Biocatalysis for sustainable solutions

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Unlocking The Magic of Nature.

Natural microorganisms

Molecular Biotechnology

Large-scale production

Industrial applications

novozymes
Novozyme’s Toolbox

- **Protein expression and comparison**
- **Proteomics**
  - mRNAs expression profiling, strain comparison
- **Microarrays**
  - Rational design
  - Regio-mutagenesis
  - Random mutagenesis
  - Screening
- **Directed Evolution**
  - Protein purification, characterization, and testing
- **Bioinformatics**
  - Homologue identification & analysis
- **Fungal Expression**
  - New gene expression
  - Fungal development
  - Fermentation opt.
- **Biochemical Characterization**
  - Protein expression and comparison

Novozymes
Ethanol market drivers

- Reduction of oil dependence
- Energy diversification
- Rural economic development
- Agribusiness development
- MTBE and lead replacement
- Kyoto protocol
- Sustainable transport fuel
Enzymes are key factor in Bioethanol:

- **1st generation biofuel, from starch (or sugar)**
  - starch: only two enzymes needed
  - enzymes have easy access to the substrate;
  - fungal amylases: eg from *Talaromyces*, produced in *Aspergillus oryzae*

- **2nd generation biofuel, from lignocellulose agricultural waste**
  - **Challenge: more efficient blends; and better access**
  - Numerous enzymes needed; fungal enzymes are superior
  - Several cellulases and numerous hemicellulases
  - Mostly used genes are from *Trichoderma, Aspergillus, Humicola* and wood-degrading Basidiomycetes
1.gen: Just two enzymes needed!
But starch binding domains (SBD`s) play a significant role!

- The enzymes attach on the surface via the SBD
- SBD`s dissolve the starch structure
- Efficient enzymes drills holes in the starch granule
- Fungi are the main SBD donor organisms
Success of 1st gen biofuel enzymes due to significantly improved (e.g. faster reduction of viscosity)

Method used: Falling ball timed in 85°C mash.

The novel *B. stearothermophilus* amylase

*B. licheniformis* amylase
Plant cell walls are complex => many enzymes needed for 2nd generation biofuel!

- Xyloglucan
- Cellulose fibres
- Pectic Substance

[Diagram of plant cell walls with various components labeled]
Novozymes R&D: 2nd generation biofuel

• Novozymes in US, Davis & Franklinton:
  • corn stover (lignocellulose)
  • NREL preprocessing
  • Abengoa collaboration

• Novozymes, DK:
  • wheat straw
  • wet oxidation or IBUS pre-processing
  • DTU, Risø and KVL university collaborations
  • Biogasol and Elsam collaboration (DONG Energy)
DANISH BIOETHANOL CONCEPT

The technology

**Wet Oxidation**
- In: Biomass
- Out: Ethanol

**SSF Fermentation**
- In: Enzyme
- Out: Ethanol

**Xylose Fermentation**
- In: Enzyme
- Out: Ethanol

**Anaerobic Treatment**
- In: Manure
- Out: Biogas

DTU: Birgitte Ahring

Risø: Belinda Thomsen
At least 4 types of enzymes to break down the cellulose!

In *T. reesei*:
- CBH I: 60%
- CBH II: 15%
- EG: 20%
- BG: 2%

and many more hemicellulases!
Proteomic Analysis of Cellulolytic Fungi

Charge →

Mass ↑

CBH I

CBH I

CBH I

CBH I

EG

EG

T. reesei

novozymes
Now much stronger discovery technology!

TAST signal trapping:
= the most efficient method for discovery(*) of secreted enzymes

TAST advantages:
- positive selection of secreted hits
- independent of available assay
- independent of known sequences

*) from species with no available whole genome sequence data
OBS secreted proteins only ca 4% of mRNA
Transposon assisted Signal Trapping = TAST

- Gene library
- Gene for secreted protein
- Gene 1
- Gene 2
- Gene for non-secreted protein
- + transposon (β-lactamase /no signal)

- secreted protein
- active
- + Ampicillin
- no secretion => inactive
Many fungal sources of CBH1 genes

- *Acremonium thermophilum*
- *Chaetomium thermophilum*
- *Scytalidium thermophilum*
- *Thermoascus aurantiacus*
- *Trichophaea saccata*
- *Sporotrichum pruinosum*

*Thermophiles*
Discovery of a fungal Cyanovirin!

- **Citrinovirin** (11kda) was found by Signal Trapping of a cDNA library of *Penicillium citrinum* (Lange & Schnorr, 2003)

- **Citrinovirin** has a signal peptide and is a secreted protein both in the wild type *P. citrinum* and in heterolog expression in *Aspergillus oryzae*

- heterolog expression was successful from both gDNA and cDNA

Conserved S-bridges on both!
TAST is done in *E.coli* but works for all groups of Eukaryotes (signals processed correctly!)

TAST-screening of cDNA libraries made successfully on:

- Ascomycetes
- Basidiomycetes
- Zygomycetes
- Plants
- Invertebrates
- Mammals
- G+ bacteria
- G- bacteria
- Archaea

=> signal peptides are recognized by *E.coli* across all the biological kingdoms
Interesting break through for biocatalysis of arabino xylan

• TAST gene discovery combined with
• improved understanding of enzyme / substrate interaction

• (acknowledgement: Hanne Sørensen, post doc, Novozymes; published: PhD thesis, DTU, 2006)
Arabinoxylan degrading enzyme activities

α-L-arabinofuranosidase
EC 3.2.1.55

Ferulic acid esterase
EC 3.1.1.73

Acetyl xylan esterase
EC 3.1.1.72

endo-β-1,4-xylanase
EC 3.2.1.8

β-xylosidase
EC 3.2.1.37
Mode of action of α-L-arabinofuranosidase

Studied by $^1$H NMR

αAra1,3$\text{_{ mono}}$

αAra1,2$\text{_{ di}}$

αAra1,3$\text{_{ di}}$

GH43 AraF

Minimal enzyme cocktails

Water soluble and water insoluble wheat arabinoxylan

- **GH51 α-L-arabinofuranosidase** from *M. giganteus*
- **GH43 α-L-arabinofuranosidase II** from *H. insolens*
- **GH10 Endo-1,4-β-Xylanase III** from *H. insolens*
- **GH3 β-xylosidase** from *T. reesei*
Addition of ferulic acid esterases:
Feruloyl esterase from *A. niger*
Feruloyl esterase from *H. insolens*

Total enzyme dosage: 0.5 g enzyme protein · kg\(^{-1}\) DM
Reaction parameters: 24 hours at pH 5, 50°C

Release of 69% arabinose and 76% xylose

= Additional release of 12% arabinose and 12% xylose

Why not also use plant genes?
Heterologue Expression of non-fungal genes in filamentous fungi is a bottle neck

- The commercialized enzymes originate almost exclusively from fungi and bacteria
- Ample new and unexploited enzyme diversity is available from plants
- Heterologue expression of plant genes in filamentous fungi has proven to be difficult (poor yields)
- A msc thesis at Novozymes studied the reason why

Results published:

- Hamann T & Lange L., 2006: "Discovery, cloning and heterologous expression of secreted potato proteins reveal erroneous pre-mRNA splicing in Aspergillus oryzae"
- J. Biotechnol. 2006 June 28
mRNA of plant gene expressed in *Aspergillus oryzae* found to be of several lengths
What happened with the plant gene in *A. oryzae*?

- *Aspergillus oryzae* recognizes certain parts of the coding region as splicing sites
- => erroneous pre-mRNA splicing
- => several lengths mRNA
- => no full length and no functional protein produced

- *Aspergillus oryzae*, the major production organism for Novozymes fungal enzymes
Details of splicing sites and branch points

Hamann & Lange
J. Biotech. 2006
Enzymes are eco-friendly solutions, used in numerous market segments => need for broad spectrum discovery

- Replacing chemicals
- Saving energy
- Diminishing pollution
- Better use of raw materials
- Saving water

Enzymes replace bromate in bread production

Detergents with enzymes wash the clothes at lower temperatures

Enzymes in animal feed reduce the amount of phosphorus in the dung

Enzymes squeeze out all of the juice from the fruits

With enzymes, tanneries use less rinse water in leather production

- Bread
- Detergent
- Feather
- Orange
- Leather
Novozymes A/S

- Largest Industrial Enzymes Producer (45% market share)
- More than 600 products sold in 130 countries
- Revenues (2005): ~$1 billion
- Employees: ~4,000 (> 600 in R&D)
- Subsidiaries in the U.S.
  - Novozymes, Inc., Davis, CA
  - Novozymes North America, Franklinton, N.C.
- Other Major Subsidiaries in Japan, China, Brazil, France, Switzerland
- Production sites in Denmark, U.S., China, Brazil
- Over 4300 active patents
- Largest number of recombinant protein products in the world
- Additional business areas in Novozymes: Biologicals and Biopolymers
Future needs for Novozymes, in relation to 
- HTScreening with Nanoapproach.

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<thead>
<tr>
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<th>Conventional Screening</th>
<th>New Technology</th>
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<tr>
<td>Volume per assay</td>
<td>5-50 μL</td>
<td>0.000001 μL</td>
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<tr>
<td>Protein per assay</td>
<td>10 - 100 ng</td>
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<tr>
<td>Cells needed per assay</td>
<td>500-5000</td>
<td>1-500</td>
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<tr>
<td>Assays per time period</td>
<td>1000 per hour</td>
<td>1000 per second</td>
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Emission of greenhouse gases from production and use of one kg enzyme product from Novozymes

Enzyme production:
1-10 kg CO₂

- Leather
  -30 kg CO₂

- Forest products
  ...

- Food
  ...

- Oil and fats
  -1300 kg CO₂

- Cereal products
  ...

- Animal feed
  -150 kg CO₂

- Detergent
  ...

- Brewing
  ...

- Cosmetics
  ...

- Textile
  -100 kg CO₂
Novozymes’ vision

“We imagine a future where our biological solutions create the necessary balance between better business, cleaner environment and better lives”.

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• Thomas Hamann, KVL (phd student)

• Birgitte Ahring and Claus Hviid, DTU
Conclusion:

- Biological production and Biocatalysis are cornerstones for development of products, processes and solutions, building a knowledge-based bioeconomy.

- Biology is back in focus, for products and processes (genomics and nanotech are tools, not the goals).
Thanks for your attention!