Improved xylose fermentation in *Zymomonas mobilis* through metabolic engineering and adaptation

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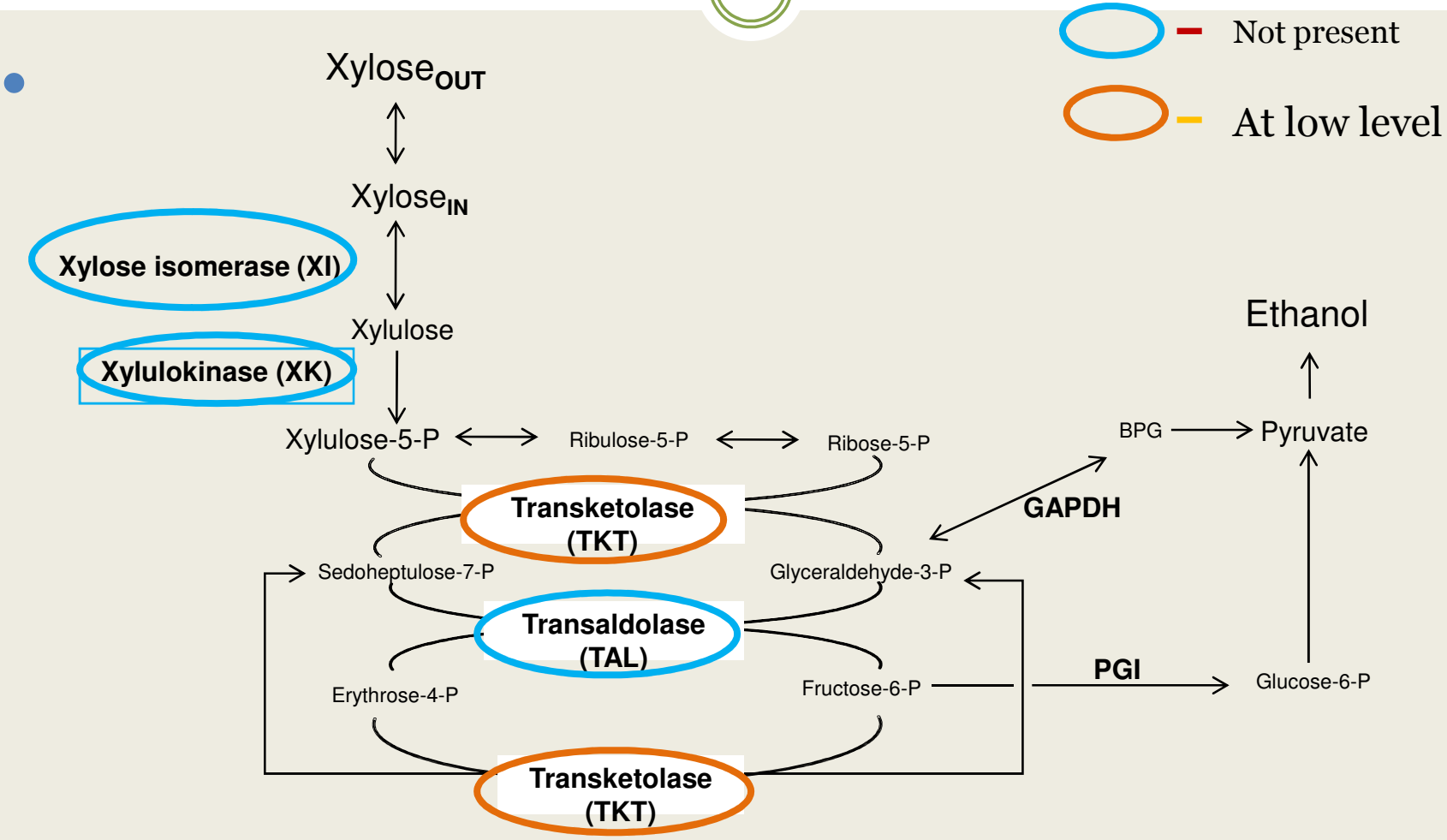
Zymomonas mobilis as cellulosic ethanol producer

- 2-5 fold higher productivity than *S. cerevisciae*
- High tolerance to ethanol and glucose
- High yield of ethanol from glucose (97%)

- Engineered strains are able to utilize xylose
- Rate of xylose consumption ~4 fold lower than glucose
- Incomplete xylose utilization when initial concentration >6%

Metabolic engineering for xylose fermentation

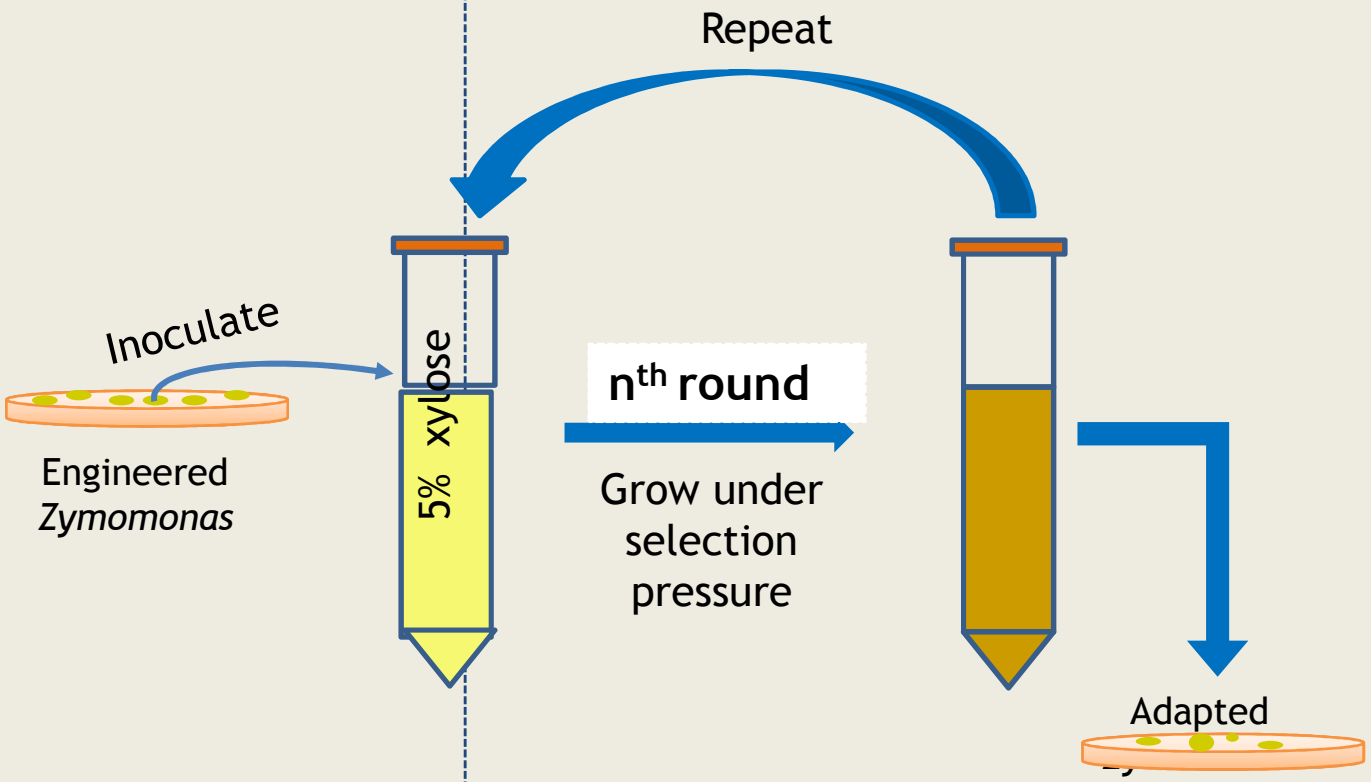


Adaptation is necessary

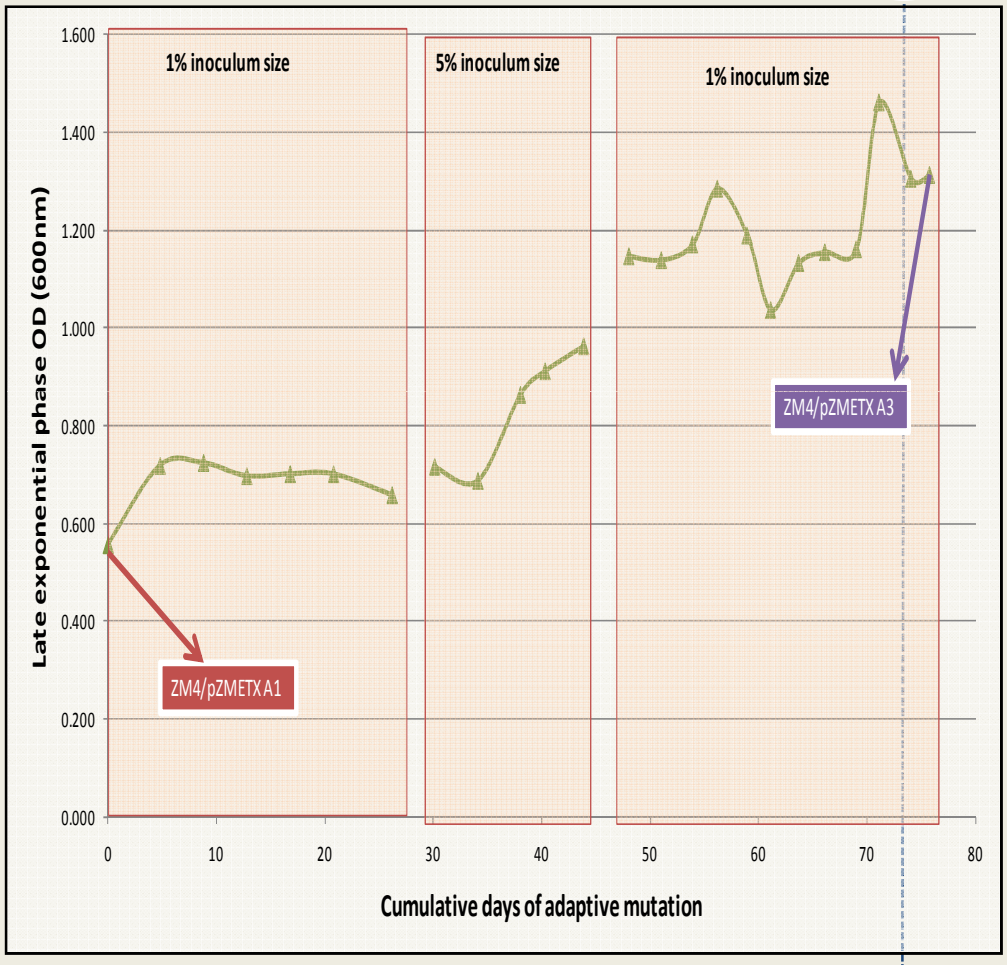
To obtain an initial strain capable of use xylose –A1

- 4%-G-1% X
- 3%-G-2% X
- 2%-G-3% X
- 1%-G-4% X
- 0%-G-5% X

To obtain a much improved strain-A3

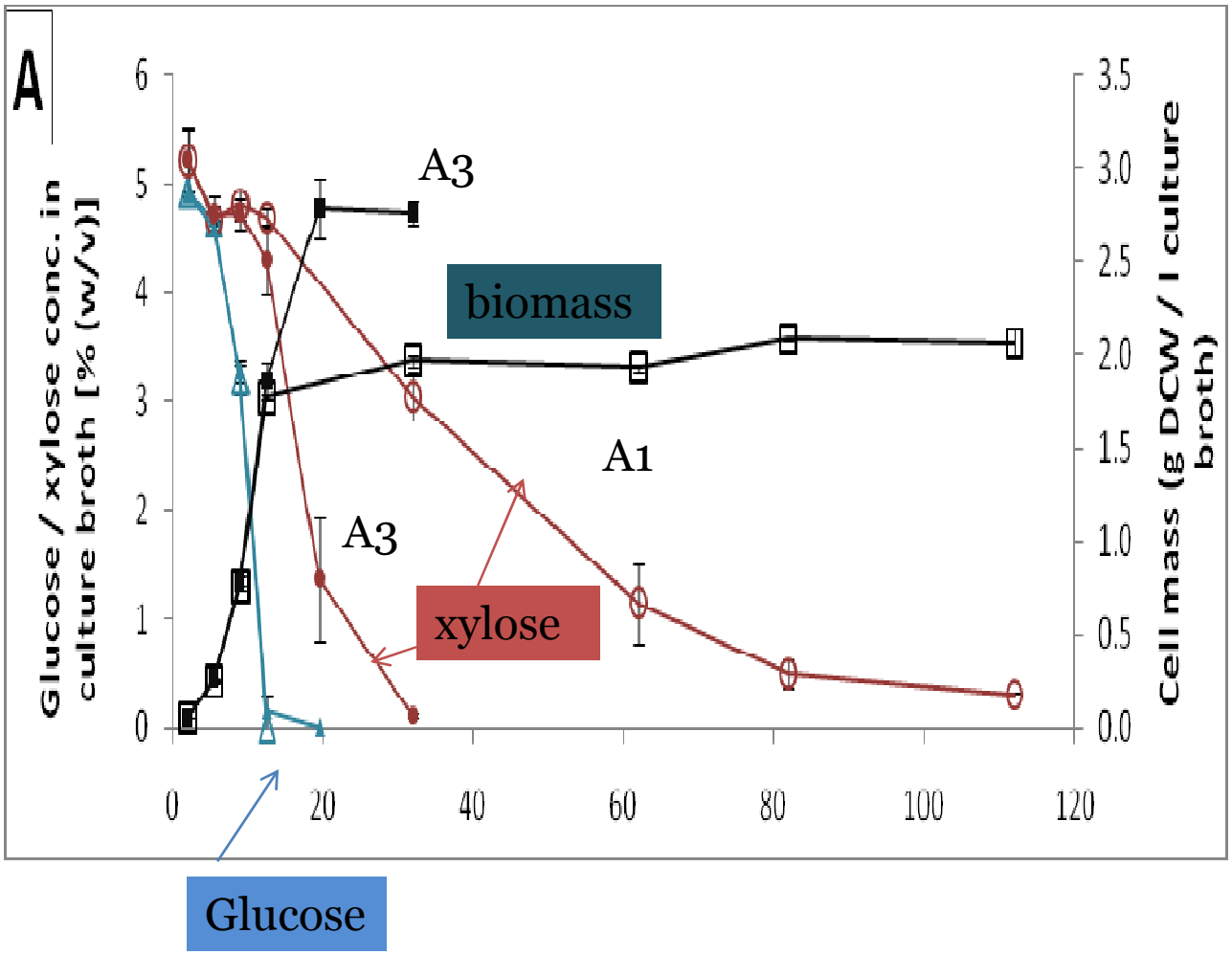


The progression of adaptation



- Adaptation over 2.5 months (31 serial transfers)
- Improvement in cell growth
  - (OD_{600} doubled from 0.6 to ~1.3).
- Improvement in consumption rate of xylose
  - (xylose consumed in 2.5 days from originally 4 days initially).

A3 vs.A1 in 5%G-5%X fermentation

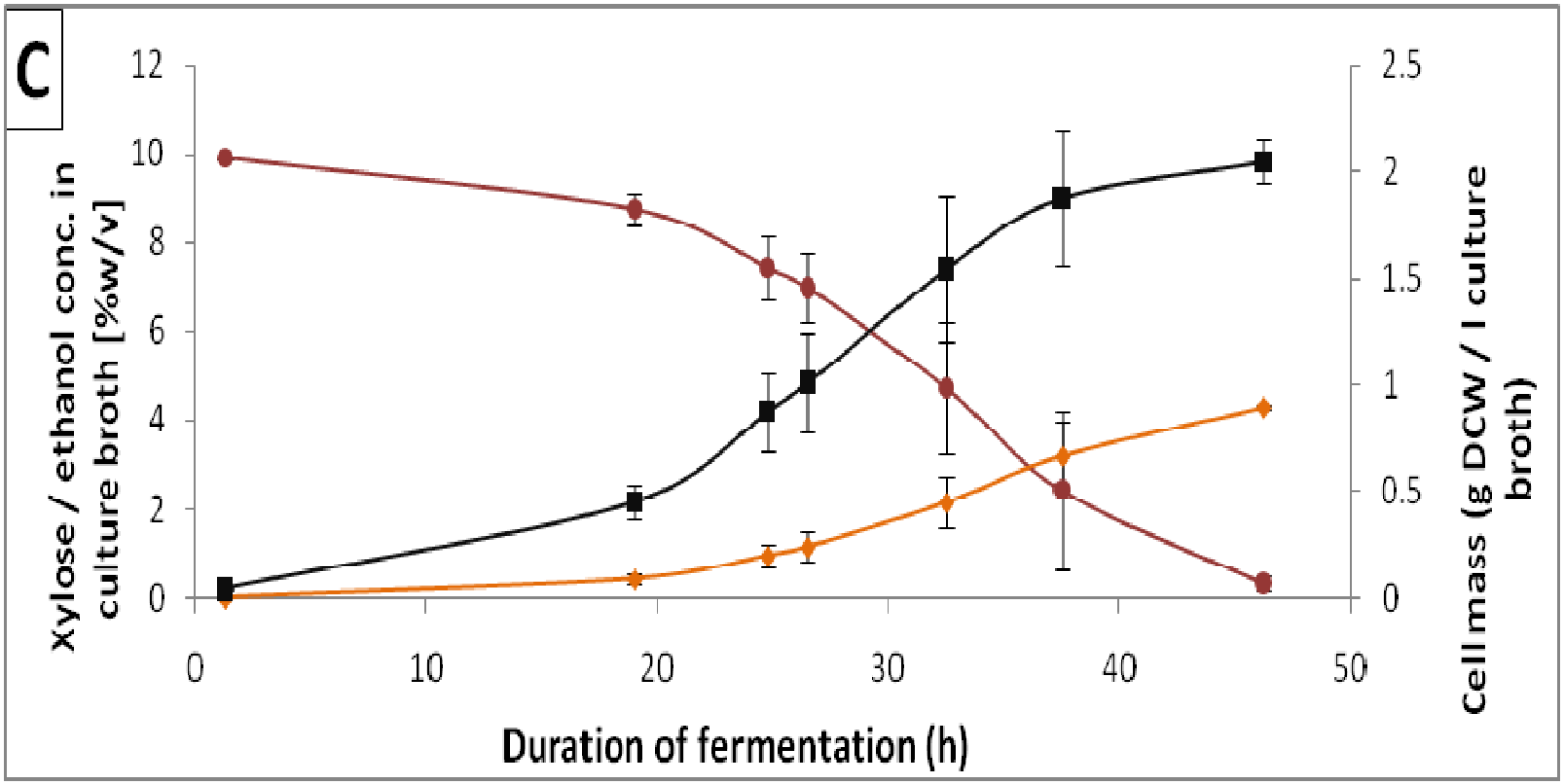


No change in gluclose metabolism

3 fold increase in xylose consumption (xylose consumed after ~35 hrs compared to > 110 hr for A1)

50% increase in cell biomass for A3

A3 was able to grow on 10% xylose

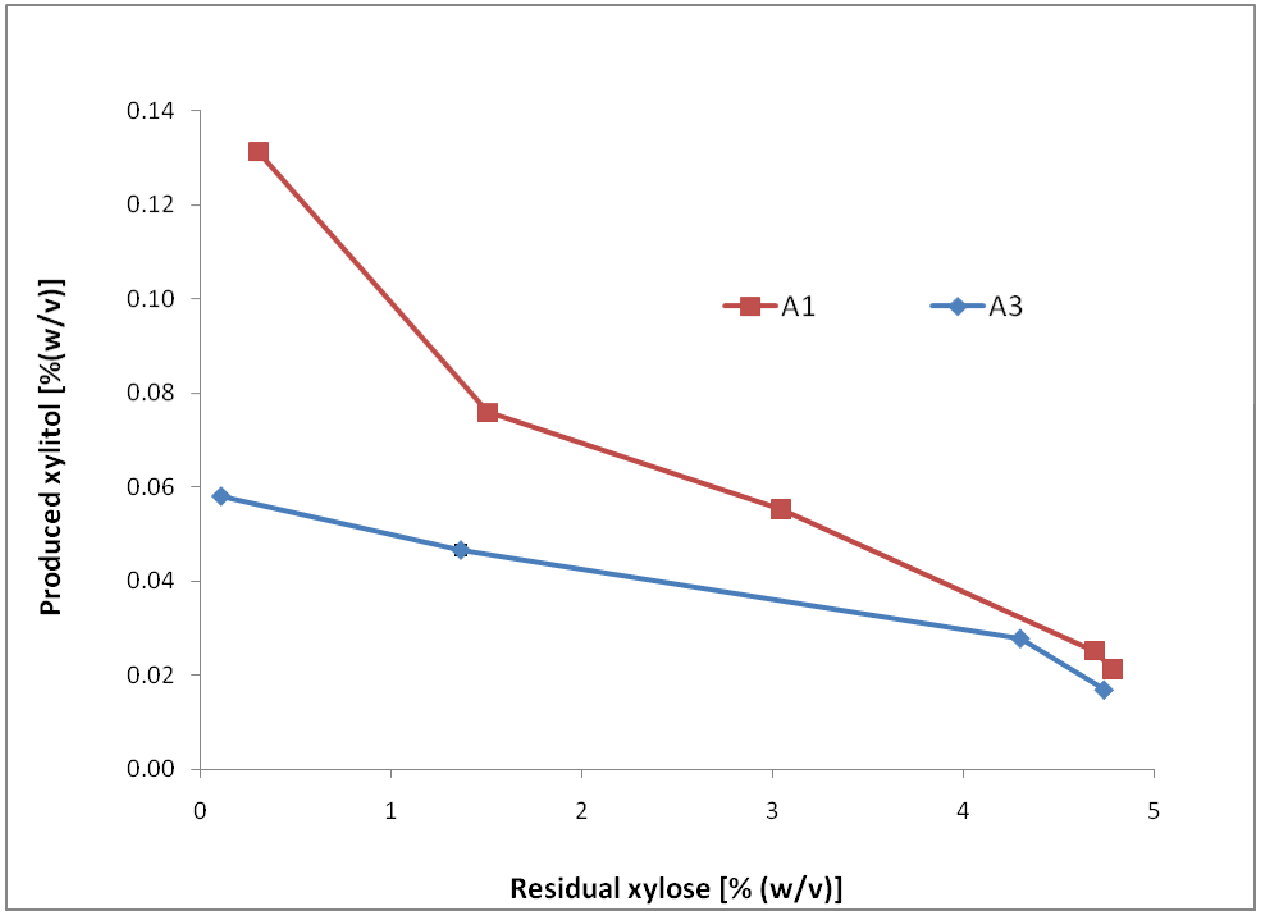


10% xylose was consumed within 2 days.

A3 vs. A1

Strain	sugar conc. [% (w/v)]	Max sp. rate of xylose consumption (g/g/h)	Final ethanol titer [% (w/v)]	Ethanol yield (%)[a]
A1	Mixed sugar (5%G-5%X)	0.45 ± 0.05	4.73 ± 0.06	93.7 ± 1.8
A3	Mixed sugar (5%G-5%X)	1.80 ± 0.05	4.99 ± 0.06	96.6 ± 4.8
A3	10% xylose	3.44 ± 0.25	4.31 ± 0.04	88.2 ± 0.1
A3	10%G-10%X		9.0	90%

What is responsible for the improvement?



A3 produces much less xylitol

A3 tolerates exogenous xylitol better than A1

	Avg[a] specific growth rate (h^{-1})	Reduction in sp. growth rate for cells cultured in 0.1% xylitol	Xylose consumption at 46 hr
A1 (0% xylitol)	0.069 ± 0.002	47.8%	70%
A1 (0.1% xylitol)	0.036 ± 0.001		24%
A3 (0% xylitol)	0.094 ± 0.001	20.2%	100%
A3 (0.1% xylitol)	0.075 ± 0.005		74%

Enzyme activities in xylose assimilation pathway

Strain	XI	XK	Tal	Tkt	GAPDH	PGI
A1	23 ± 0	1700 ± 200	610 ± 30	350 ± 70	1200 ± 100	1900 ± 200
A3	105 ± 7	2300 ± 100	540 ± 40	430 ± 70	1500 ± 200	2400 ± 100
A1	31 ± 1	1800 ± 200	520 ± 70	280 ± 10	1500 ± 200	3300 ± 200
A3	162 ± 28	2200 ± 400	700 ± 40	380 ± 70	1800 ± 400	3500 ± 200

XI is the rate-limiting step with activity being the lowest

XI activities were increased 4-8 times in A3

Adaptation selectively accelerates the rate- limiting step.

? reductase

Xylose → Xylitol

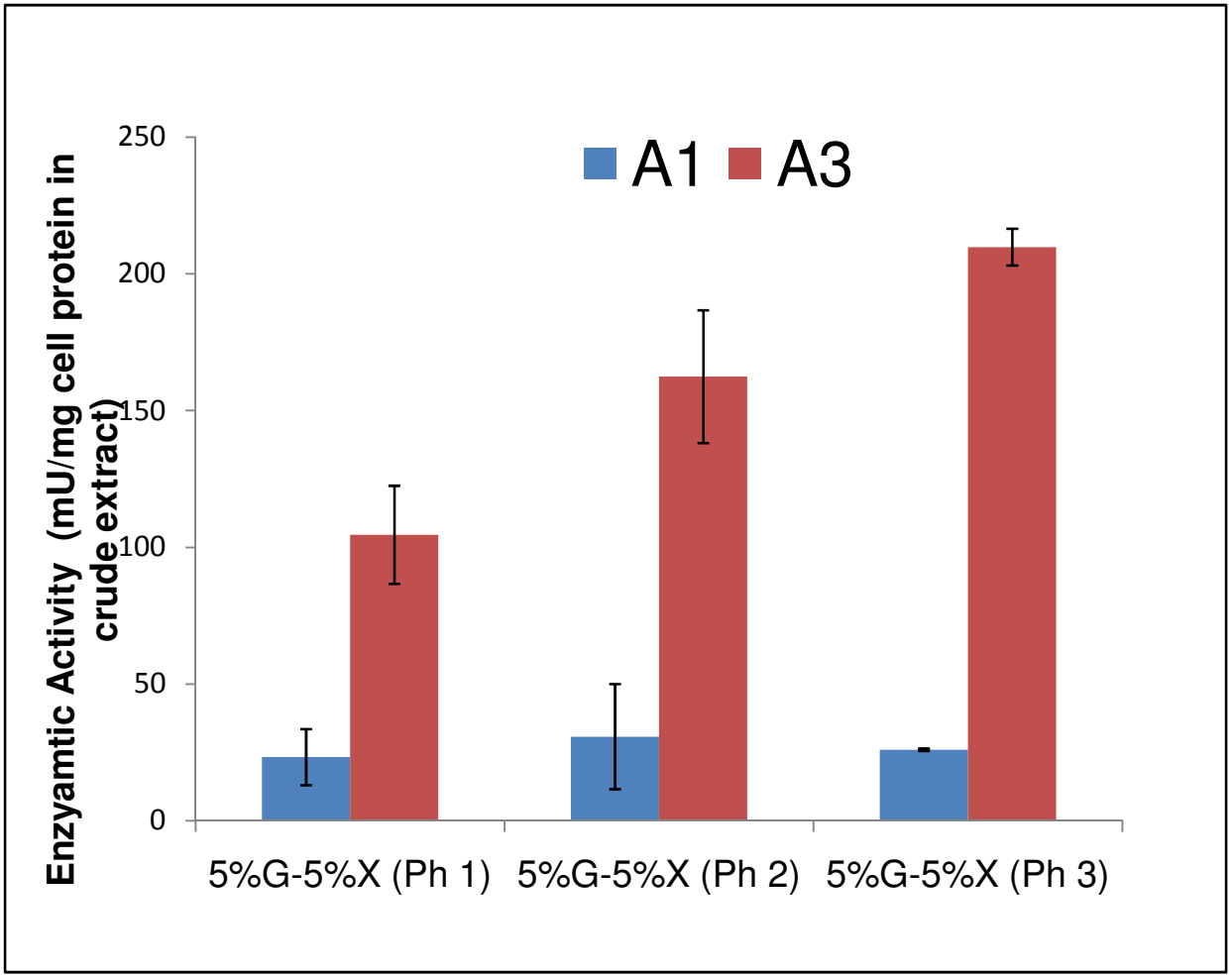
XI

↓

Xylulose

Increased XI help chennaling more carbon to ethanol production.

A mutation was found on a plasmid from A3.



Why A3 produces less xylitol?

Identification of xylose reductase

1. Whole genome sequence was searched against a known fungal XR
2. Six genes with significant homology were identified
3. These 6 genes from A1 were sequenced and compared to their counterparts in A3
4. One gene (ZMO0976) was found to have a mutation

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  1 mntstqkpah fdkisikgid ksatrvalgt waiggwmwgg tdddasikti hraidlgini
 61 idtapaygrg haeevvgkai kgqrdnliia tkvgldwtlt pdqsmrrnss asrikkeied
121 slrrlgtdyi dlyqvhwpdp lvpieetati lealrkegki rsigvsnysv qqmdefkkya
181 elavsqspyn lfereidkdi lpyakkndlv vlgygalcrg llsgrmtadr aftgddlrkt
241 dpkfqkprfe hylaaveelk klakehynks vlalairwml eqgptlalwg akkpeqidgi
301 devfgwqisd edlkqidail aknipnpiga efmappprdk
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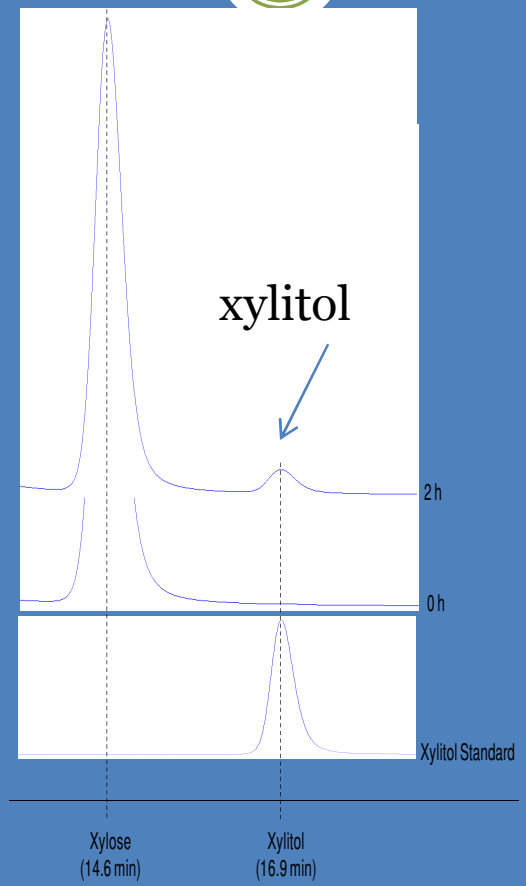
c

Could this gene encodes the XR?



Single mutation drastically reduces XR activities.

Samples	Activity (mU/mg protein)
E. coli expressing ZMO0976 (CE)	460 ± 50
E. coli expressing mZMO0976 (CE)	8 ± 2
Purified recombinant ZMO0976	3400 ± 200
Purified recombinant mZMO0976	140 ± 50

In vitro confirmation of xylose reductase activity of ZMO 0976

Conclusions

- Adaptation is a powerful tool for strain improvement
  -much improved xylose-fermenting strain
  - Pinpoint rate-limiting steps
  - Identify an elusive enzyme for xylitol production
- Mechanistic studies led to a better understanding about xylose fermentation in *Z. mobilis*
  - Reduced xylitol formation
  - Increased XI activity
  - A single amino acid mutation in XR gene
- Xylitol metabolism holds the key for the improvement.

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