Simultaneous Co-Fermentation of Mixed Sugars: A Promising Strategy for Producing Cellulosic Biofuels and Chemicals

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Corn ethanol vs. Cellululosic ethanol

Corn starch
- Gelatinization
- Amylases
- Glucose
- **yeast**
- Ethanol + CO₂

- Single sugar fermentation
- No fermentation inhibitors
- Easy high loading

Cellulosic biomass
- Pretreatment + Cellulases
- Glucose + Xylose + Acetate + Fermentation inhibitors
- **yeast**
- Ethanol + CO₂

- **Mixed** sugar fermentation
- Fermentation inhibitors
- Difficulties in high loading
**Saccharomyces cerevisiae**: a workhorse strain for industrial ethanol production

- The most widely used yeast since ancient times in baking and brewing
- Osmotolerant and ethanol-tolerant
- Numerous genetic/genomic tools are available
  - Overexpression / Knockout
  - Expression of heterologous enzymes

- **Cannot utilize xylose**
  - Not suitable for producing cellulosic biofuels
Basic strategy in metabolic engineering of xylose fermentation in *S. cerevisiae*

### Scheffersomyces stipitis

- Xylose
  - XYL1
  - Xylitol
  - XYL2
  - Xylulose
  - XYL3
  - X-5-P
  - PPP and Glycolysis
  - Ethanol

- **Natural xylose fermenting**
- **Low** ethanol tolerance

### Saccharomyces cerevisiae

- Xylose
- Xylitol
- Xylulose
- X-5-P
- PPP and Glycolysis
- Ethanol

- **High** ethanol tolerance
- Amenable to metabolic engineering
Laboratory evolution of an engineered *S. cerevisiae* strain for further improvement

Enrichment by serial culture in 80 g/L of xylose

Single colony isolation

Evaluation

DA24

$n$
Comparison of xylose fermentation capability between engineered *S. cerevisiae* and *S. stipitis*

The engineered *S. cerevisiae* strain consumed xylose almost as fast as *S. stipitis*, the fastest xylose-fermenting yeast.

Ha et al. *PNAS*, 108:504-509
Why we want to co-ferment cellobiose and xylose?

Typical fermentation profile of glucose and xylose mixture
Engineered *S. cerevisiae* strains ferment xylose only after glucose depletion

Lau M. W., Dale B. E. *PNAS* 106:1368-1373
Grand scheme of co-fermentation of cellobiose and xylose in cellulosic hydrolysate

Cellulosic biomass

Pretreatment

Hemicellulose

Xylose

Cellobiose

Cellulose

1. Lower enzyme cost

2. Higher productivity

3. Enable a continuous process

4. Facilitate efficient and rapid chemical production

S. cerevisiae DA24-16BT3

Xylose consumption ↑
Supply of NADPH ↑

Time

Xylose

XR
Xylitol

Glucose

Cellobiose transporter

β-glucosidase

Cellodextrin transporter (cdt-1)
β-glucosidase (gh1-1)

Xylose

XYL1 and mXYL1

Xylose

Xylitol

XYL2

Xylose

Xylulose

PPP

XKS1

β-glucosidase

Glycolysis

Ethanol

Ha et al. PNAS, 108:504-509
Synthesis of engineered yeast capable of co-fermenting cellobiose and xylose simultaneously

Cate group at UC-Berkeley

Transporters from *N. crassa*  
NCU00801 (*cdt-1*)  
NCU00809  
NCU08114  

β-Glucosidase  
NCU00130 (*gh1-1*)

Glycolysis

Galazka et al. *Science* **330**:84-86

Jin group at UIUC  

*XYL1/XYL2/XYL3*

PPP & Ethanol Production

Ha et al. *PNAS*, **108**:504-509

+ Xiaomin Yang at BP
Co-fermentation of cellobiose and xylose by an engineered S. cerevisiae (DA24-16BT3)

<table>
<thead>
<tr>
<th></th>
<th>OD</th>
<th>Ethanol</th>
<th>$Y_{\text{EtOH}}$</th>
<th>$P_{\text{EtOH}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylose 40</td>
<td>16</td>
<td>13</td>
<td>0.33</td>
<td>0.28</td>
</tr>
<tr>
<td>Cellobiose 40</td>
<td>17</td>
<td>13</td>
<td>0.33</td>
<td>0.28</td>
</tr>
<tr>
<td>Cellobiose/xylose 40/40</td>
<td>23</td>
<td>32</td>
<td>0.40</td>
<td>0.70</td>
</tr>
</tbody>
</table>
Co-fermentation of glucose, cellobiose, and xylose by strain DA24-16BT3 and S. stipitis

<table>
<thead>
<tr>
<th></th>
<th>OD ($A_{600}$)</th>
<th>Ethanol (g/L)</th>
<th>$Y_{\text{EtOH}}$ (g/g)</th>
<th>$P_{\text{EtOH}}$ (g/L·hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DA2416-BT3</td>
<td>25</td>
<td>48</td>
<td>0.38</td>
<td>0.99</td>
</tr>
<tr>
<td>S. stipitis</td>
<td>19</td>
<td>25</td>
<td>0.38</td>
<td>0.55</td>
</tr>
</tbody>
</table>
Co-fermentation by an engineered industrial strain (HP111BT)

Low Initial OD (OD ~1.0)

- $Y_{E/S} = 0.38 \, \text{g/g}$
- $P_E = 1.11 \, \text{g/L-h}$

High Initial OD (OD ~10.0)

- $Y_{E/S} = 0.39 \, \text{g/g}$
- $P_E = 2.00 \, \text{g/L-h}$
Xylitol: a functional sweetener and chemical

- A very popular food additive in Asian market
  - Sugar substitute with lower calorie (2.4 cal/g)
  - Better sensory with a cooling effect
  - Good for diabetic patients and prevents dental caries
- Selected as one of the top value-added chemicals from biomass by US-DOE
Xylitol production through co-utilization of xylose and cellobiose

**Current process**

- Xylose
- Glucose
- XR
- NADPH
- Xylitol
- D-10

**Co-fermentation process**

- Xylose
- XR
- NADPH
- Xylitol
- D-10-BT
- Cellulose transporter
- β-glucosidase
- Cellobiose
- Glucose

Xylose consumption ↑
Supply of NADPH ↑
Enhanced production of xylitol without glucose repression

**Glucose/Xylose**

- Time (h): 0, 12, 24, 36, 48
- Glucose and Xylose (g/L): 0, 5, 10, 15, 20
- A$_{600}$, Ethanol and Xylitol (g/L): 0, 5, 10, 15, 20

**Cellobiose/Xylose**

- Time (h): 0, 12, 24, 36, 48
- Cellobiose and Xylose (g/L): 0, 5, 10, 15, 20
- A$_{600}$, Ethanol and Xylitol (g/L): 0, 5, 10, 15, 20

<table>
<thead>
<tr>
<th></th>
<th>OD (A$_{600}$)</th>
<th>Xylitol (g/L)</th>
<th>P$_{\text{xylitol}}$ (g/L·hr)</th>
<th>Xylitol production per sugar consumed (g/g)</th>
<th>Fermentation conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose/Xylose 20/20</td>
<td>10</td>
<td>13</td>
<td>0.28</td>
<td>0.67</td>
<td>80 rpm, 50mL</td>
</tr>
<tr>
<td>Cellobiose/Xylose 20/20</td>
<td>13</td>
<td>19 (46%↑)</td>
<td>0.40 (43%↑)</td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>
pH controlled bioreactor fermentation

<table>
<thead>
<tr>
<th></th>
<th>Cell mass (g/L)</th>
<th>Xylitol (g/L)</th>
<th>$P_{\text{Xylitol}}$ (g/L-hr)</th>
<th>Xylitol production per sugar consumed (g/g)</th>
<th>Fermentation conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>glucose/xylose 40/100</td>
<td>5.5</td>
<td>49</td>
<td>0.92</td>
<td>0.77</td>
<td>500 rpm, 2vvm ph 5.5</td>
</tr>
<tr>
<td>cellobiose/xylose 40/100</td>
<td>7.4</td>
<td>85 (73%↑)</td>
<td>1.60 (74%↑)</td>
<td>1.4</td>
<td></td>
</tr>
</tbody>
</table>
Why do we study galactose metabolism?

- Galactose is a major sugar in marine biomass

- Marine plant biomass has several attributes that would make it an attractive renewable source for the production of biofuels
  - Higher production yields per unit area
  - Can be depolymerized relatively easily compared to lignocellulosic biomass
  - Higher carbon dioxide fixation rates than terrestrial biomass
Galactose metabolism is tightly regulated in *S. cerevisiae* (strong glucose repression)

Improvement of galactose fermentation through co-fermentation with cellobiose

Diagram showing the metabolism pathways for glucose, cellobiose, galactose, and the resulting products (ethanol and CO₂) over time.
Comparison of sequential fermentation (A) and co-fermentation (B)

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<tr>
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<th>( Y_{\text{ETOH}} ) (g/g)</th>
<th>( P_{\text{ETOH}} ) (g/L·hr)</th>
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<tr>
<td>glucose/galactose</td>
<td>16</td>
<td>21</td>
<td>0.34</td>
<td>0.60</td>
</tr>
<tr>
<td>(40 g/L and 40 g/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cellobiose/galactose</td>
<td>22 (38% ↑)</td>
<td>27 (29% ↑)</td>
<td>0.36 (6% ↑)</td>
<td>0.74 (23% ↑)</td>
</tr>
<tr>
<td>(40 g/L and 40 g/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Ha et al. Appl. Environ. Microbiol. 77,5822-5826
Numerous applications of co-fermentation for producing fuels and chemicals

- Cell
- Fuels
  - Ethanol
  - Butanol
  - Fatty acid
  - Hydrocarbon
- Diversification of products
- Chemicals
  - Organic acids
  - Diacids
  - Dialcohols
  - Sugar alcohols (xylitol)
  - Sugar acids
- Extension of substrates
- Glucose
- Cellobiose + Xylose
- Cellobiose + Galactose
Summary

- Optimization of the xylose metabolic pathway and laboratory evolution drastically improved ethanol yield and productivity of engineered *S. cerevisiae*

- Co-fermentation of non-fermentable carbon sources (cellobiose and xylose) is possible by metabolic engineering
  - Cellodextrin transporter and intracellular β-glucosidase

- Engineered industrial *S. cerevisiae* showed impressive ethanol production capability

- Cellobiose and galactose co-fermentation is also feasible

- Various chemicals can be produced using the co-fermentation technology
  - Enhanced production of xylitol from cellulosic hydrolysate
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