Ethanol production from second generation feedstocks by yeasts

Jan de Bont
IUMS, Sapporo, Japan
September 8, 2011
C5 Yeast Company towards Fuel Ethanol

- C5 Yeast Company originates from the former Royal Nedalco, which belonged to Royal Cosun

- Potential seen in second generation (=cellulosic) ethanol

- It has invested in R&D for second generation ethanol
  - In house (Bergen op Zoom, The Netherlands)
  - Universities (Delft, Groningen, Wageningen, Stuttgart)
  - Technology providers
  - R&D departments of Companies

- The company very recently has been acquired by DSM
DSM strengthens yeast technology leadership for 2G biofuels
Heerlen, NL, 28 June 2011 08:15 CET

Royal DSM, the global Life Sciences and Materials Sciences company, today announces that it has reached an agreement to acquire C5 Yeast Company B.V. (Bergen op Zoom, Netherlands) from Royal Cosun. The acquisition will allow DSM to combine C5 Yeast Company’s business with its own advanced yeast and enzyme technologies for second generation biofuels, further increasing its leadership position in this field.

The C5YC R&D-team

Official start under DSM ownership
August 25, 2011
DSM:
Presence in the world
DSM: 100 years of successful transformations

Classical Biotechnology

Technological competences
- Mechanical engineering
- Chemical engineering
- Polymer technology
- Material science
- Fine chemicals
- Modern Biotechnology
DSM: Transformation completed

Breakdown DSM sales (%)

2000
- Petrochemicals
- Engineering Plastic Products
- Base Chemicals & Materials
- Others

2005
- Base Chemicals & Materials
- Others

2010*
- Polymer Intermediates
- Performance Materials
- Pharma
- Nutrition

* Excluding discontinued
DSM: Focused platforms and pipelines

Global shifts
Climate & Energy
Health & Wellness

Innovation Platforms (Examples - Business Groups)

- Bio processing ingredients
- Food/feed ingredients
- (Bio) Manufacturing
- Bio-based clean / green materials
- Sports & Life Protection Materials

Emerging Business Areas (DSM Innovation Center)

- Bio-based Products & Services
- Advanced Surfaces
- Biomedical
DSM: Focused platforms and pipelines

Global shifts
Climate & Energy
Health & Wellness

Innovation Platforms (Examples - Business Groups)

Bio processing ingredients
Food ingredients
(Bio) Manufacturing
Bio-based clean / green materials
Sports & Life Protection Materials

Emerging Business Areas (DSM Innovation Center)

Bio-based Products & Services
Advanced Surfaces
Biomedical
EBA Bio-based Products and Services

Strategy

Bio-based Products & Services
- License and service models to cellulosic ethanol industry
- Production of bio-based chemicals from traditional crops
- Focus on green materials with better performance

Aspirations
- Sales in 2020 > € 1bn
- High EBITDA margin
1. General aspects ethanol production

2. Hemicellulolytic enzymes

3. Industrial yeast capable of xylose/arabinose (C5-sugars) fermentations
Background to 1st generation ethanol

- 1st generation ethanol is currently produced from digestible C6 sugars from:
  - Corn
  - Wheat
  - Sugar cane
  - Sugar beet

- This ethanol production is based on mature yeast fermentation technology
Sugar composition of wheat and of corn kernels

**Wheat (kernel):**
- Arabinose, 3%
- Xylose, 4%
- Glucose 2nd generation, 3%
- Glucose 1st generation, 68%

**Corn (kernel):**
- Arabinose, 2%
- Xylose, 3%
- Glucose 2nd generation, 1%
- Glucose 1st generation, 76%
Ethanol plant locations in the USA
Background to 2nd generation ethanol

• Prices of 1st generation biomass are increasing while the market for ethanol is growing

• Producers and governments consider alternative sources of biomass to produce ethanol

• 2nd generation biomass sources are widely available and they provide a huge opportunity in producing ethanol
Background to 2\textsuperscript{nd} generation ethanol

- 2\textsuperscript{nd} generation feedstocks include:
  - Stover, cobs and fiber from corn
  - Straw and bran from wheat
  - Bagasse from sugar cane
  - Pulp from sugar beet
  - Grasses
  - Empty fruit bunches from oil palm and glycerol
  - Woody materials

\begin{itemize}
  \item \textbf{Wheat bran}
  \item \textbf{Grasses}
  \item \textbf{Sugar beet pulp}
  \item \textbf{Bagasse}
\end{itemize}
Plant cell wall architecture

Corn and co-products

- Corn fiber (outer layers)
- Starch (endosperm)

- Corn Cobs (after removal of kernels)
- Corn Stover (remaining plant material on land)
Feedstock composition

Examples of feedstocks under consideration for cellulosic ethanol production

- corn fiber
- corn cobs
- corn stover
- switch grass
- bagasse (sugar cane)

Legend:
- Starch
- Cellulose
- Hemicellulose
- Lignin
- Protein
- Lipid/ Ash
Feedstock composition of Corn Fiber and Corn Cobs

**Corn Fiber**
- C6
- C5
- Glucose 1st gen.
- Arabinose
- Xylose

**Corn Cobs**
- C6
- C5
- Glucose 2nd gen.
- Arabinose
- Xylose
Major Technological Issues

1. Pretreatment

2. Hydrolytic polymer-degrading enzymes

3. Industrial yeast capable of C5 fermentations
Pretreatment & enzymatic hydrolysis

- On Site Enzyme production
- Efficiency conversion approximately 80%
- Remaining polymers

Pretreatment 37 °C, Low pH

Fermenting microorganisms
Simplified scheme production ethanol from second generation feedstocks

Second Generation Feedstock

Physical and/or chemical pretreatment

Enzymatic hydolysis

Yeast fermentation

Distillation

Industrial yeast fermenting both C₅ and C₆ sugars

Mild and effective pretreatment technologies

Enzyme cocktails hydrolyzing (hemi)cellulose

Co-products

Ethanol
Enzymes and hemicellulose

Pretreatment can liberate substantial amounts of sugars from hemicellulose, but application of enzymes holds promise in establishing production processes with less severe conditions.

- $\beta$-1,4-endo-xylanases (EC 3.2.1.8)
- $\beta$1,3-xylosidase?
- AXH-d3 (EC 3.2.1.55)
- $\alpha$-(4-O-methyl)glucuronosidases
- AXH-s1,2 (EC 3.2.1.55)
- $\alpha$-1,5-arabinofuranosidase?
- Galactosidase + xylosidase?
- $\beta$-xylosidase (EC 3.2.1.37)

Extra: Feruloyl esterase (EC 3.1.1.73)
Approach enzyme work

- Determine remaining oligomeric structures after pretreatment and enzymatic hydrolysis
- Interactions with enzyme companies for testing preparations
Fraction I upon enzyme treatment: Many pentose containing oligomers enriched with O-acetyl and/or Glucuronic acid side groups

H = hexose
P = pentose
Ac = O-acetyl
GlcA\textsubscript{me} = 4-O-methyl-Glucuronic acid

Additional Enzymes Required
Producing Ethanol with Yeast

- The production of ethanol with yeast is a proven process for 1st generation feedstocks.

- The bulk of the fuel ethanol producers produce feed, and occasionally food products as well. Application of yeast (S. cerevisiae) is commonly accepted in this industry.

- Agricultural biomass such as corn fibers, corn cobs and corn stover can be made available at ethanol plants that now run on yeast.

- Relatively low capital investment is required when these second generation feedstocks can be fermented also by yeast in conjunction with yeast 1st generation processes.
Fermentation by natural *S. cerevisiae*

Second generation feedstock

- Cellulose
- Hemicellulose
- Xylose and arabinose (C5)
- No C5 capabilities

- Starch
- Sucrose
- Glucose
- Fructose
- Ethanol (C6)
Fermentation by modified *S. cerevisiae*

Second generation feedstock

- Cellulose
- Hemicellulose
- Xylose and arabinose (C5)
- C5-Technology!

Starch

Sucrose

Glucose

Fructose

C6

ETHANOL

Evolutionary engineering of mixed-sugar utilization by a xylose-fermenting *Saccharomyces cerevisiae* strain.

FEMS Yeast Research 5, 399-409 (2005).
Key in xylose-fermenting *S. cerevisiae*

Many biochemical steps, optimizations required

Technology C5
Yeast Company

Ethanol

**Diagram:****

- **NADPH**
- **Xylose**
- **Xylitol**
- **NADH**
- **Xylulose**
- **Xylose isomerase**

A pathway involving NADPH and Xylose leads to Xylitol, while another pathway involving NADH and Xylulose is represented. The text notes the complexity of the biochemical steps required for this process.
Enabling C5 Metabolism in *S. cerevisiae*

**D-glucose**

- **HXT** (Plasma membrane)
  - Glucose
  - HXK
  - G6P
  - PGI
  - F6P
  - PFK
  - F16DP
  - FBA
  - DHAP
  - TPI
  - G3P
  - TDH PGK PGM
  - ENO PYK PDC
  - ADH
  - EtOH

**D-xylose**

- **HXT**
- Xylitol
- XI
- XKS
- X5P
- RPE
- RKI
- D-ribulose-5P
- D-ribose-5P

**L-arabinose**

- **GAL2**
- Arabinose
- araA
- araB
- araD
- L-ribulose
- L-R5P

**Metabolites**

- G6P
- F6P
- F16DP
- G3P
- X5P
- D-ribose-5P
- L-arabinose
- L-ribulose
- arabinose
Xylose isomerase

• Xylose isomerase (EC 5.3.1.5)
  – alternative name glucose isomerase
  – widely spread in Nature
  – not found in yeast
  – tetramer: 4 identical subunits
  – one subunit: around 440 amino acids

• Xylose isomerase is difficult to express in *S. cerevisiae*
  – Functional Expression of a Bacterial Xylose Isomerase in *Saccharomyces cerevisiae*
    D. Brat, E. Boles and B. Wiedemann
    Applied and Environmental Microbiology, Apr. 2009, p. 2304
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• Rationale for expression behavior?
Amino acid sequence Xylose Isomerase

Primary structure with conserved regions (Prosite)

MAKEMYFPQIKIKFKEGKDSKKNPLAFHYDAAEKEVMGKMKDKWLRFAMAMAWWHTLCAEGADQ

FGGTKSFPWNEGTDIAEIAKQKVDAFGFEIMQKLPGIYPYCFHDVDLVSSEGNSIEEYESNL

KAVVAYLKEKQKETGILKLLWSTANVFGHKRYMNGASTNPDVDVARAIVQIQNAIDAGIE

LGAEVYFPGRENGYMSLLNTDQKRKEKETHMATMLTMARDYARSKGFKGTFLIEPKPMETT

KHQYDVDTETAIIGFLKAHNLDKDFKVNIEVNHAFTLAGHTFEHEHELACAVDAGMLGSIDANR

GDYQNGWDTDQFIIDQYELVQAWEIIIRGGGFVTTGGTNFDAKTRRNSTDLEDIIIAHVSG

MDAMARALENAAKLLOESPYTKMKKERYASFDGSIGKDGEDGKLTTLEQVYEYGKKNGEPK

QTSGBKQELYEAIVAMYQ

Active site: conserved in all xylose isomerases

Metal-binding site: conserved in all xylose isomerases
Comparison of xylose isomerase from various sources

<table>
<thead>
<tr>
<th>Schematic representation</th>
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- : conserved amino acids active site
- : conserved amino acids metal-binding site
Comparison of xylose isomerasers from various sources

Eight sequences of xylose isomerase

Schematic representation

- ────  ────  ────
- ────  ────  ────
- ────  ────  ────
- ────  ────  ────
- ────  ────  ────
- ────  ────  ────
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- : conserved amino acids active site
- : conserved amino acids metal-binding site
## Xylose isomerase expression in *S. cerevisiae*

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*: conserved amino acids active site
*: conserved amino acids metal-binding site
*: conserved amino acids in xylose isomerase active in yeast
Amino acid sequence

Primary structure with conserved regions

MAKEYFPQIQKIKFEGKDSKNLAFHYYDAEKEMGKKMKDWLRFAIMAWWHTLCAEGADQMGGGTKSFPWNEGTDIAEIAKQKVDAFGEIIMQKLGIPYYCFHDVDLvSEGNSIEEEYESNLKAVVAYLKEKQKETGKLLWSTANFGHKRYMNGASTNPFDVDVVARAIQVIKNIAIDAGIE

LGAENYVFWGREGYMSLLNTDQKREKEHMATMLTMARDYARSKGFKGTFLLEEPKPMEPT

KHQYDVDTETAIGFKLKAHNLDKDFKVNIIEVNHAFLAGHTFEEHELACAVDAGMLGSIDANR

GDYQNGWDTDQFPIDQYLELVQAWM ElIRGGGFVTGGTNFDAKTRRNSTDLEDIIIAHVS

MDAMARALENAALQLQESPYTKMKKERYASFDSGIGKDGEDGKLTLLEQVYEYGKKNGEPK

QTSGKQELYEAIVAMYQ

Active site: conserved in all xylose isomerases

Metal-binding site: conserved in all xylose isomerases

Conserved in Xylose Isomerases active in yeast (patented)
Arabinose-fermenting RN1015

Mineral medium
0.2 g/l yeast pitch
Xylose-fermenting RN1001

Mineral medium
0.2 g/l yeast pitch

Ethanol
Xylose
Glucose

Marker-free strain
## Fermentation rates in 2008

<table>
<thead>
<tr>
<th>Strain</th>
<th>Compound</th>
<th>Rate (g sugar.(g yeast. h)^{-1})</th>
</tr>
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<tbody>
<tr>
<td>RN1001/1015</td>
<td>Glucose</td>
<td>3.0</td>
</tr>
<tr>
<td>RN1001</td>
<td>Xylose</td>
<td>1.4</td>
</tr>
<tr>
<td>RN1015</td>
<td>Arabinose</td>
<td>0.7</td>
</tr>
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Several routes have been taken to optimize the performance of the original xylose-fermenting strain.

Key aspects:
- Rate of xylose consumption
- Tolerance to acetic acid
Xylose consumption and ethanol production at 3g/l Acetic acid

Ethanol Production

Xylose consumption

Mineral medium
0.5 g/l yeast pitch
Application test at ABNT (York, NE, USA)
• The current C5 Yeast Company strain is performing very well
• Several tons of yeast that exhibit C5 Yeast Company’s Technology have been produced successfully by a commercial partner
• This yeast has been tested successfully at pilot scale

Nevertheless:
• Better hosts may be available that are more suitable in toxic hydrolysates
Industrial hosts

• Several strains have been tested for their potential as better hosts

• Promising strains have been obtained

• These strains are used as hosts for the C5-technology
Other strain on corn stover hydrolysate

Glucose only for RN1026
Glucose plus xylose for RN1016

Both strains are diploid organisms
Conclusions

• C5 Yeast Company yeast is available and has been produced at ton scale.

• C5 Yeast Company collaborates with first-movers in the ethanol industry around the world.

• C5 Yeast Company yeast is being successfully trialed at pilot scale at leading ethanol producers and technology suppliers.

• C5 Yeast Company continues to further develop and improve its yeast strains to resist higher inhibitor concentrations, etc.