Optimizing Pentose Utilization in Clostridia for Improved Solvents Production from Lignocellulosic Hydrolysates

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Shanghai Research and Development Center of Industrial Biotechnology

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Optimizing pentose utilization in Clostridia for improved solvents production from lignocellulosic hydrolysates

Sheng Yang

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Shanghai Institutes for Biological Sciences
Chinese Academy of Sciences
Locations & historic changes of Chinese solvent plants

1st period plants, 1955-1965

2nd period plants, 1965-1985

3rd period plants, 1985-1996

Ni and Sun, BBSRC China partner Workshop, 2009
Semi-continuous fermentation process for ABE production

Air
Steam
Mash
Seed
Gas Recovery
Distillation

Clostridium acetobutylicum EA2018

Ni and Sun, BBSRC China partner Workshop, 2009
Restoration of ABE fermentation in China from 2006

<table>
<thead>
<tr>
<th>Companies</th>
<th>Ton/y</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laihe Chemical Co. Ltd</td>
<td>100 000</td>
<td>Jilin</td>
</tr>
<tr>
<td>Jinyuan Ethanol Co. Ltd</td>
<td>50 000</td>
<td>Guangxi</td>
</tr>
<tr>
<td>Jiangsu Lianhai Biological Technology Co., Ltd.</td>
<td>50 000</td>
<td>Jiangsu</td>
</tr>
<tr>
<td>Lianyungang Lianhua</td>
<td>40 000</td>
<td>Jiangsu</td>
</tr>
<tr>
<td>Tianguan group</td>
<td>30 000</td>
<td>Henan</td>
</tr>
<tr>
<td>Cathay Industrial Biotech Ltd.</td>
<td>30 000</td>
<td>Jilin</td>
</tr>
<tr>
<td>The Jingmaoyuan Biochemical Company Ltd.</td>
<td>30 000</td>
<td>Jiangsu</td>
</tr>
<tr>
<td>Tongliao Zhongketianyuan Starch Chemical Corp.</td>
<td>10 000</td>
<td>Mengolia</td>
</tr>
<tr>
<td>Jilin Zhonghai Chemical</td>
<td>5 000</td>
<td>Jilin</td>
</tr>
<tr>
<td>Heilongjiang Haocheng Chemical</td>
<td>5 000</td>
<td>Heilongjiang</td>
</tr>
<tr>
<td>Jidong Solvent</td>
<td>5 000</td>
<td>Hebei</td>
</tr>
<tr>
<td>Hebei Jizhou Solvent</td>
<td>3 000</td>
<td>Hebei</td>
</tr>
</tbody>
</table>

Modified from Ni and Sun (2009) Appl Micrbiol Biotechnol
Gene disruption tools for Clostridia

**Group II Intron-based TargeTron**

1. **pSY6** L1.LtrB group II intron
2. Transform host with ligation reaction (or cloning necessary)
3. Express the Intron and reverse transcriptase
4. ORF is permanently disrupted by intron insertion.
5. RNP inserts intron RNA and reverse transcribes cDNA copy.

**I-SceI based markerless gene deletion**

- Single cross: two possibilities
- Double cross: two possibilities

**Acetogenesis**

- Glucose → Lactate → Pyruvate → Ethanol
- Acetate → Acetyl-CoA → Ethanol
- Acetoacetyl-CoA → Acetone → CO₂

**Solventogenesis**

- Ethanol → Acetone → Butanol

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Heap J. et al. (2007) J Microbial Method
Shao L. et al. (2007) Cell Research
Jiang Y. et al. (2009) Metabolic Engineering
Feedstock dominates the variable cost for solvent production

1.5 generation feedstock – non-grain hexose

Distribution of variable cost for solvent production data from NCPC

1.5 generation feedstock – non-grain hexose

- Sweet sorghum
- Cassava
- Wheat amylopectin
- Molasses
Seeking lignocellulosic feedstock for ABE production

Sweet sorghum

300 t/a pilot

2.0 generation feedstock: pentose rich

Sweet sorghum residue
Corn stover
Corn cob
Corn fiber
Glucose repression in *Clostridium acetobutylicum*

**Carbon catabolite repression (CCR) in *Bacillus subtilis* and other Firmicutes**

Disruption of \textit{glcG} relieve CCR effect in \textit{C. acetobutylicum}
Rate-limiting steps of D-xylose metabolism occurs before PPP

Fig. A Individual pentose consumption of 824WT

Fig. B Individual pentose consumption of 824gIcG in presence of glucose
Candidate regulatory sites of XylR from ROK family are shown by red circles. Genes predicted by genome context analysis are marked by asterisks. Homologous genes are marked by matching colors.

Gu et al. BMC Genomics 2010
Overexpression of \textit{xylT}, \textit{xylA} and \textit{xylB} improved D-xylose consumption in the presence of D-glucose
Cofermentation of D-glucose, D-xylose and L-arabinose by 824glicG-TBA

Xiao et al 2011 AEM
Corncob-based xylose mother liquor is a pentose rich feedstock.
Fermentation profiles of strains 2018WT, 2018glcG and 2018glcG-TBA in xylose mother liquor

0.22 g/g

0.33 g/g
Can ABE be made from corn stover hydrolysates?
NCIMB8052 is promising starter strain in corn stover hydrolysates

<table>
<thead>
<tr>
<th>Clostridia</th>
<th>growth</th>
<th>solvent (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C. acetobutylicum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EA 2018</td>
<td>+</td>
<td>3.56</td>
</tr>
<tr>
<td>ATCC 824</td>
<td>+</td>
<td>3.14</td>
</tr>
<tr>
<td><strong>C. beijerinckii</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCIMB 8052</td>
<td>+</td>
<td><strong>11.2</strong></td>
</tr>
<tr>
<td>ATCC 55025</td>
<td>+</td>
<td>1.99</td>
</tr>
<tr>
<td>CGMCC 1.2127</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>CICC 22954</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>DSM 51</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>DSM 791</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><strong>C. saccharobutylicum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCP 262</td>
<td>+</td>
<td>6.37</td>
</tr>
<tr>
<td><strong>C. saccharoperbutylacetonicum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N 1-4</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
## ABE fermentation of lignocellulose hydrolysates

<table>
<thead>
<tr>
<th>Strain</th>
<th>Feedstock</th>
<th>Initial sugar (g/L)</th>
<th>Yield on total sugar (g/g)</th>
<th>Cost of medium (Yuan/per ton sugar)</th>
<th>Detoxification or not</th>
<th>Sugar utilization</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. beijerinckii</em> ATCC55025</td>
<td>Wheat bran</td>
<td>45</td>
<td>0.21</td>
<td>1190</td>
<td>Y</td>
<td>Insufficient use of D-xylose and L-arabinose</td>
<td>(Liu, Ying et al. 2010)</td>
</tr>
<tr>
<td><em>C. Acetobutylicum</em> ATCC824</td>
<td>Corn stover</td>
<td>45</td>
<td>0.27</td>
<td>1060</td>
<td>Y</td>
<td>Insufficient use of D-xylose and cellobiose</td>
<td>(Wang and Chen 2011)</td>
</tr>
<tr>
<td><em>C. beijerinckii</em> P260</td>
<td>Barley straw</td>
<td>60</td>
<td>0.44</td>
<td>900</td>
<td>Y</td>
<td>Full use</td>
<td>(Qureshi, Saha et al. 2010)</td>
</tr>
<tr>
<td><em>C. beijerinckii</em> P260</td>
<td>Corn stover</td>
<td>60</td>
<td>0.44</td>
<td>900</td>
<td>Y</td>
<td>Full use</td>
<td>(Qureshi, Saha et al. 2010)</td>
</tr>
<tr>
<td><em>C. beijerinckii</em> IB4</td>
<td>Corn fiber</td>
<td>30</td>
<td>0.31</td>
<td>9800</td>
<td>N</td>
<td>Insufficient use of D-xylose and L-arabinose</td>
<td>(Guo, Tang et al. 2012)</td>
</tr>
<tr>
<td><em>C. beijerinckii</em> P260</td>
<td>Wheat straw</td>
<td>25.4</td>
<td>0.37</td>
<td>2200</td>
<td>N</td>
<td>Full use</td>
<td>(Qureshi, Saha et al. 2008)</td>
</tr>
<tr>
<td><em>C. beijerinckii</em> BA101</td>
<td>Corn fiber</td>
<td>25</td>
<td>~0.35</td>
<td>2130</td>
<td>N</td>
<td>Full use</td>
<td>(Qureshi, Ezeji et al. 2008)</td>
</tr>
<tr>
<td><em>C. beijerinckii</em> P260</td>
<td>Wheat straw</td>
<td>28.4</td>
<td>0.42</td>
<td>2100</td>
<td>N</td>
<td>Full use</td>
<td>(Qureshi, Saha et al. 2008)</td>
</tr>
<tr>
<td><em>C. acetobutylicum</em> P262</td>
<td>Corn stover</td>
<td>81</td>
<td>0.27</td>
<td>1300</td>
<td>N</td>
<td>74% of total sugars was consumed</td>
<td>(Parekh, Parekh et al. 1988)</td>
</tr>
<tr>
<td><em>C. beijerinckii</em> P260</td>
<td>Wheat straw</td>
<td>52</td>
<td>0.35</td>
<td>1080</td>
<td>N</td>
<td>87% of total sugars was consumed, with D-xylose, and D-glucose left</td>
<td>(Qureshi, Saha et al. 2008)</td>
</tr>
</tbody>
</table>

### The technical successful criteria

- Undetoxified, additive <200 yuan/t ABE, unsterilized, < 48 hour, titer>12g/L, yield>0.3 g/g total sugar
Unconsumed sugar
Consumed sugar

8052 only give a yield of 0.24 g/g in CSH

Unwashed NREL PCS, CTec2 hydrolysates

<table>
<thead>
<tr>
<th>Cellobiose (g/L)</th>
<th>Glucose (g/L)</th>
<th>Xylose (g/L)</th>
<th>Acetic acid (g/L)</th>
<th>SO₄²⁻ (g/L)</th>
<th>Furfural (g/L)</th>
<th>HMF (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.9</td>
<td>85.8</td>
<td>56.6</td>
<td>8.4</td>
<td>10.0</td>
<td>0.07</td>
<td>2.6</td>
</tr>
</tbody>
</table>

diluted to 40 g/L sugar, with 2 g/L ammonia sulfate, 5 g/L calcium carbonate fermentation at 37°C for 48 hour
Disruption of gene xyI/R improves D-xylose utilization by C. beijerinckii

Candidate regulatory sites of XylR from ROK family are shown by red circles
Gu (2010) BMC Genomics

8052WT  8052ΔxylR

xylT (cbei0109)

xylAI (cbei2383)

xylAII (cbei4681)

xylB (cbei2384)

16S rRNA
XylT overexpression in strain 8052ΔxylR further enhances D-xylose consumption
8052ΔxylR-xylT (MM1643) produce 20% more solvent in CSH

8052wt in CSH

MM1643 in CSH

Unconsumed sugar
Consumed sugar

0.24 g/g
0.30 g/g

unwashed NREL PCS, CTec2 hydrolysates
diluted to 40 g/L sugar, 2g/L ammonia sulfate, 5g/L calcium carbonate fermentation at 37°C for 48 hour
Summary

• ABE fermentation in China is shifting from hexose to pentose-rich lignocellulosic feedstock.

• The genes of pentose metabolic pathway of two model strains, *C. acetobutylicum* ATCC 824 and *C. beijerinckii* NCIMB 8052, were identified and improved to enhance solvent yield by elimination the negative regulation as well as strengthen the xylose pathway.

• *Clostridium acetobutylicum* EA2018 can be engineered to coferment hexose and pentose of XML at solvent yield>0.3 by disruption of *glcG* and overexpression of xylose uptake and metabolism genes.

• CSH tolerant *Clostridium berjerinckii* NCIMB 8052 can be engineered to coferment hexose and pentose of undetoxified CSH at solvent yield>0.3 by disruption of *xylR* and overexpression of xylose transporter.
Strategy for Firmicutes pentose metabolic engineering

1. Disrupt Enzyme II of PTS system to release glucose repression
2. Disrupt repressor protein to enhance individual secondary substrate pathway
3. Overexpression secondary substrate uptake and pathway genes
Acknowledgement

Dr Han Xiao  Dr Yang Gu  Prof ET Papoutsakis
Dr Yu Jiang  Dr Wilfrid Mitchell  Prof Peter Durre
Dr Zhilin Li  Prof Yunliu Yang  Prof Nigel Minton
Jun Chen  Prof Weihong Jiang  Dr Xinyan Guo
Feng Dong  Prof Chen Yang  Dr Guifang Wu
XlyR or AraR-regulated genes in Clostridium genomes

Candidate regulatory sites of XylR or araR from ROK family are shown by red circles. Genes predicted by genome context analysis are marked by asterisks. Homologous genes are marked by matching colors.

Gu et al BMC Genomics 2009
Zhang et al J Bacteriol 2012
I. The glucose-PTS activity was only slightly reduced in strain 824glcG and its methyl α-glucoside-PTS activity was lost.
I. Non-PTS-mediated of D-glucose transport and metabolism might play a greater role in train 824glcG

TABLE 2. Comparison of specific activities of glucose kinase for strain 824WT and strain 824glcG in P2 medium containing 40 g/L glucose.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Specific activity (U/mg) (^a)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acidogenic phase</td>
<td>Solventogenic phase</td>
<td></td>
</tr>
<tr>
<td>824WT</td>
<td>0.16 ± 0.02</td>
<td>0.23 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>824glcG</td>
<td>0.31 ± 0.13</td>
<td>1.05 ± 0.36</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) OD\(_{600}\) of samples taken in acidogenic phase and solventogenic phase were around 1.8 and 4.0, respectively.
I. Transcriptional fold-changes of *xylT*, *xylA* and *xylB* of strain 824glcG-TBA

<table>
<thead>
<tr>
<th>Genes</th>
<th>Transcriptional level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acidogenic phase&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>xylT</em></td>
<td>1.17 ± 0.08</td>
</tr>
<tr>
<td><em>xylA</em></td>
<td>132 ± 27</td>
</tr>
<tr>
<td><em>xylB</em></td>
<td>119 ± 5</td>
</tr>
</tbody>
</table>

<sup>a</sup> OD<sub>600</sub> of samples taken in acidogenic and solventogenic phase were 3.8 and 7.0, respectively. Fermentations were performed in P2 medium containing 40 g/L glucose and 20 g/L xylose.