Maximizing Ethanol Production by Engineered Pentose-Fermenting *Zymomonas mobilis*

Dhinakar S. Kompala

Department of Chemical Engineering
University of Colorado, Boulder

James D. McMillan and Min Zhang

Biotechnology Center for Fuels and Chemicals
National Renewable Energy Laboratory

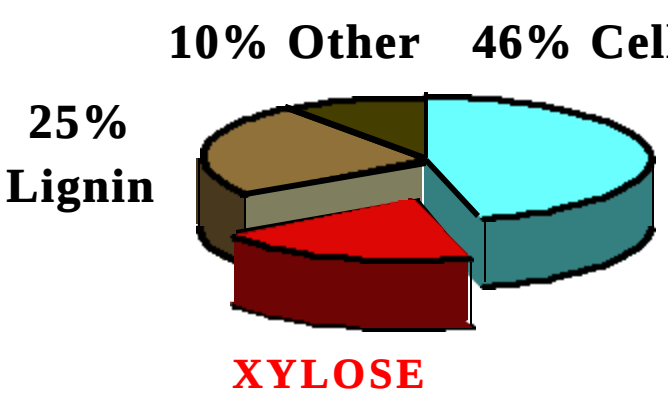
Objective

Develop robust microbial biocatalysts capable of effectively conversion of a variety of sugar feedstreams to fuels (and chemicals)

Strategic Targets

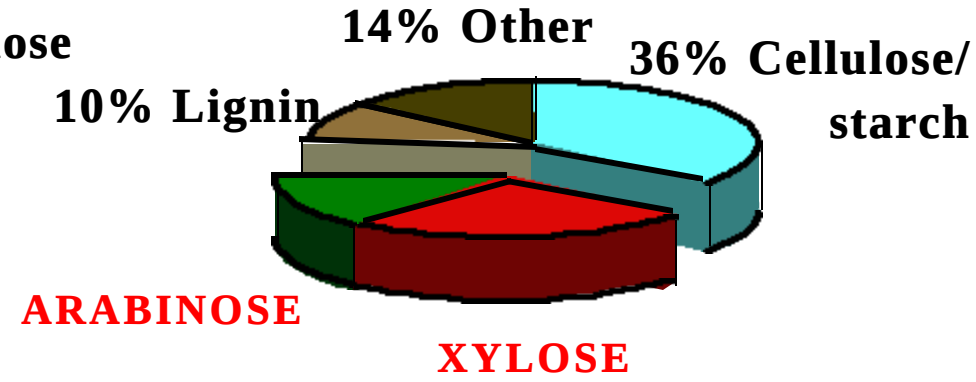
- **Increase Ethanol Yield**
- **Increase Ethanol Concentration**
- **Reduce Fermentation Time**

Biomass Composition



19% Hemicellulose

- Hardwood Species



40% Hemicellulose

- Agricultural Residues

(i.e., Corn Fiber, Corn Stover)

Fermentation Performance Criteria

Essential Microbial Traits

- High Conversion Yield
- High Ethanol Tolerance
- Resistance to Inhibitors in Hydrolysates
- No Oxygen Requirement
- Low Fermentation pH
- Broad Substrate Utilization Range
- GRAS Status

Desirable Traits

High sugar consumption rate
High specific growth rate
High volumetric productivity
High specific productivity
C5/C6 cofermentation
Minimal nutrient requirements
High salt tolerance (acetate)
Entner-Doudoroff pathway
Facilitated sugar transport
Non spore forming
Non-conjugative
Amenable to scale-up
Availability of "industrial"strains
Compatibility with SSF
Cellulase producer
Thermotolerance
High shear tolerance
Available gene transfer system

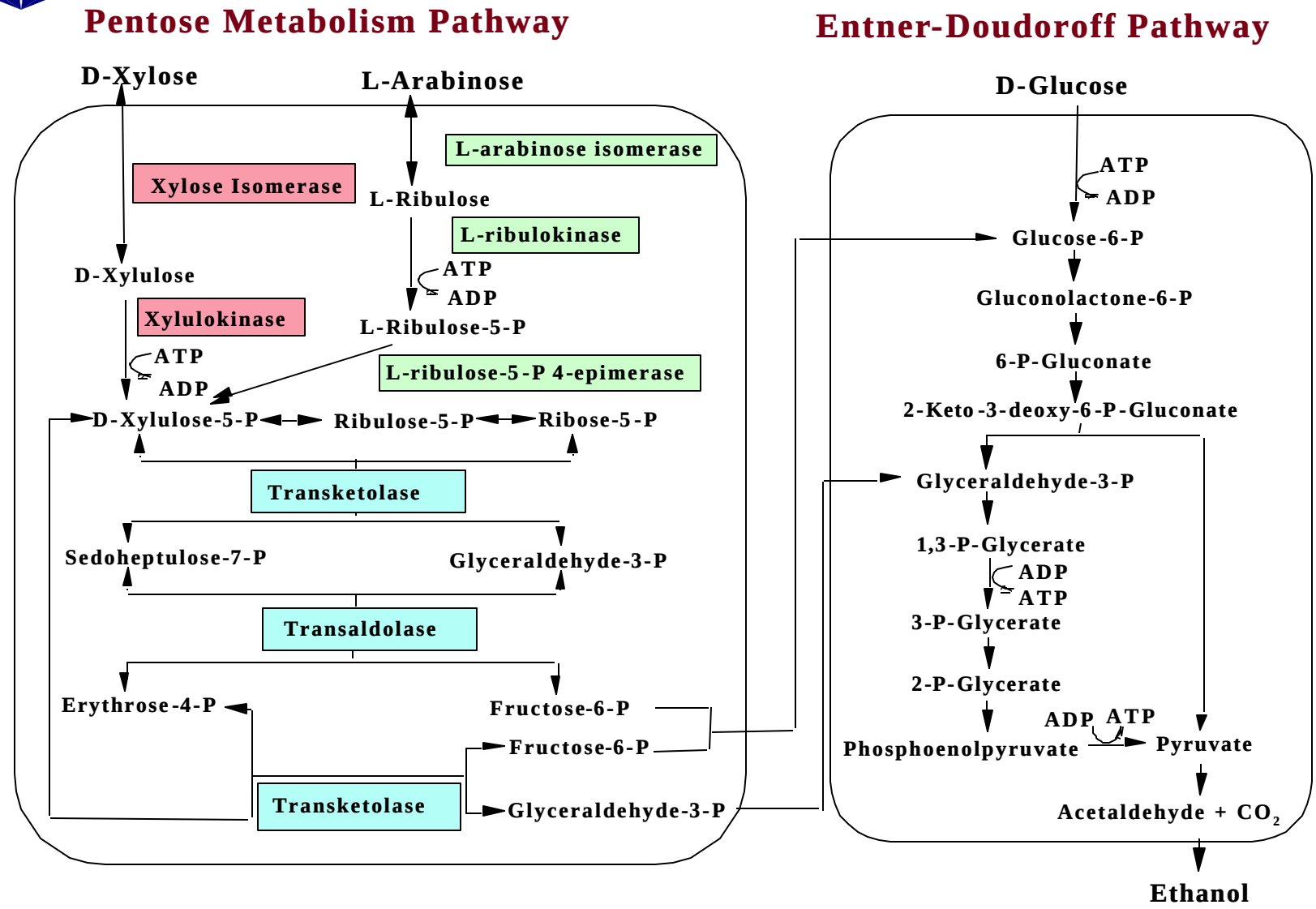
Promising Biocatalysts

- *Zymomonas*
- Recombinant *Saccharomyces*
- Recombinant *E. coli*
- *Lactobacillus*
- Xylose-Assimilating Yeasts
- *Clostridium*

Highlights of *Zymomonas mobilis*

- **Advantages:**
  - **Natural fermentative microorganism (GRAS)**
  - **Near theoretical ethanol yield from glucose**
  - **Reduced yield loss to biomass formation**
  - **No oxygen requirement**
  - **Tolerant to inhibitors in hydrolysates**
  - **High ethanol tolerance**
  - **Fermentation at low pH**
  - **Grows at high sugar concentrations**
  - **High specific productivity**
- **Limitations**
  - **Narrow substrate utilization range**

Pathways Required for Pentose Fermentation



Cloning Strategy for Pentose Metabolism Pathway

Xylose Assimilation Genes

Z. mobilis genome *E. coli* genome

PCR Synthesis Subcloning

XhoI/NotI XhoI SalI NotI

P_{gap} P_{xyl} *xylA* *xylB*

NotI NotI

P_{gap} *xylA* *xylB*

P_{gap}-*xylA/xylB* Operon

Arabinose Assimilation Genes

Z. mobilis genome *E. coli* genome

PCR Synthesis

P_{gap} *araB'* *araB* *araA* *araD*

PCR-mediated overlap extension and Subcloning

P_{gap} *araB* *araA* *araD*

P_{gap}- *araBAD* Operon

Pentose Metabolism Genes

Z. mobilis genome *E. coli* genome

PCR Synthesis

BglII XbaI XbaI BglII

P_{eno} *tal* *tktA*

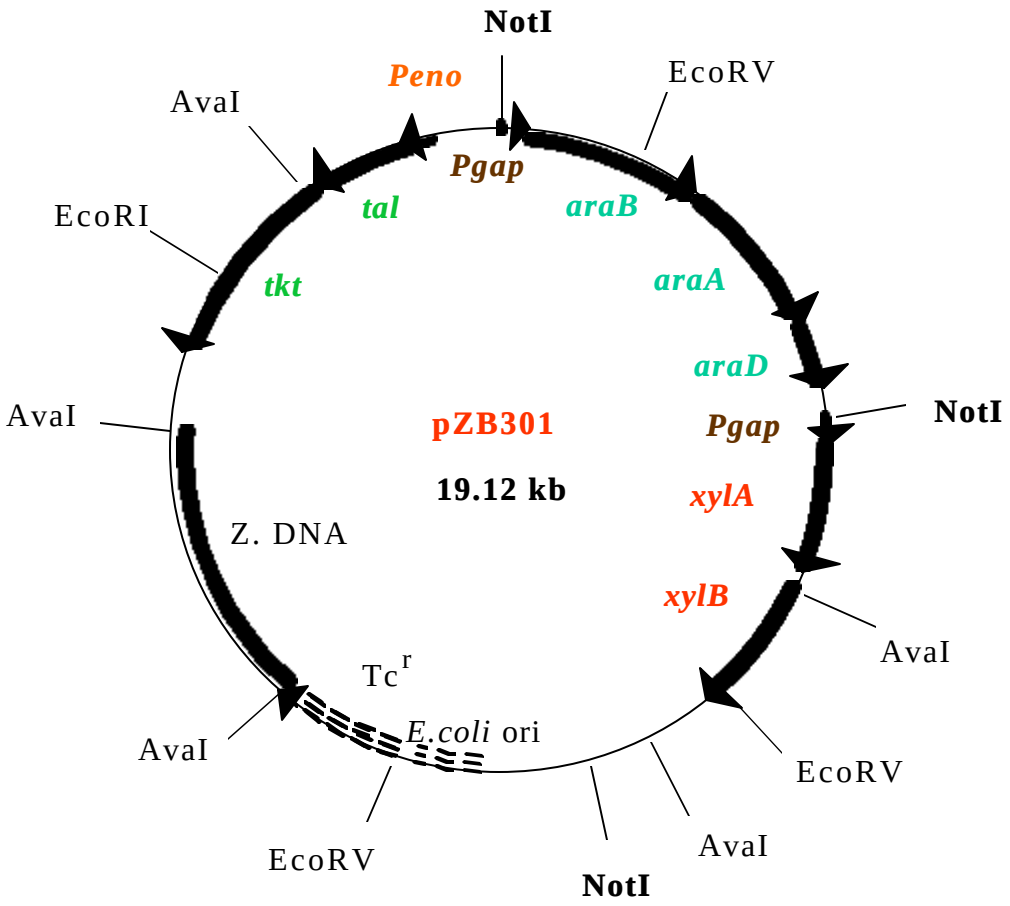
BglII BglII

P_{eno} *tal* *tktA*

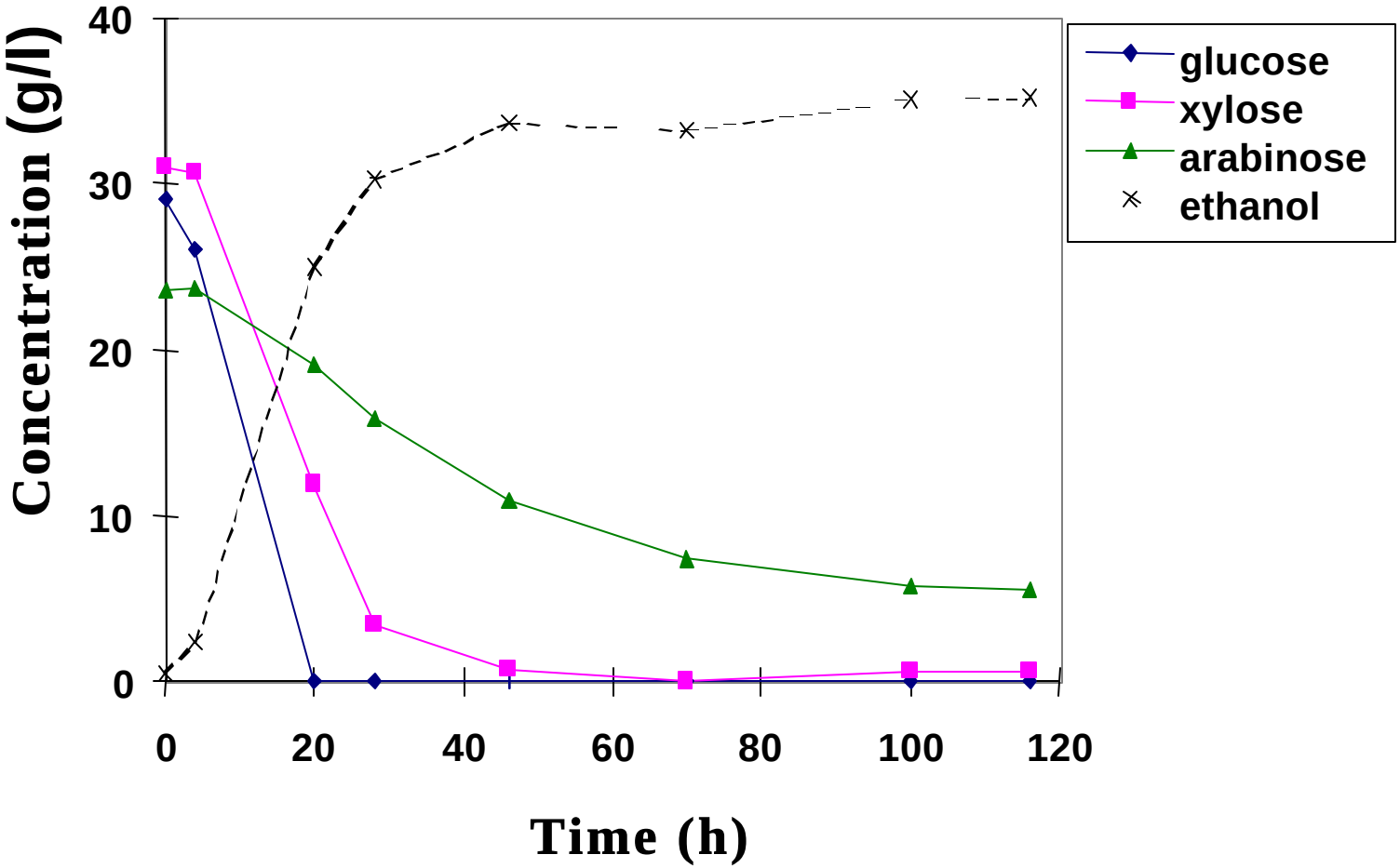
P_{eno}-*tal/tktA* Operon

Subcloning

Plasmid Containing Xylose- and Arabinose-Fermenting Genes



Fermentation Profile by *Z. mobilis* 206C/pZB301 grown on Glu:Xyl:Ara (30:30:20 g/l) at pH = 5.5, T = 31.5°C



Specific Aims

1. Develop new genetic engineering tools for the control of gene dosage and expression, and construct improved strains of *Z. mobilis* for efficient ethanol fermentation.

2. Measure intracellular activity through enzymatic assays, HPLC and NMR spectroscopy and evaluate ethanol production by the new strains on mixed sugars.

3. Develop a structured kinetic model for the integrated pathway network and identify potential improvements through dynamic simulations.

Strategy to Develop More Stable r*Zymomonas* Strains

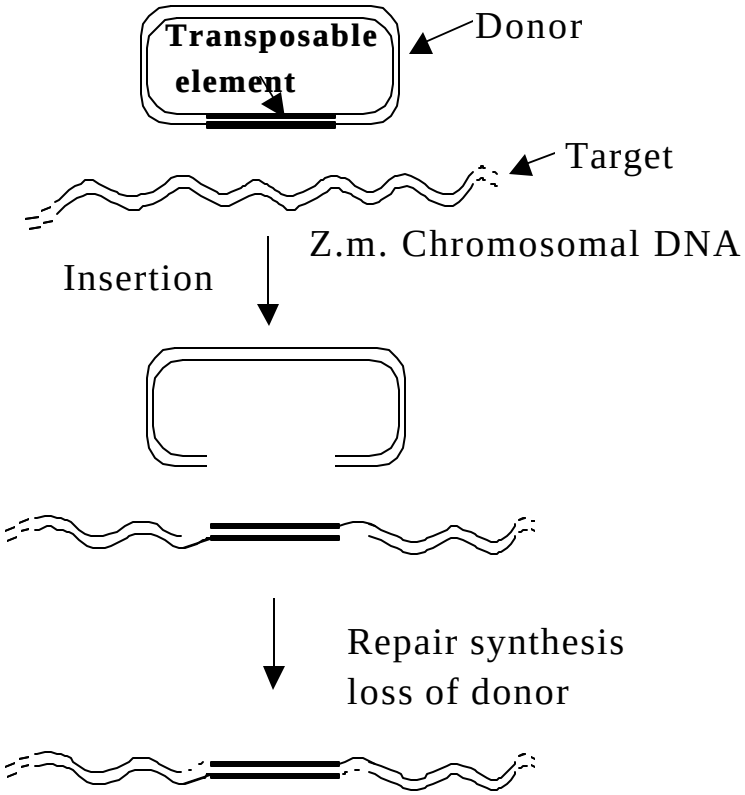
- ✓ **Develop integration systems.**

- ✓ **Integrate xylose assimilation genes (*xylA*, *xylB*)**
  pentose-phosphate pathway genes (*tal*, *tkt*)
  into the *Zymomonas* genome.

- ✓ **Integrate xylose assimilation genes (*xylA*, *xylB*)**
  arabinose assimilation genes (*araA*, *araB*, *araD*)
  pentose-phosphate pathway genes (*tal*,*tkt*)
  into the *Zymomonas* genome.

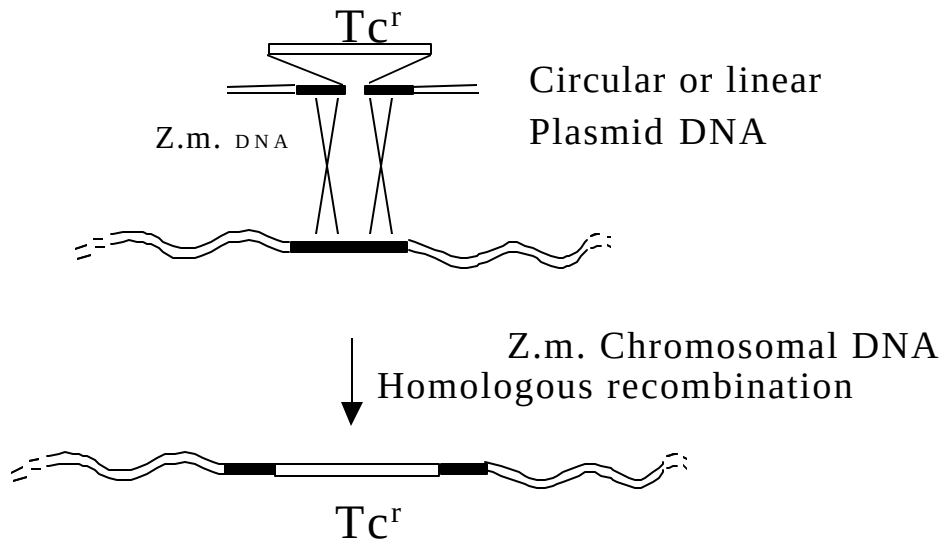
Strategy for Gene Integration

Gene Integration by Transposon



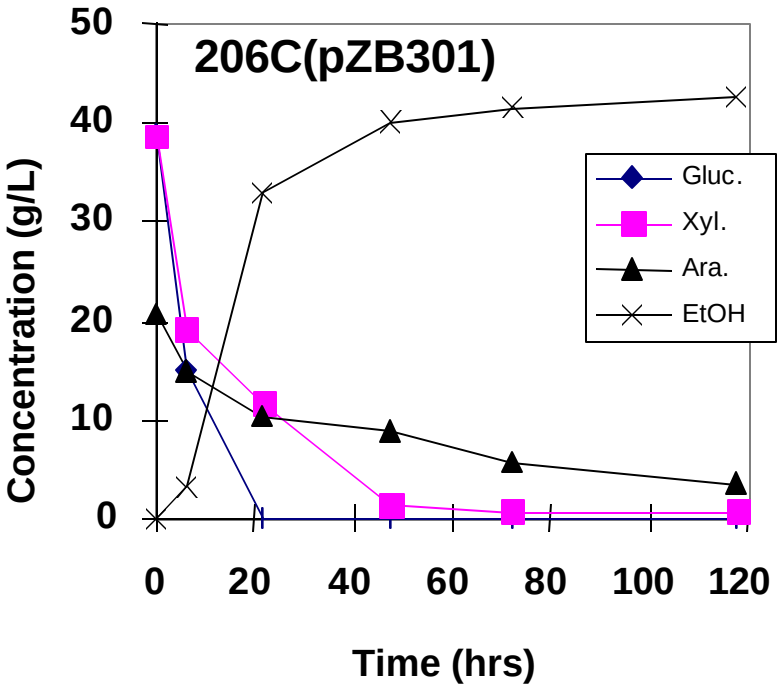
Random insertion
Large DNA fragment

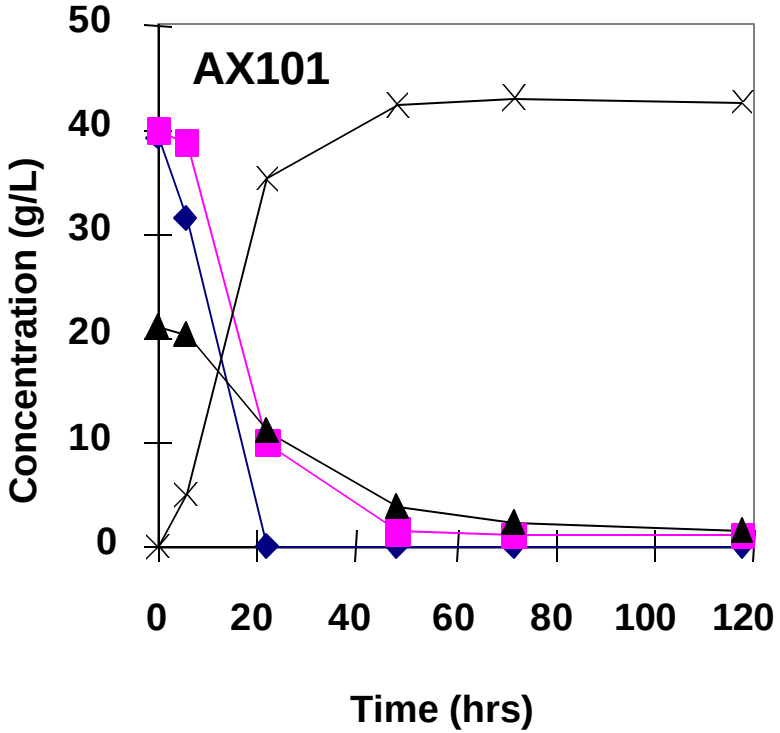
Homologous Recombination



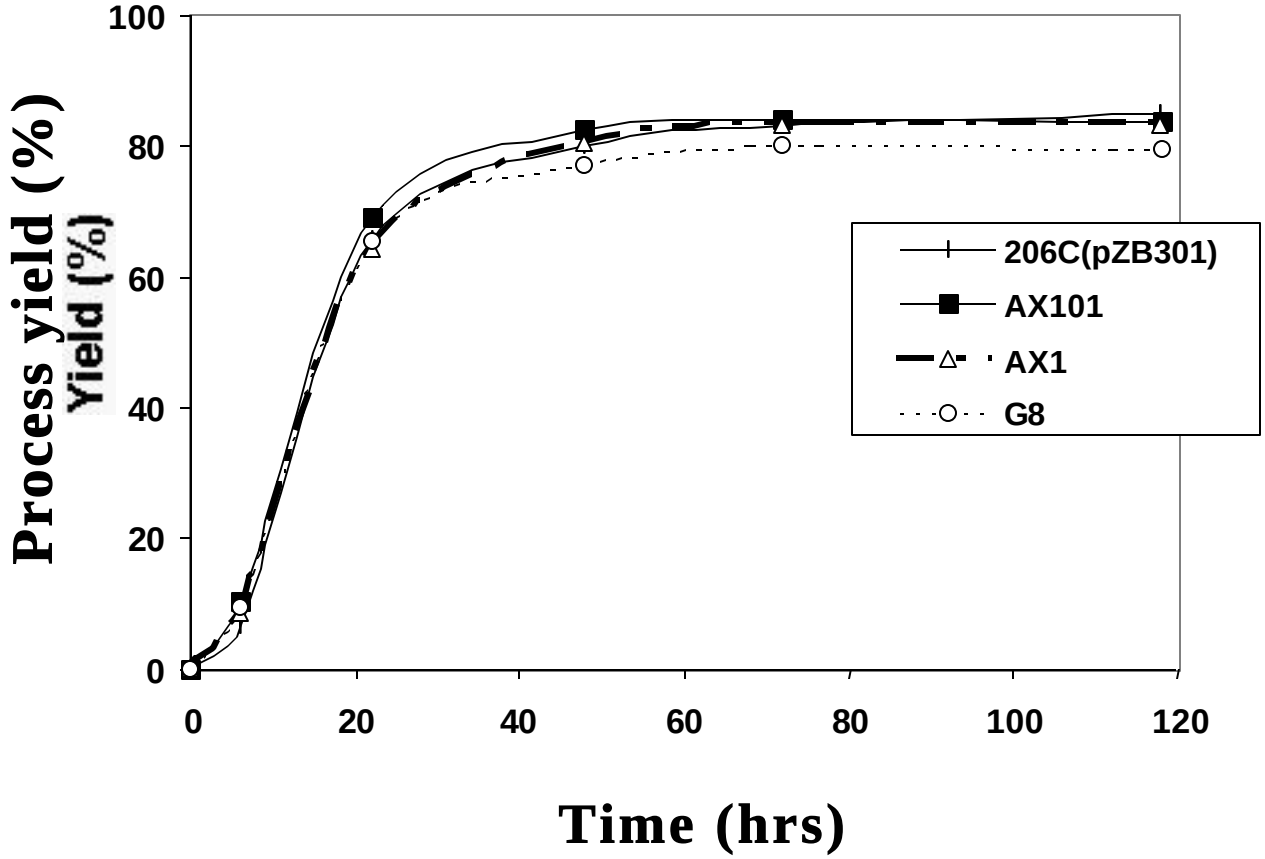
Site specific recombination
Gene inactivation

Comparison of fermentation performance of plasmid-bearing and integrated *Z. mobilis* strains on mixed sugars (G:40, X:40, A:20 g/l) at pH=5.5, T=30°C

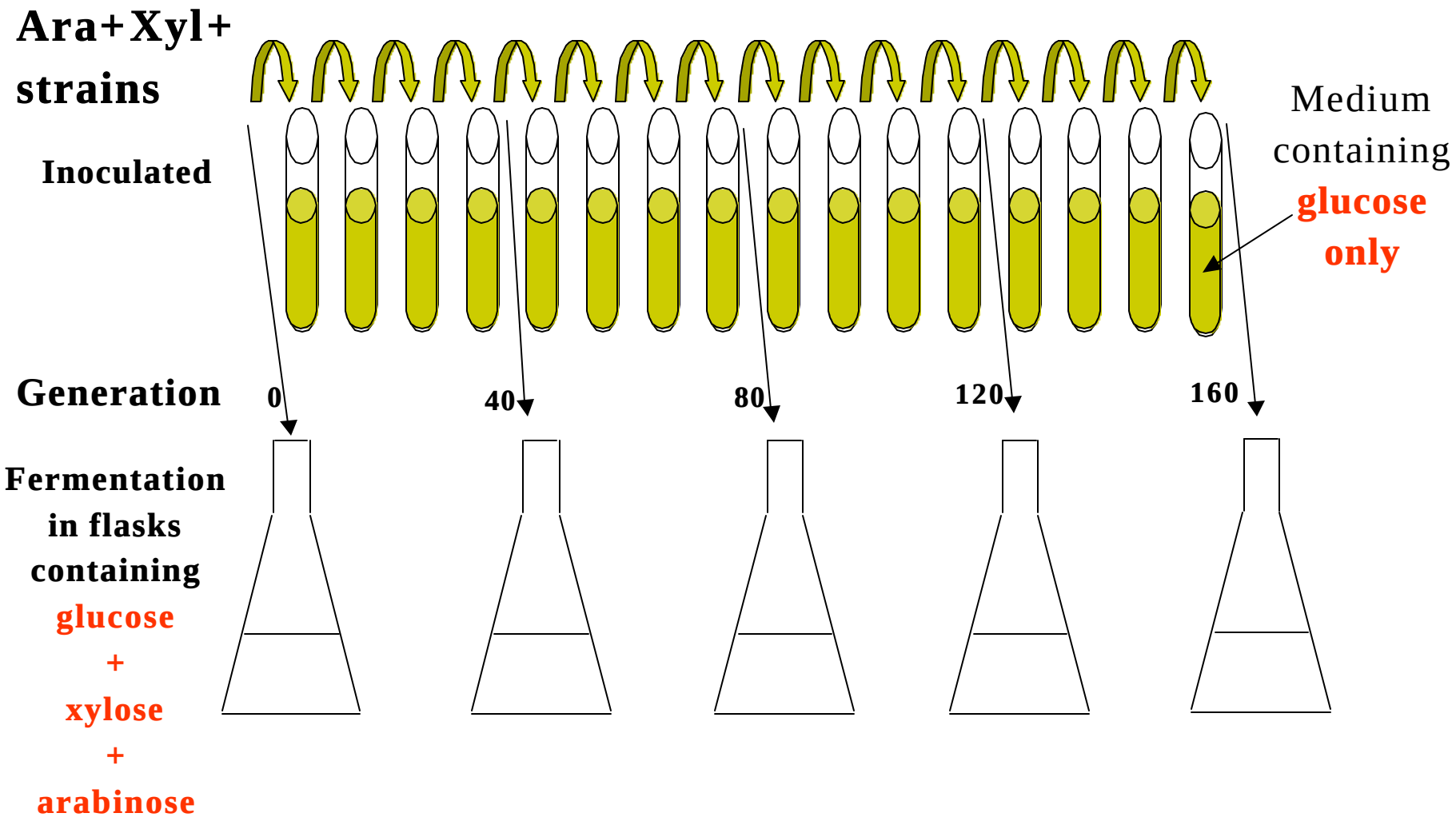




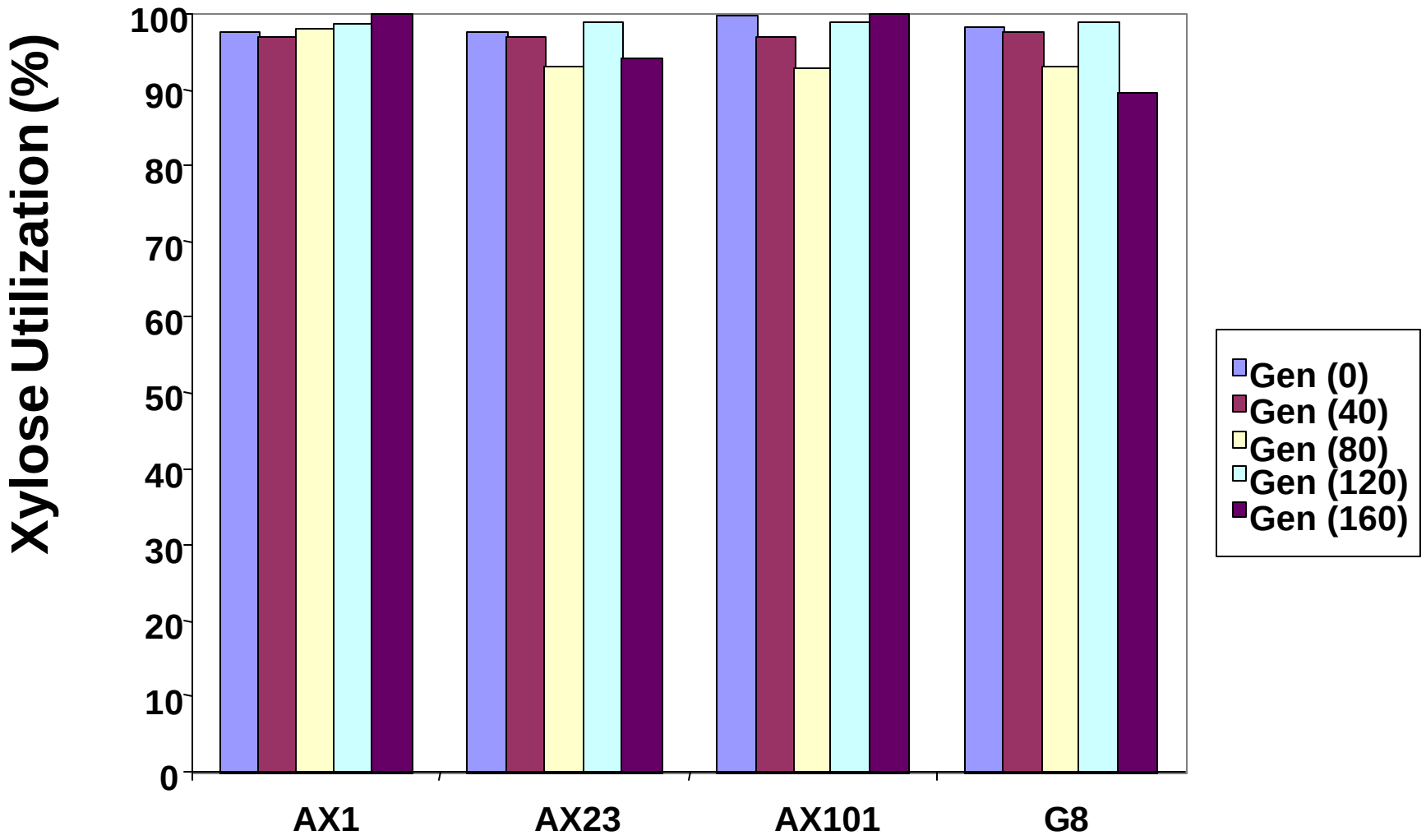
Process yield on mixed sugars (G:40, X:40, A:20 g/l) at pH=5.5, T=30°C



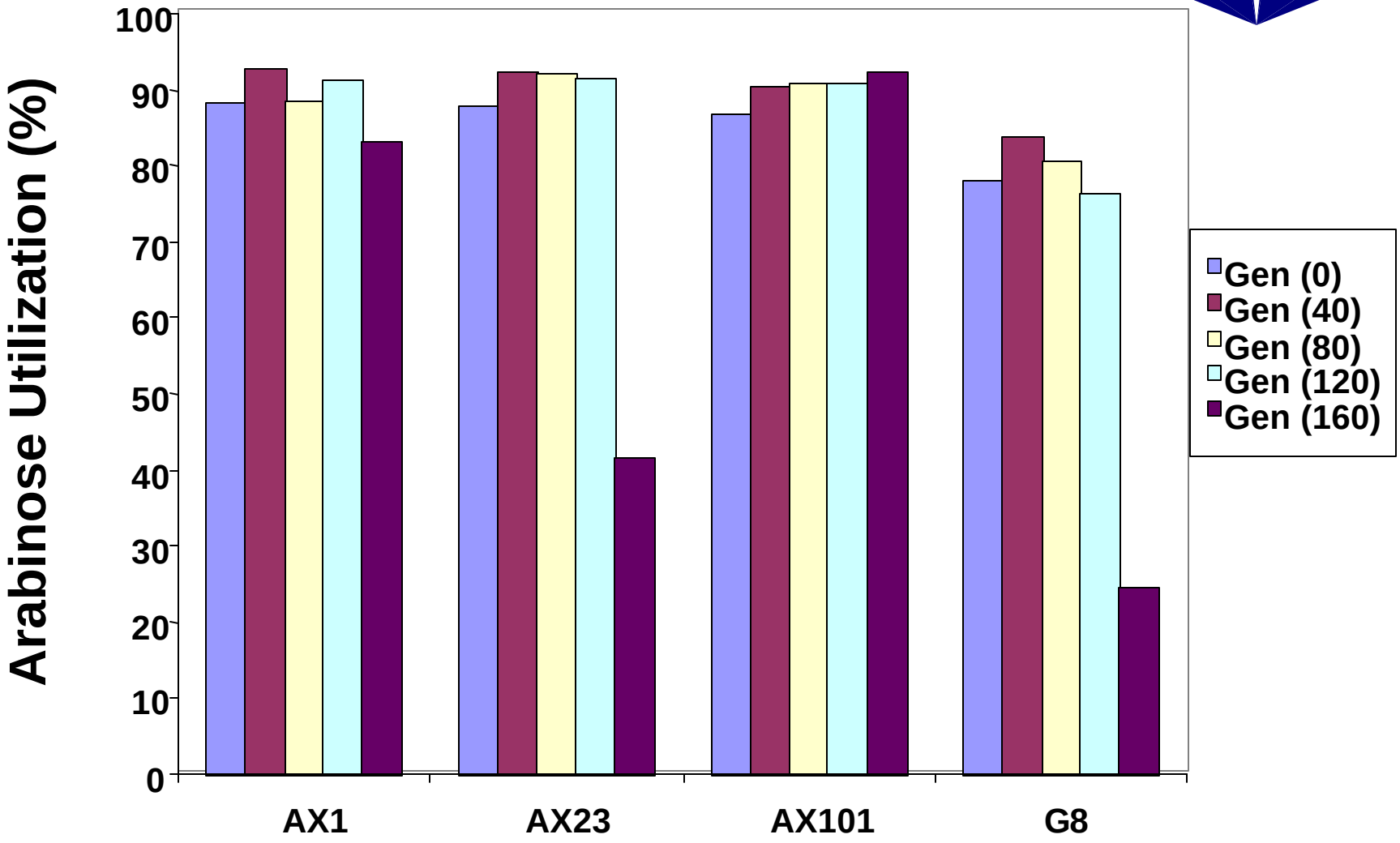
Stability Test



Xylose Utilization



Arabinose Utilization



Progress along Specific Aim 1

- **Integrated the seven genes necessary for xylose and arabinose fermentation into the *Zymomonas mobilis* genome and coordinately expressed the seven genes.**

- **The chromosomal integrated strains demonstrated similar ethanol process yield (83%) to the plasmid-bearing strain from a mixture of glucose, xylose and arabinose.**

- **The strains demonstrated stability for 160 generations on non-selective medium (in the absence of Tc).**

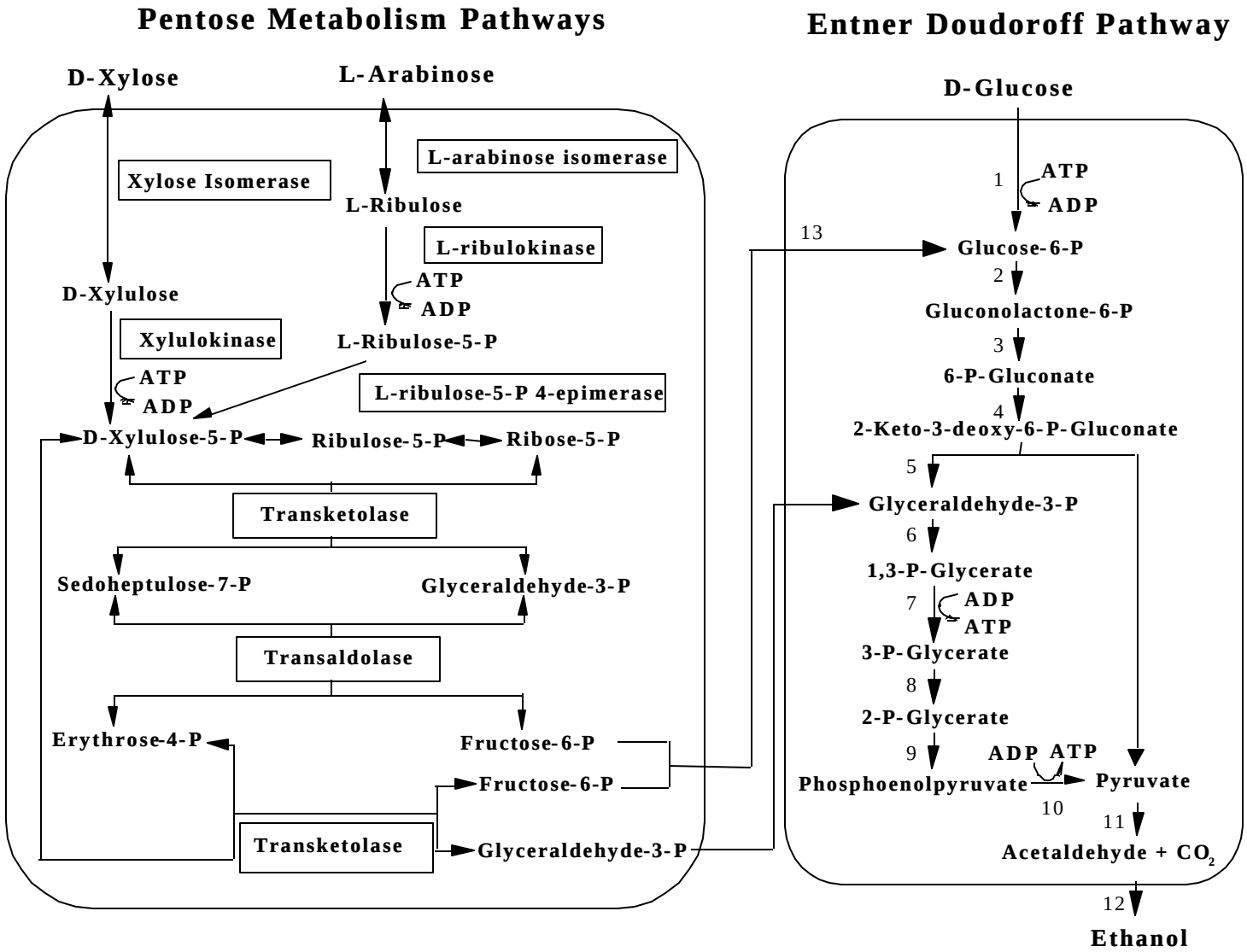


Fig. 1. Postulated reaction pathways required for *Zymomonas* pentose fermentation. The left side of the diagram shows the putative pentose metabolism pathways and the cloned enzymes (shown in rectangular boxes) that have been demonstrated to confer pentose fermentation capability. The right side of the diagram shows the native Entner Doudoroff (ED) pathway. The numbered enzymes in the ED pathway are: glycokinase (1); glucose-6-phosphate dehydrogenase (2); 6-phospho gluconolactonase (3); 6-phosphogluconate dehydratase (4); 2-keto-3-deoxy-6-phospho gluconate [KDPG] aldolase (5); glyceraldehyde-3-phosphate dehydrogenase (6); 3-phosphoglycerate kinase (7); phosphoglycerate mutase (8); enolase (9); pyruvate kinase (10); pyruvate decarboxylase (11); alcohol dehydrogenase (12); and glucose-6- phosphate isomerase (13).

Recombinant Zymomonas Strains

1. **C25**: Chromosomally integrated strain containing *xylA/xylB* and *tal/tktA* genes in two distinct synthetic operons

2. **Adapted 39676/pZB4L**: same genes carried on a plasmid, isolated from long-term continuous culture on hardwood hemicellulose hydrolyzate.

7 enzyme activity profiles measured in batch culture

XI: xylose isomerase (EC5.3.1.5)

XK: xylulokinase (EC 2.2.1.17)

TAL: transaldolase (EC 2.2.1.2)

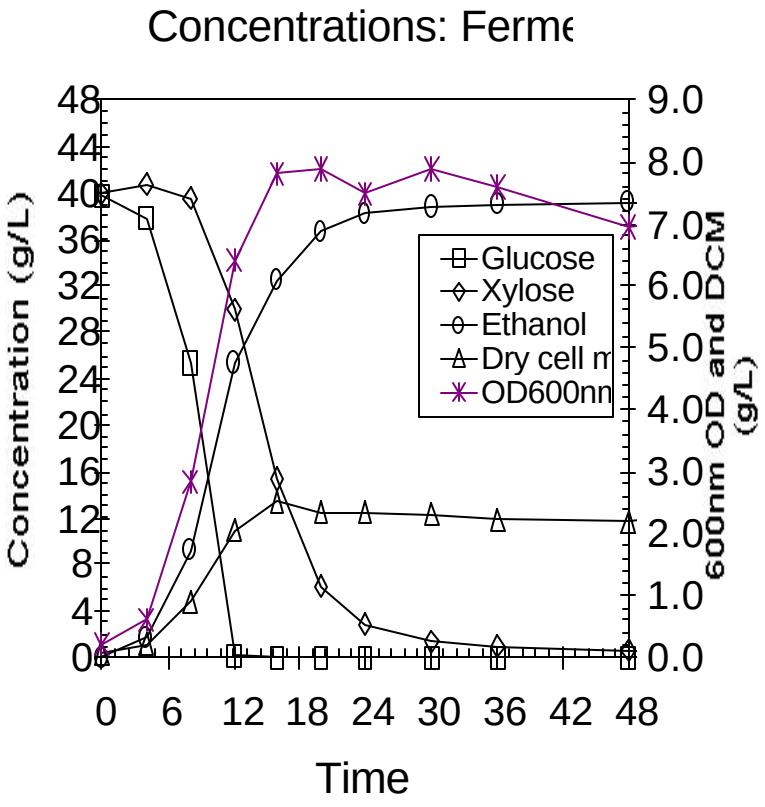
TKT: transketolase (EC 2.2.1.1)

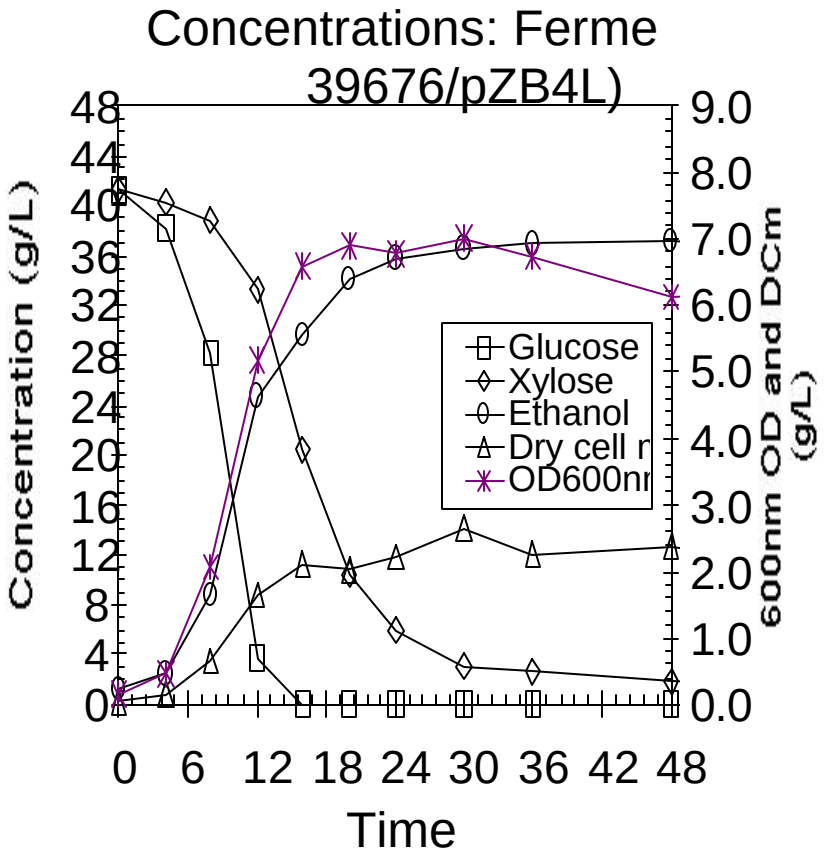
PGI: phosphoglucose isomerase (EC5.3.1.9)

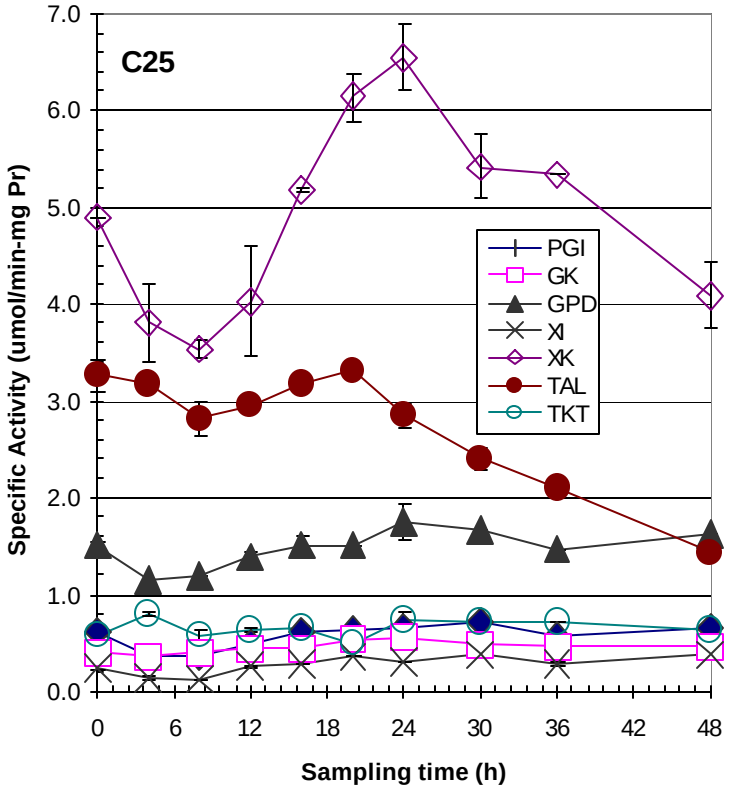
GK: glucokinase (EC 2.7.1.2)

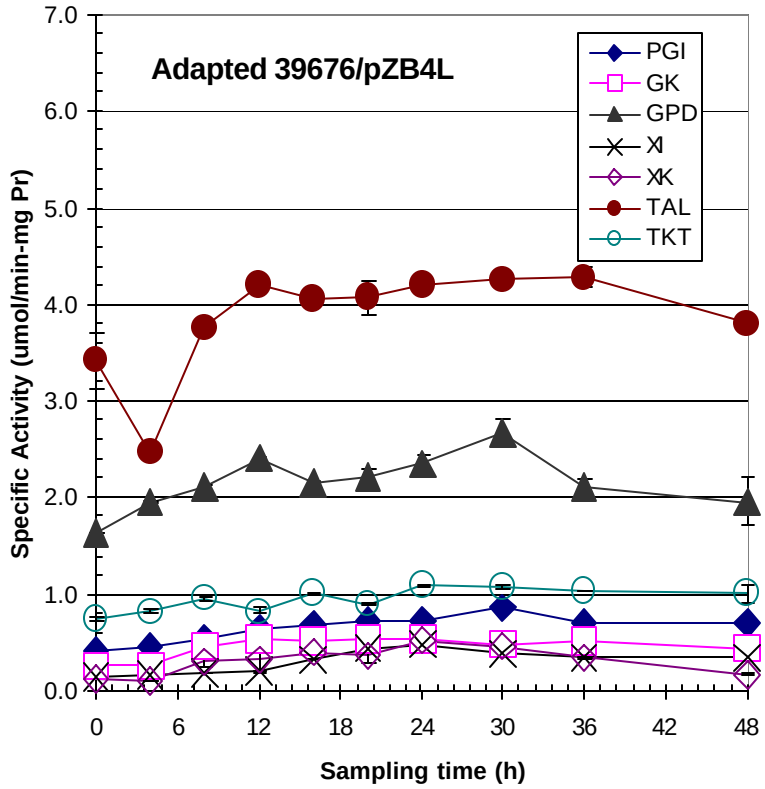
GPD: glucose-6-phosphate dehydrogenase (EC 1.1.1.49)

Fermentation Dynamics on Glucose and Xylose

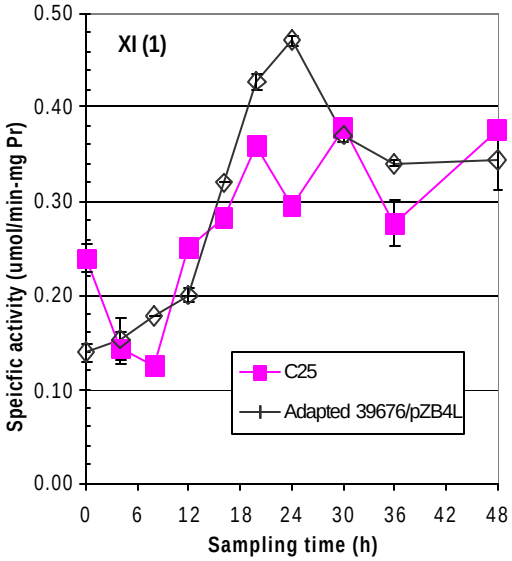


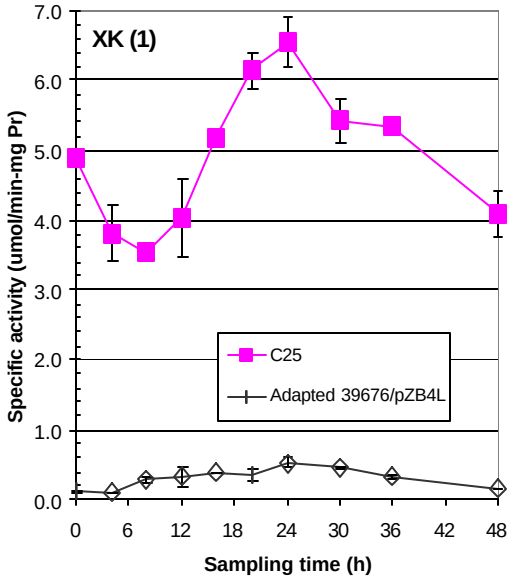


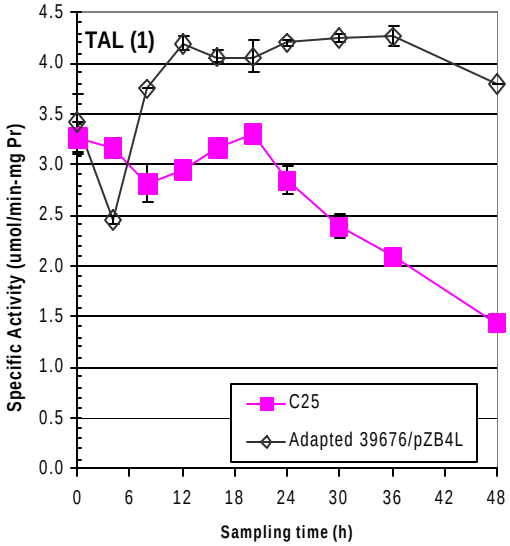


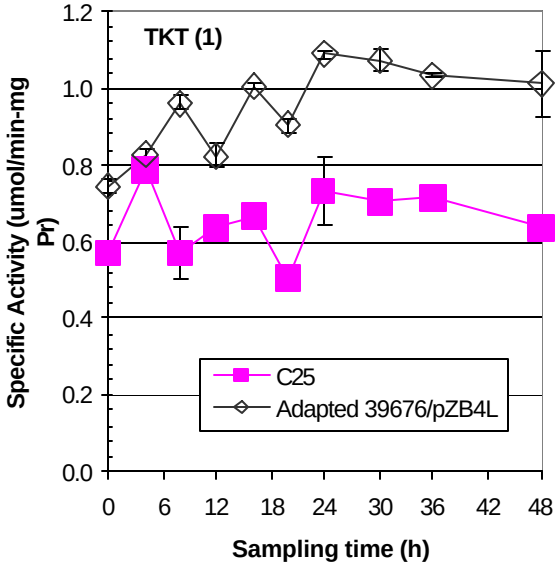


7 enzyme profiles for the 2 tested strains

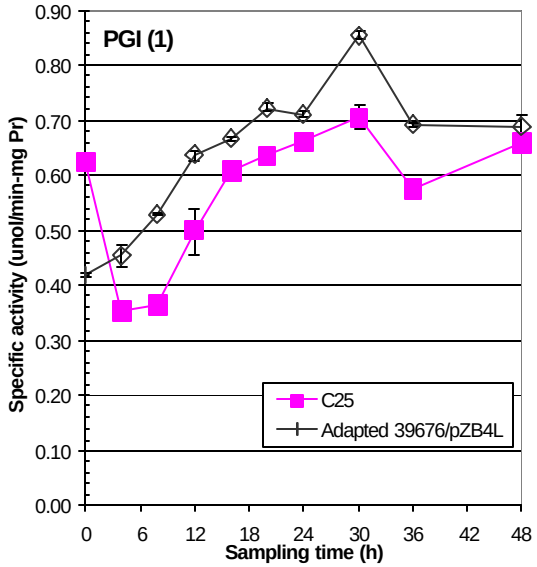


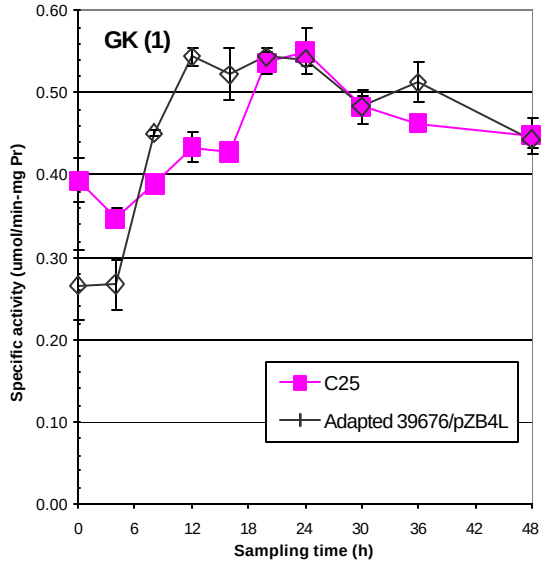


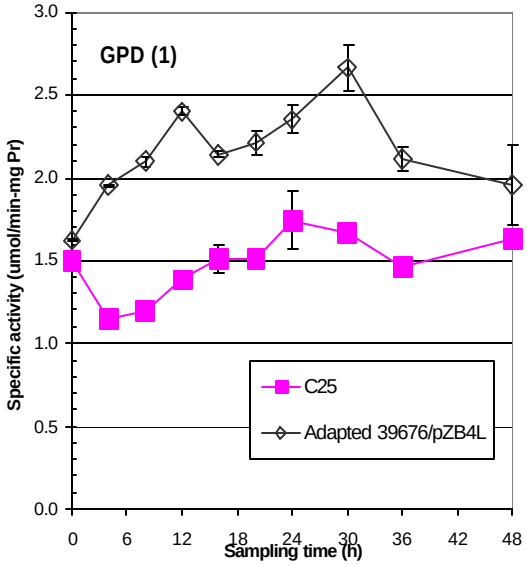




4 Xylose utilization enzyme dynamic profiles







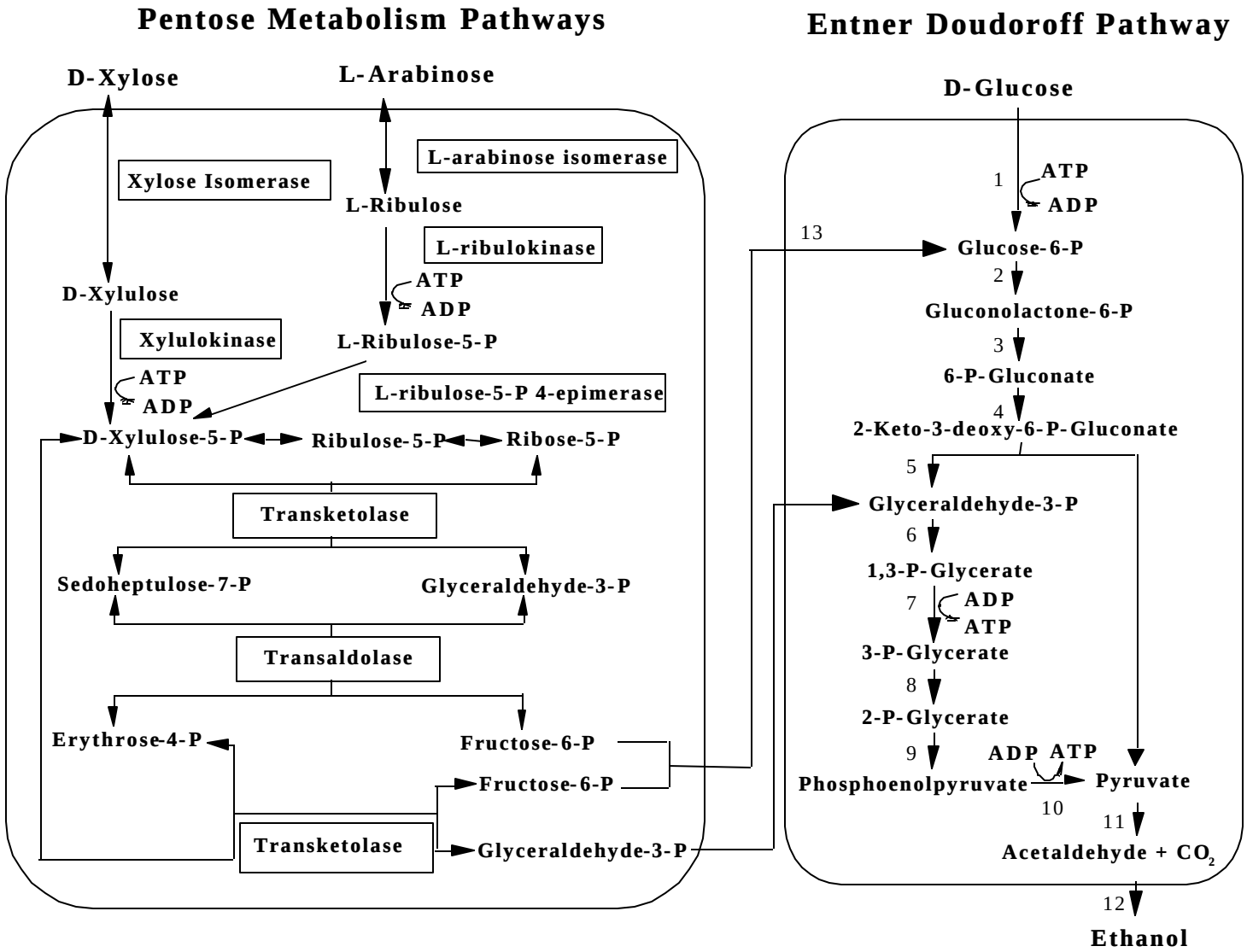
3 native glycolytic enzyme dynamic profiles

Progress along Specific Aim 2

Systematic variations have observed in key enzymatic activities during batch fermentation on sugar mixtures.

NMR spectroscopic measurements currently underway for the phosphorylated intermediates along the pentose pathway.

Proteomic analysis through 2D gel electrophoresis to identify significant variations in the expression levels of these enzymes.



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Progress along Specific Aim 3

Kinetic parameters K_m and k_{cat} being determined for the individual enzymes in the network of PP and ED pathways

Hypothesized reaction network being evaluated with new bioinformatics tools under development by Dr. Imran Shah

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