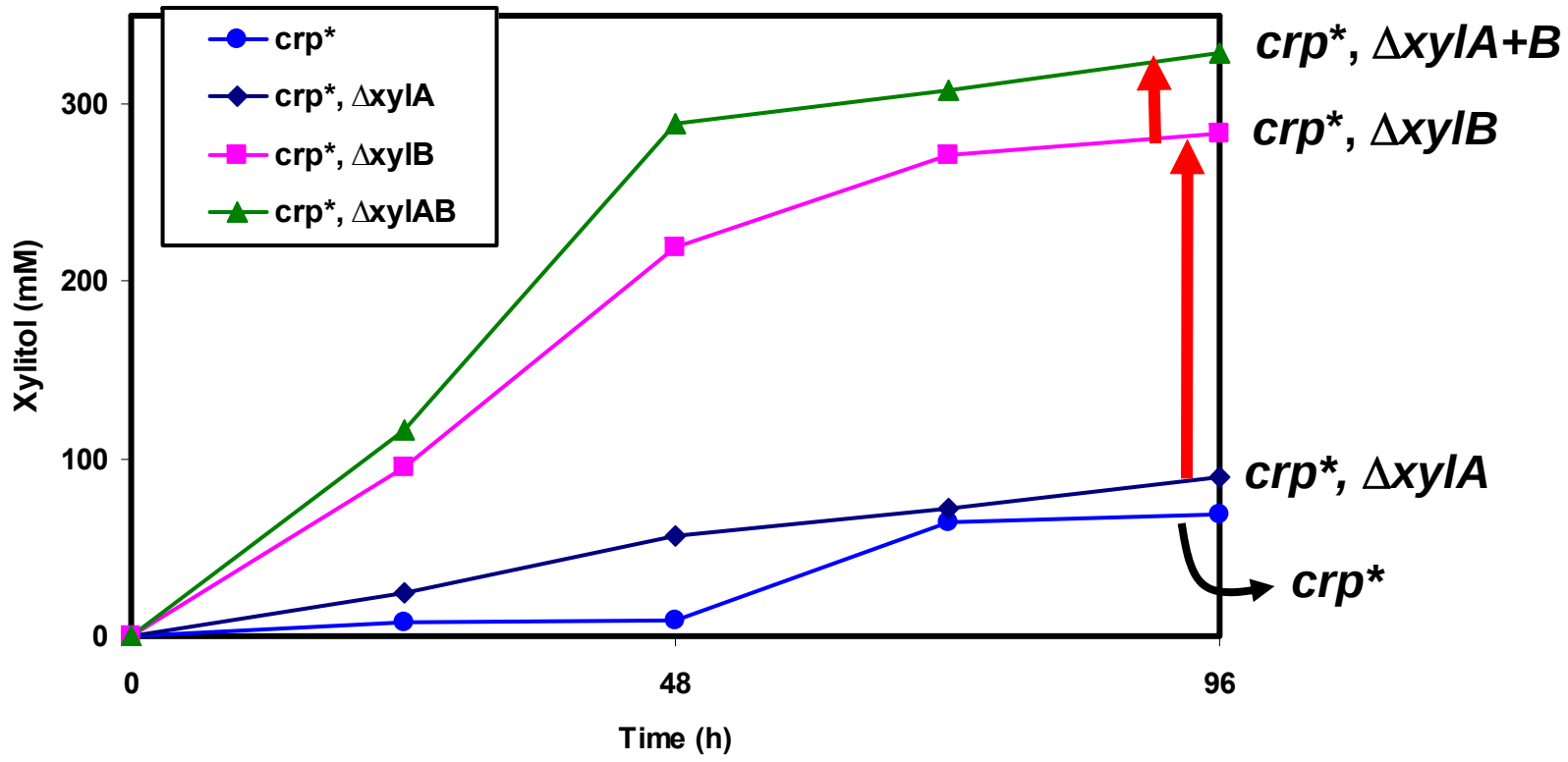
- Obtain reducing equivalents from glucose
- Utilize xylose solely for xylitol production



ΔXylA vs. ΔXylB vs. ΔXylA+B

Xylose $\xrightarrow{xylA}$ Xylulose $\xrightarrow{xylB}$ Xylulose-5-P $\longrightarrow$ Metabolism

Shake-flask cultures in rich medium supplemented with glucose and xylose

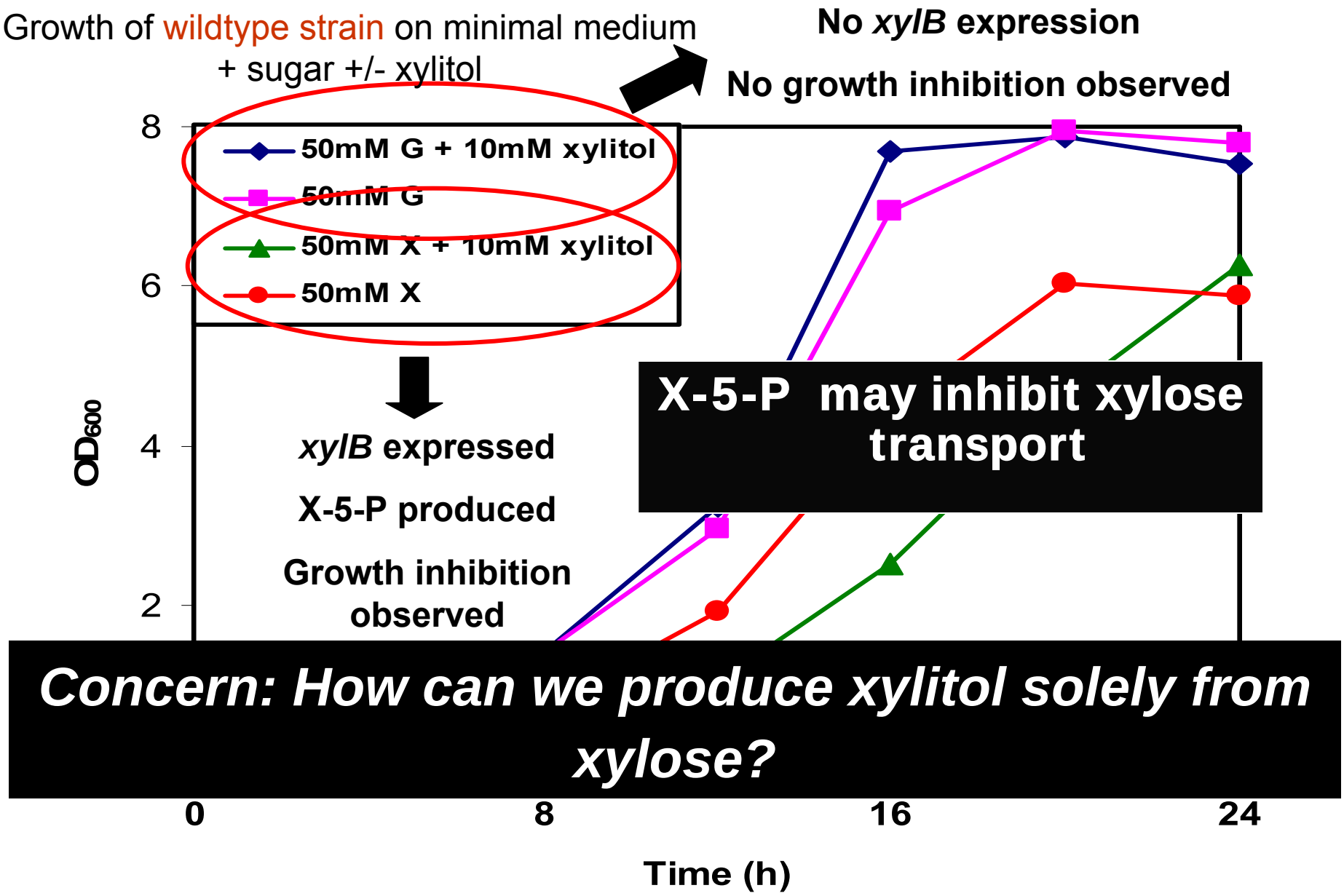


XylB deletion is necessary for high xylitol titer in strains!

Hypothesis: Xylitol + ATP $\xrightarrow{Xyl\ B}$ Xylitol-5-P + ADP

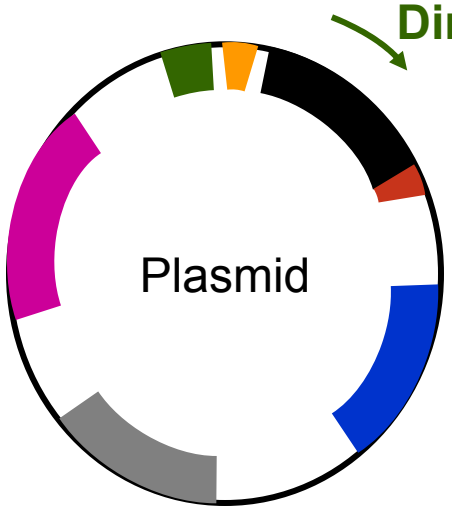
Xylitol-5-phosphate (X-5-P) may be toxic to cells. Mechanism of toxicity?

Possible Mechanism for X-5-P Toxicity



Concern: How can we produce xylitol solely from xylose?

Xylitol Production from Xylose



- *Tac* promoter sequence
- Multiple cloning site (MCS)
- Desired gene sequence
- Terminator sequence
- Antibiotic resistance sequence
- Origin of replication site
- *LacI* (repressor) gene

- Recruited xylulokinase (XK) from another organism
  - Xyl3 from *Pichia stipitis yeast*
  - Natural xylitol producer
  - 23% Amino acid sequence similarity with XylB
  - Cloned *P. stipitis xyl3* and *E. coli xylB* to plasmids inducible by IPTG

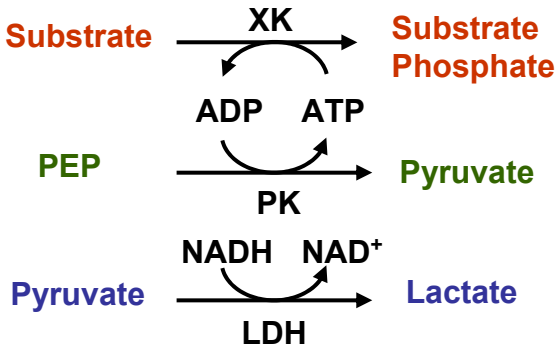
Protein Expression Analysis

- Functional expression of XK confirmed in Δ*xylB E. coli* strain (PC07)

Strain + cloned gene	OD_{600} t = 24
PC07 + control plasmid	0.08
PC07 + XylB	4.26
PC07 + Xyl3	5.22

- Activity of XKs on xylulose (native substrate) and xylitol was investigated

3 rxns coupled in assay



	Specific activity on xylulose (units/mg protein)	Specific activity on xylitol (units/mg protein)
No XK	< 0. 1	< 0. 1
XylB	1.63 ± 0.30	0.30 ± 0.11
Xyl3	1.20 ± 0.26	< 0. 1

PK: Pyruvate kinase; LDH: Lactate dehydrogenase

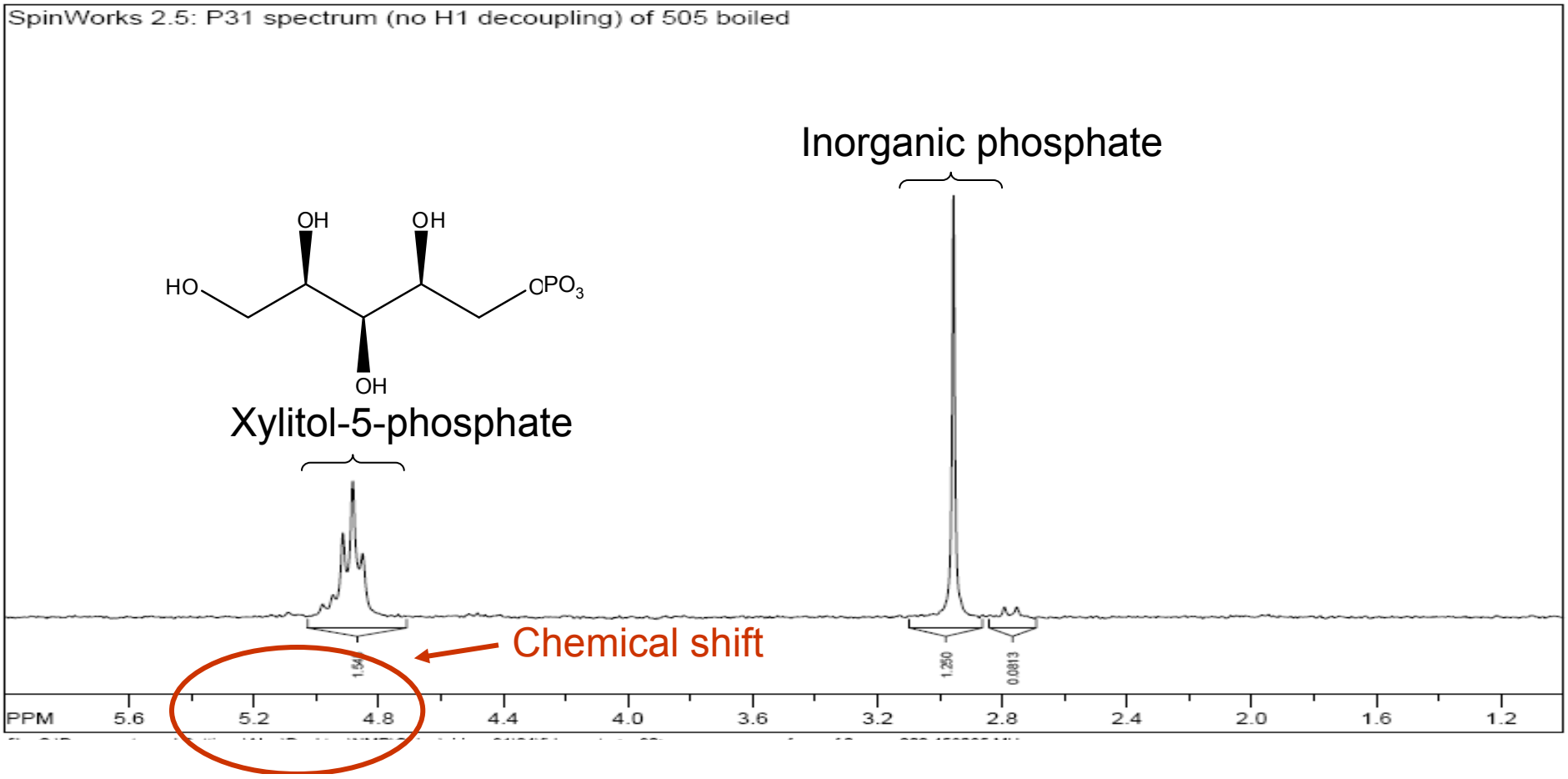
X-5-P Production Analysis Using ^{31}P NMR

Enzyme reactions performed *in vitro*

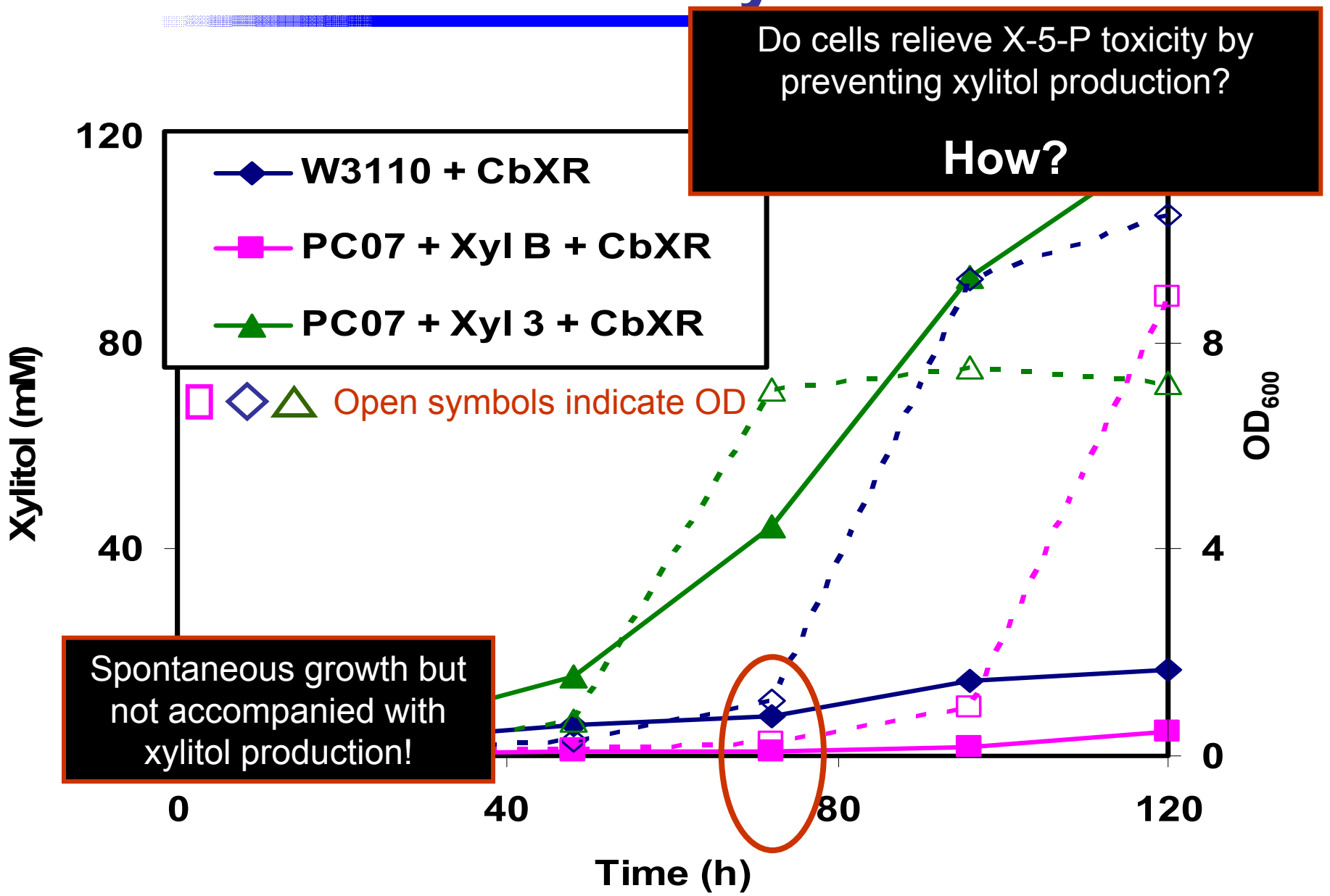
Xylitol + ATP —(**XK**)→ **X-5-P + ADP**

X-5-P peak area ~4X higher with XylB than Xyl3

*** Peak area normalized w.r.t. cell OD**



Shake-Flask Cultures:Xylitol Titers & ODs



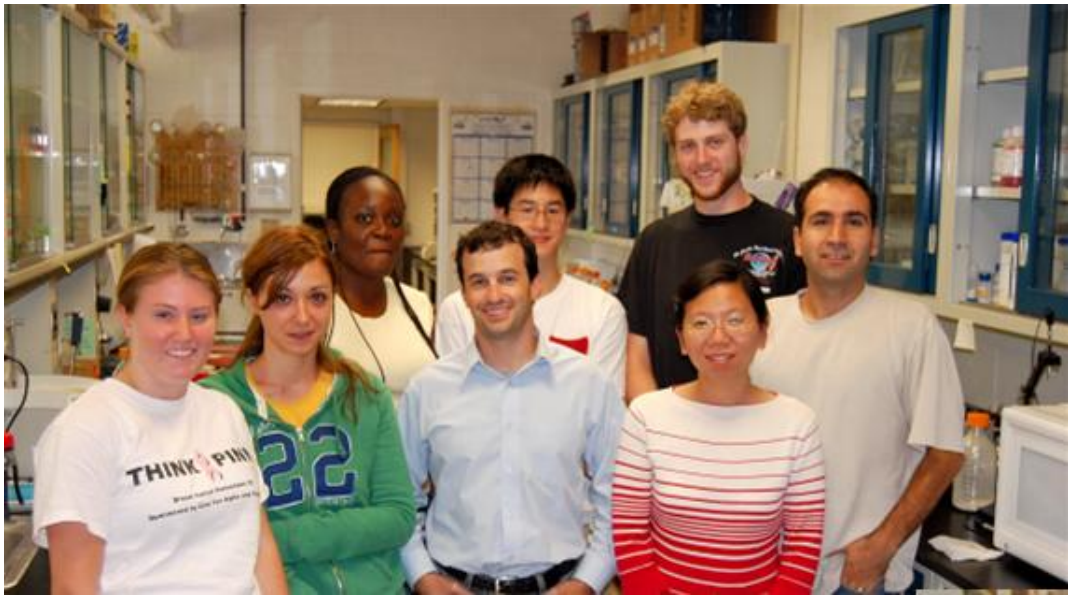
Cells conditioned in glucose minimal medium. Cultures in xylose minimal medium

Conclusions

- Deletion of *xylB* is necessary for obtaining high xylitol titers in xylitol-producing *E. coli* strains
- Expression of *xylB* results in the production of toxic phosphorylated intermediate: X-5-P
- X-5-P may inhibit xylose transport in *E. coli*
- Xylitol production from xylose is enabled by heterologous expression of *P. stipitis xyl3*

Acknowledgements

- Dr. P Cirino
- Cirino lab
- Dr A. Benesi (PSU Chem.)
- TW Jeffries, U Wisconsin



- Metabolic Engineering Working Group, BES 0519516
- Penn State Associate Vice President for Research

National Science Foundation
WHERE DISCOVERIES BEGIN

PENNSTATE
1855

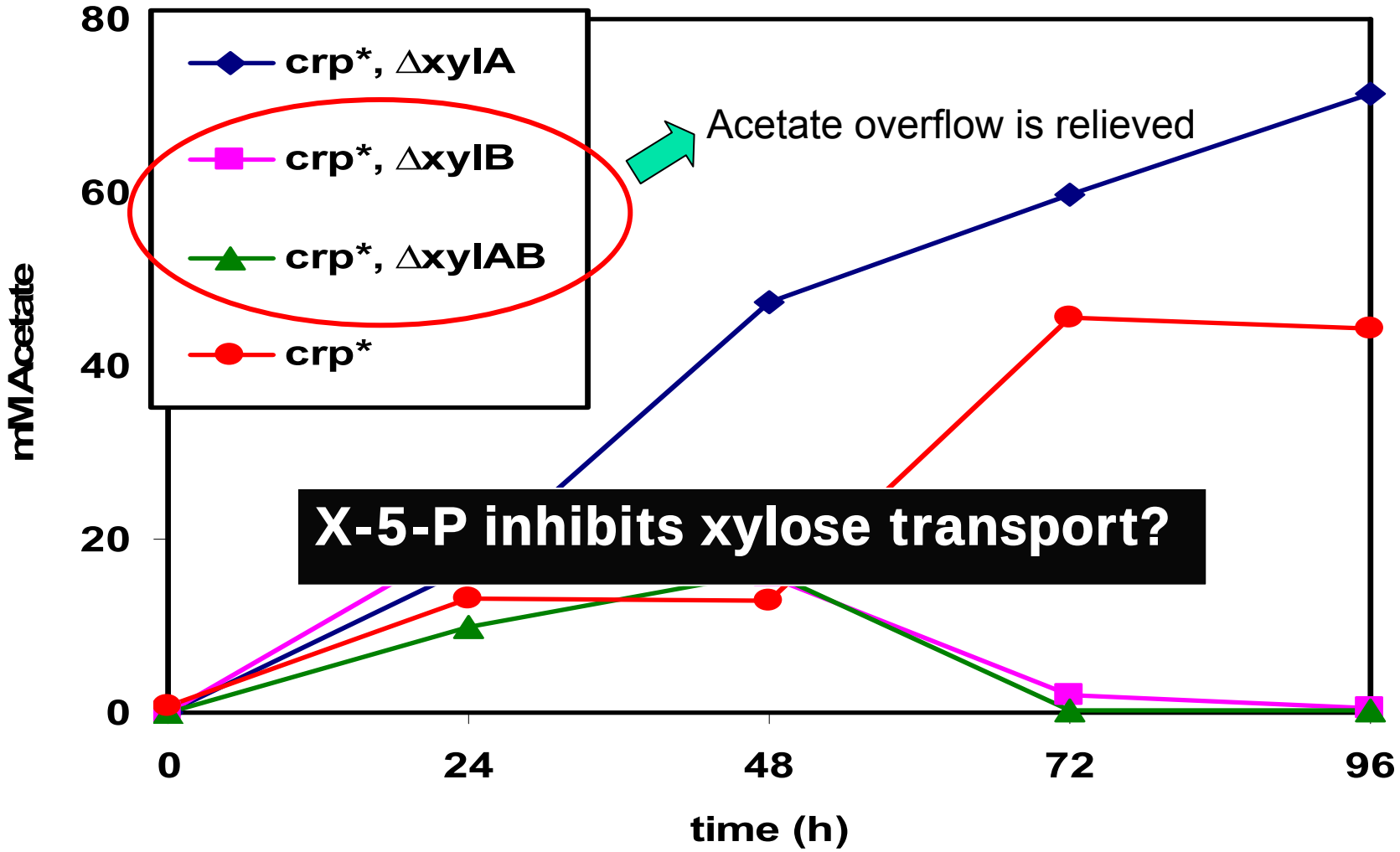
Questions

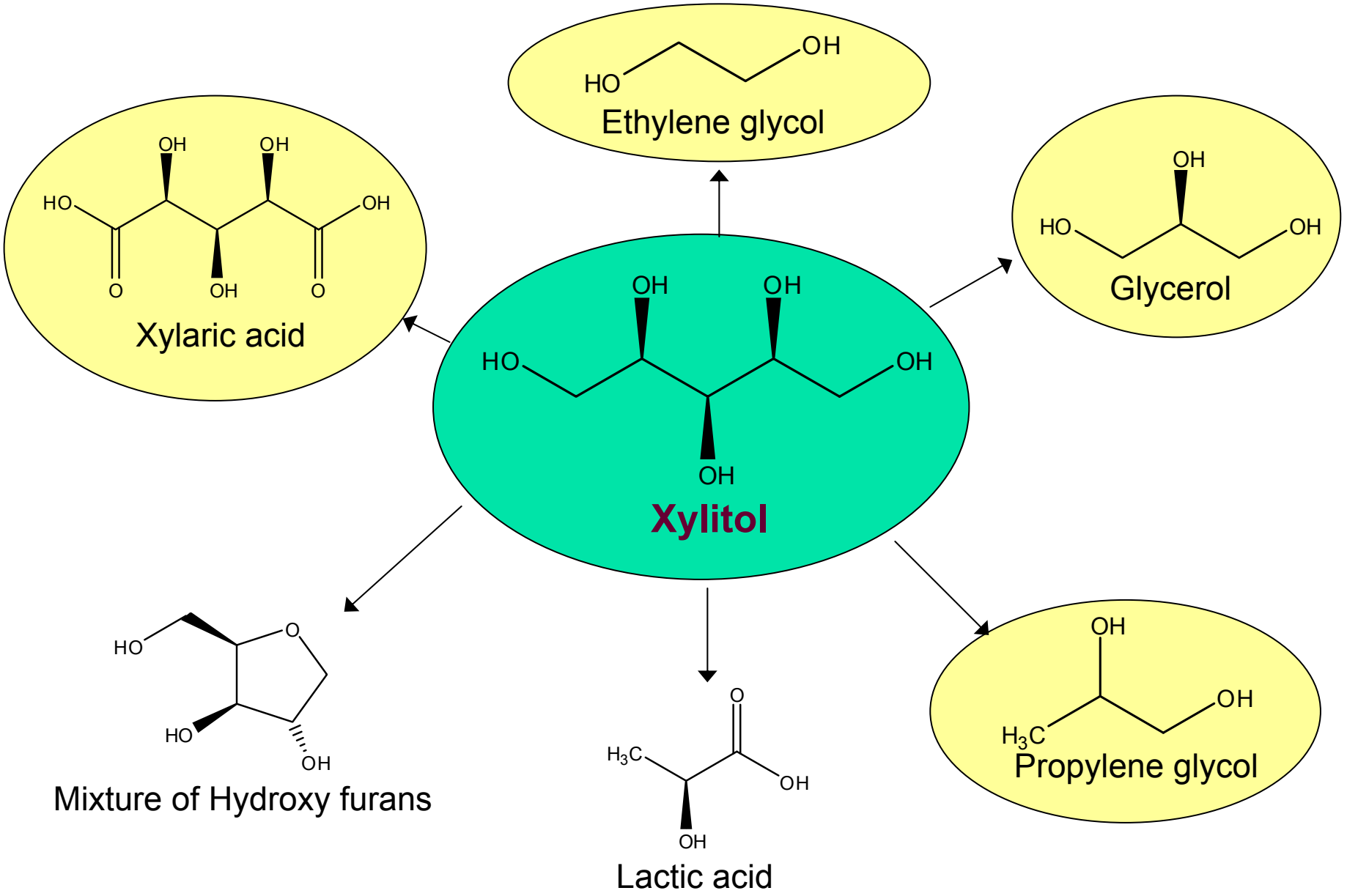
Thanks for Listening!

Acetate overflow in strains expressing XylB

Acetate overflow: Shunting of carbons from glucose to acetate

– Due to saturation of the respiratory pathways used in NADH reoxidation




Ethylene glycol
Xylaric acid
Glycerol
Xylitol
Mixture of Hydroxy furans
Lactic acid
Propylene glycol

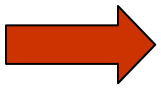
	% of total dry weight[1]	
	Hemicellulosic sugar	Xylan
Hardwoods	26	17
Softwoods	22	6
Wheat Straw	29	19
Corn Stalks	28	17
Soybean residue	19	13

[1] Jeffries TW: Advances in biochemical engineering/biotechnology 1983, 27:1-32

Batch cultures cont'd

- Batch culture inoculum conditioned on glucose minimal medium
  - Long growth lag phase
  - Switch to conditioning on xylose minimal medium
  - Results:
    - Growth lag reduced: growth in the first 24 hours
    - Xylitol production: xylose minimal medium

Strain	Xylitol t = 72 (mM)
W3110 + *Cb*XR	0.53
PC07 + XylB + *Cb*XR	0.00
PC07 + Xyl3 + *Cb*XR	143.36



Cells relieve growth inhibition/ production of X-5-P by preventing xylitol production!

How?

Xylitol yield in other organisms

Author	Host Organism	Xylitol Gene	Media	Yield
Lee et al.[1]	*S.cerevisiae*	*S.cerevisiae* GRE3	Defined	0.199 mol xylitol/mol glucose
Winkelhausen et al.[2]	*C.boidinii*	*C.boidinii* XR	Complex	0.436 mol xylitol/mol xylose
Kim et al.[3]	*S.cerevisiae*	*S.cerevisiae* GRE3	YPD	0.474 mol xylitol/mol glucose
Kim et al.[3]	*S.cerevisiae*	*P.stipitis* XR	YPD	0.921 mol xylitol/mol glucose
Bae et al.[4]	*S.cerevisiae*	*P.stipitis* XR	YPD	1.32 mol xylitol/mol glucose
Kim & Oh[5]	*C.tropicalis*	*C.tropicalis* XR	Defined	0.866 mol xylitol/mol xylose
Converti & Dominguez[6]	*Debaryomyces hansenii*	*D.hansenii* XR	Complex	0.799 mol xylitol/mol xylose
Suzuki et al.[7]	*E.coli*	*C.tropicalis* XR	LB	3.15 mol xylitol/mol glucose

1) Lee et al. Process Biochemistry 35 (2000):1199-1203
2) Winkelhausen et al. Engineering in Life Sceinces (2004): 150-154.
3) Kim et al. Enyme and Microbial Technology (2002): 862-866.
4) Bae et al. Enzyme and Microbial Technology (2004): 545-549.
5) Kim & Oh. Biotechnology Letters (2003): 2085-2088.
6) Converti & Dominguez. Biotechnology and Bioengineering (2001):39-45.
7) Suzuki et al. Journal of Biosceince and Bioengineering (1999): 280-284.