Thermostable Cellulases: Why & How?

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Royal DSM

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DSM: Company Profile 2009

Life Sciences and Materials Sciences Company

- **Global top 30 chemical industry**
- Net sales : $13,692 million
- Net earnings: $895 million
- **23,500 employees**
  - R&D: approx. 2,130
  - in the Netherlands: approx. 7,200
  - in the US: approx. 3,000
- >200 locations on 5 continents
- **Among Top Three listed in Dow Jones Sustainability Index** in 2004, 2005, 2006, 2007, 2008, 2009
- **Strong technological toolbox**:
  Integrated use of biotechnology, biocatalysis, organic chemistry, chemical and polymer technolog materials sciences

Member of Dow Jones Sustainability Indexes 06
Member of Dow Jones Sustainability Indexes 05
Member of Dow Jones Sustainability Indexes 04

World Business Council for Sustainable Development

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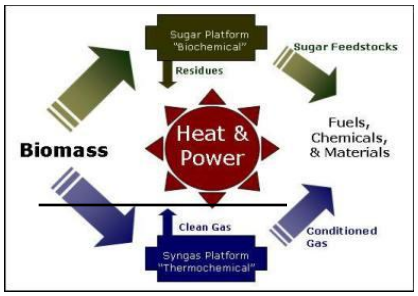
Oil-Refineries and Bio-Refineries

- *Oil*-refinery:
  - Crude oil (finite) as input
  - Established technology
  - Very efficient use of raws

- *Bio*-refinery (1st generation):
  - Starch / sugar (renewable) as input
  - Technology established
  - Efficient use of feedstock



- *Bio*-refinery (2nd generation):
  - Biomass (renewable) as input
  - Logistics?
  - Technology very much in development (Enzymes & FO)
  - Byproducts?
  - Less efficient use of feedstock

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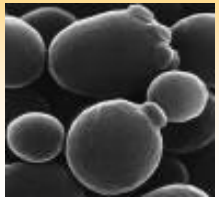
Replenishing Chemistry by Biotechnology

Carbo hydrates

Enzymes

Yeast



Bio-fuels

Oil

Chemistry

Polymerization

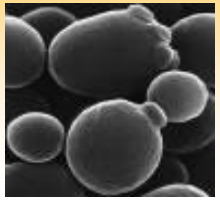
Materials

New Opportunities from the Combination

Carbo hydrates

Enzymes

Fermentation organism



Bio-fuels

BioChemicals

BioPolymers

BioMaterials

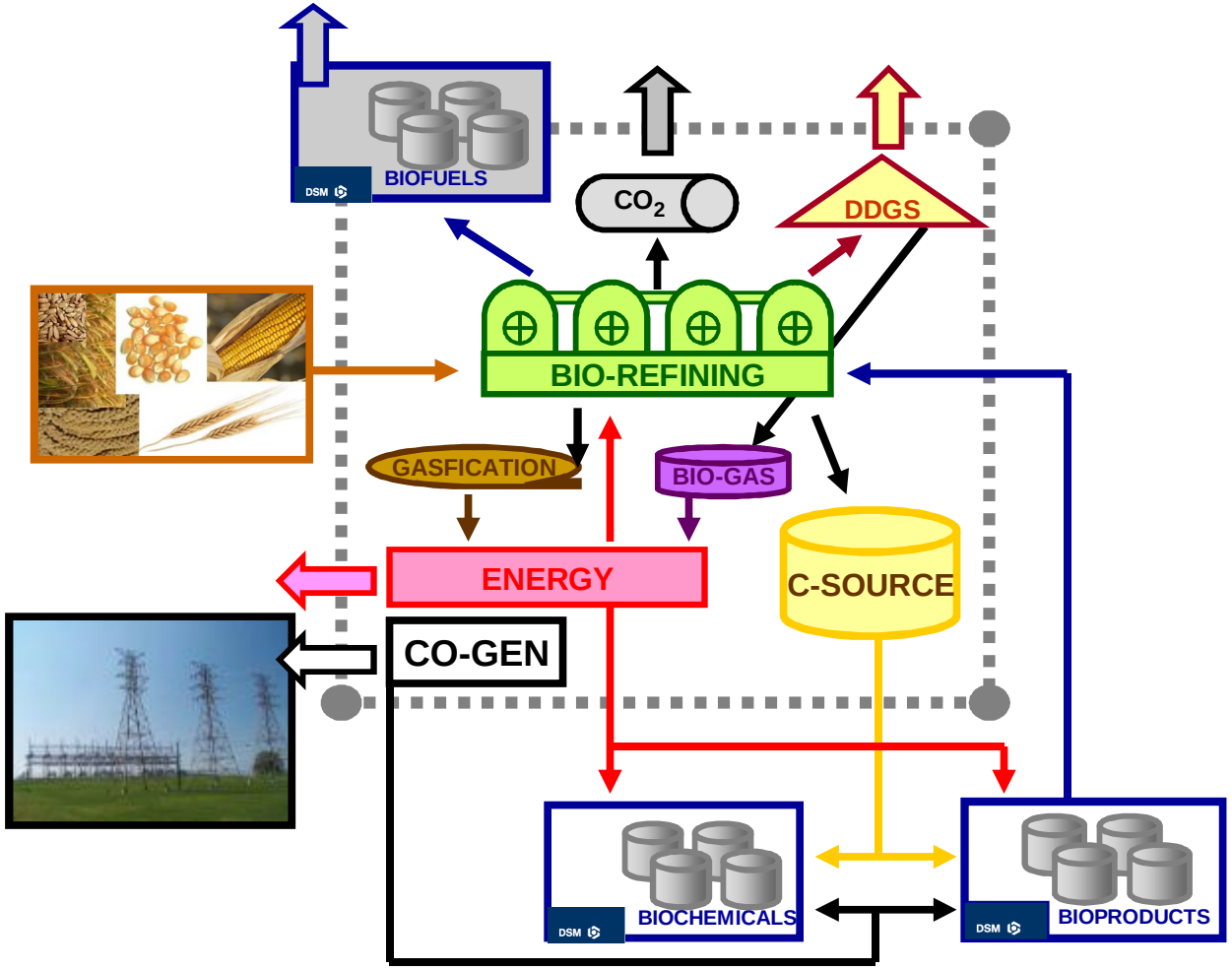
Oil

Chemistry/ Enzymology

Polymerization

Resulting in a broad range of innovative bio-based businesses

Biorefinery for Bio-based Building Blocks (Sugars)



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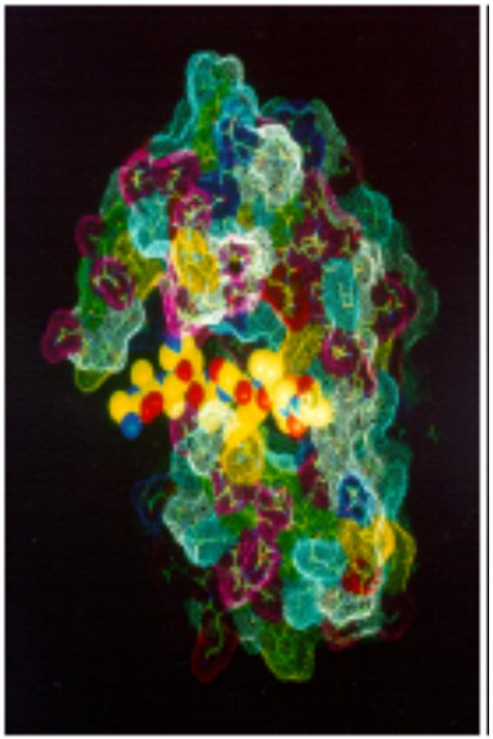
Enzymes: *A Core Bio-based Building Block Platform for DSM*



- **DSM possesses a strong knowledge base within enzyme research-, application and manufacturing**
- **DSM holds hundreds of patents based on enzyme technology including biomass deconstruction enzymes**
- **DSM sells $300MM worth of enzymes in baking, food & fruit processing, brewing, wine, dairy, animal feed, and biocatalysis**
- **DSM enzyme technology is also used in the production of other DSM products like antibiotics, flavors, animal feed, and food.**
- **DSM has global enzyme business**

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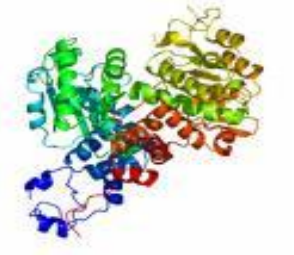
DSM Enzymes for Cellulosic Feed-Stocks:
Leveraging DSM's Enzyme Technology

- DSM has a large collection of both commercial and pre-commercial hydrolases (cellulase, hemicellulase, mannanase, pectinase) along with fermentation processes developed for various business segments
- Several existing DSM *in house* enzyme products of value for cellulose processing
- *US DoE Partnership with DSM for commercial development of cellulosic enzymes for lignocellulosic feedstocks*



Cellulase Enzymes

A. niger sequence*:* Pel *et al.* (2007) Nature Biotechnology **25**, 221

P. chrysogenum sequence: van den Berg *et al.* (2008) Nature Biotech. **26**, 1161

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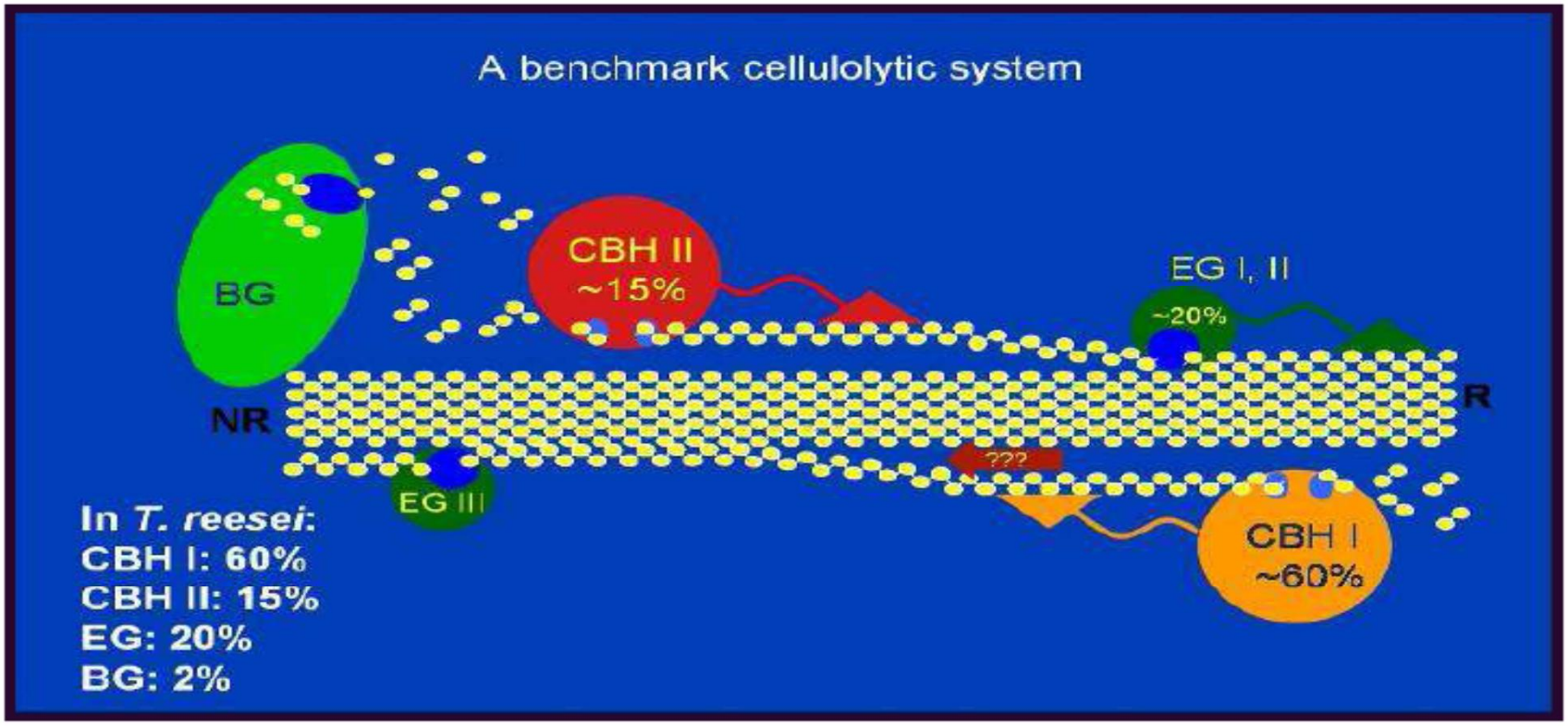
Trichoderma reesei cellulases: *Enzymes need to cooperate*



- Improve the catalytic efficiency of fungal cellulase components
- Supplement with additional components to make the system work better

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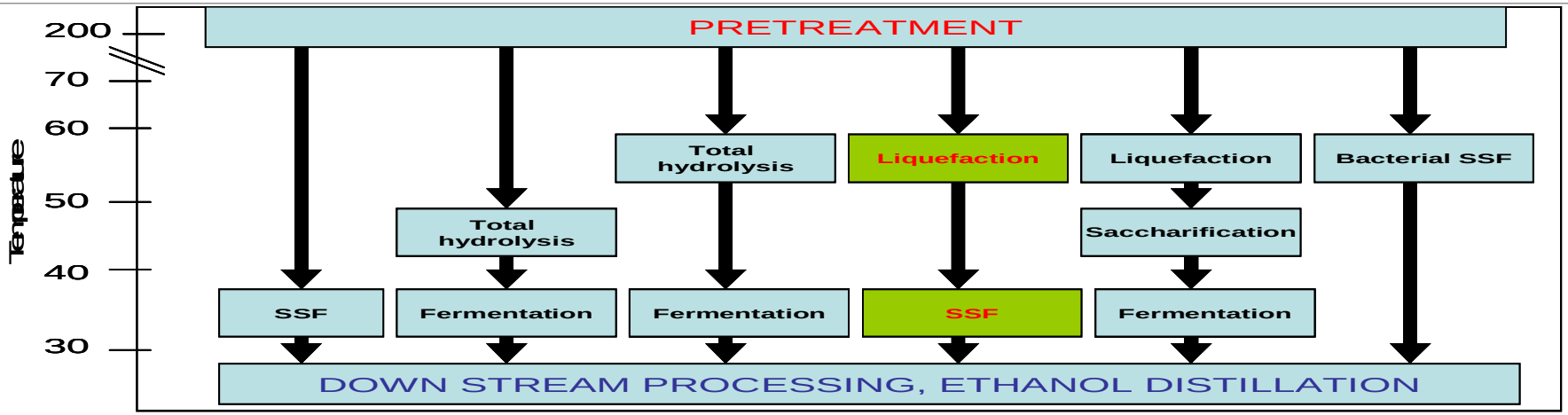
Configuration of various hydrolysis and fermentation processes at different temperatures using thermostable enzymes and DSM preferred choice. (SHF, SSF, HHF, HHCF, NSSF, SSCF, CBP)



Selected hydrolysis and fermentation strategies

Name	Description	Features
SHF: Separate hydrolysis and fermentation fermentation	Enzymatic hydrolysis and fermentation done sequentially in different vessels	Hydrolysis and fermentation at respective optimal conditions; enzyme product inhibition; separate treatment of C5 and C6 sugar streams
SSF: Simultaneous saccharification & fermentation	Enzymatic hydrolysis and fermentation done simultaneously in same vessel	Compromise in conditions for optimal hydrolysis and fermentation; improved rates and yields; separate treatment of C5 and C6 sugar streams
HHF: Hybrid hydrolysis and fermentation	Enzymatic hydrolysis and fermentation done roughly sequentially in same vessel	Hydrolysis continues after shift to fermentation conditions; process optimization difficult; separate treatment of C5 and C6 sugar streams
NSSF: Non-isothermal simultaneous saccharification and fermentation	Enzymatic hydrolysis and fermentation done roughly simultaneously in different vessels	Hydrolysis and fermentation at respective optimal conditions; process optimization difficult; separate treatment of C5 and C6 sugar streams
SSCF: Simultaneous saccharification and cofermentation	Like SSF, only both C5 and C6 sugars are fermented in same vessel	Fewer vessels, lower capital costs; requires engineered microorganism optimized for efficient C5/C6 fermentation
CBP: Consolidated bioprocessing	Enzymatic hydrolysis and fermentation carried out in single vessel by single or combination of microorganisms	Fewer vessels, lower capital costs; requires engineered microorganism optimized for enzyme production and C5/ C6 fermentation

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Ref: Liisa Viikari, 2007, Adv Biochem Eng. Biotech

Thermostable Cellulases: *Why ?*

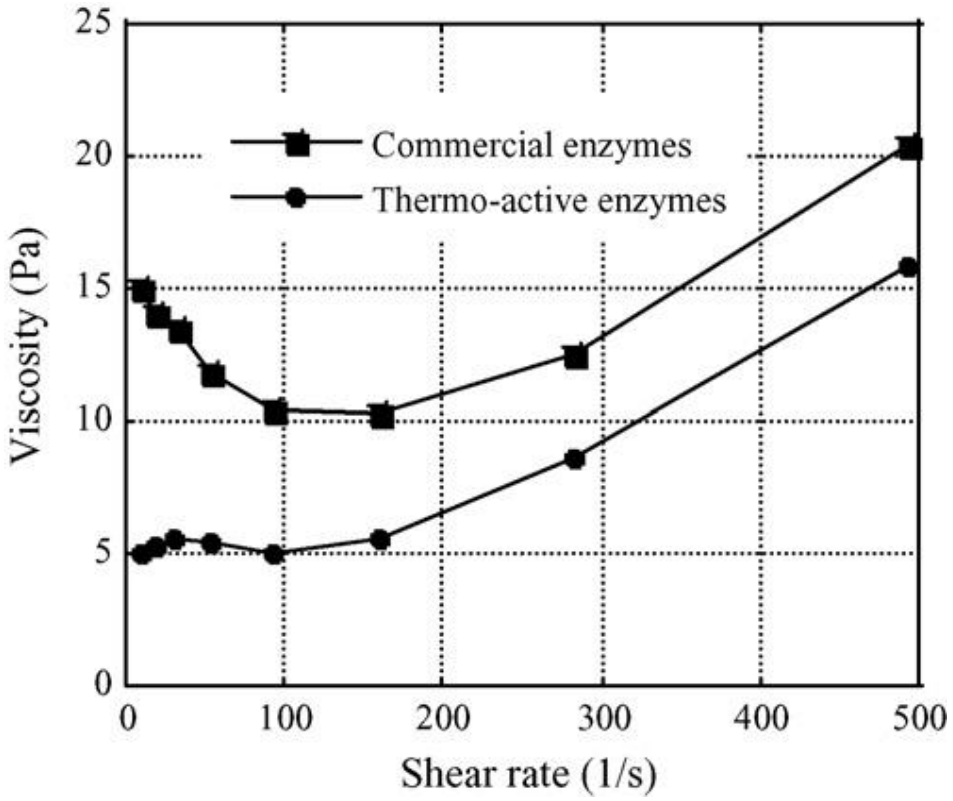
- Increased cellulase activity, less energy cost for cooling, higher DS loading, and decreased risk of contamination.
- Can be cloned and over-expressed at high levels in *fungal hosts*.
- Ease of DSP/Formulation since one can introduce heat step to precipitate *fungal host* proteins.
- Biotransformation reactions can be carried out at higher temperatures where accessibility to substrates gets better and lignin is less tightly associated with cellulose.
- Enzymes are more robust to exposure of inhibitors and product ethanol.
- More resistant to proteolysis.

Viscosity Reduction

- Positive effect of temperature on gelatinization of the solids.
  - At 65 C the solids become faster gelatin-like in comparison with 40C when treated with DSM enzyme.
    - higher absorption of water to the polymers.
  - Faster gelatinizing resulting in easier accessibility of the substrate by the enzymes
- The effect of higher temperature (65°C in comparison to 50 °C) on viscosity
  - sugar concentrations ~100 g/L. (**approx equal to complete hydrolysis of 20 % wheat straw**)
    - 50 °C: 70 Pa
    - 65 °C: 60 Pa
- Higher throughput in all steps
- Reduction in $/gallon capital installed having
  - lower capital and fixed costs, less evaporation energy per gallon produced.
- DSM enzyme effective enzyme concentration with its low absorption features to the substrate provides more available enzyme with high dosage of substrate.

Viscosity Reduction

- An effect of higher temperature on viscosity reduction of the slurry of pretreated feedstock at the start of the hydrolysis.
- This is expected as mixing same %DS at low or high temperatures is different.
- Reduced viscosity open the possibility to add more feedstock to the reaction mixture in hydrolysis thus increasing the dry solids content of the hydrolysate. High DS is crucial in order to reach economically viable ethanol concentration in the fermented slurry.



Ref: G. Zacchi, Enz. & Microb. Tech. 2006: Viscosity in slurries pre-hydrolysed for 16 h with the commercial enzyme mixture and the thermo-active enzymes.

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Contamination

- Operating at higher temperature during hydrolysis will result in a *'more sterile'* hydrolysate.
  - Hydrolysate obtained at 65°C will have a lower bioburden than hydrolysate of 45-50°C.
  - If hydrolysis time is extended and thereby fermentation time is shortened, *the risk on contamination during fermentation will also be significantly reduced.*
- An important advantage of DSM enzyme cocktail. A loss of 5% due to contamination problems is *not uncommon* for this type of industrial operations.
  - At 60 $/T feedstock and 80 gal/T ethanol yield, a 5% loss = 0.0375 $/gal.
  - At 3 $/gal installed capital and 10% depreciation = 0.015 $/gal
  - At 0.15 $/gal fixed costs = 0.0075 $/gal
- So the cost of contamination is then 0.06 $/gal. Not negligible to an enzyme cost of 0.10 $/gal.

Dosage

- Enzyme dosage is a key parameter for its cost contribution in making cellulosic ethanol.
- Lets compare DSM Enzymes at 50 and 60 °C
  - Application tests:
    - 20 % TS pWS (unwashed)
    - 72 h hydrolysis (50°C and 60°C)
    - 72 h fermentation (33 °C) with C6 yeast
- Experimental results obtained at 50 & 60 °C suggest 20-24% reduction in DSM enzyme cocktail dosage when operating at 60C vs 50 C to achieve same hydrolysis yield.

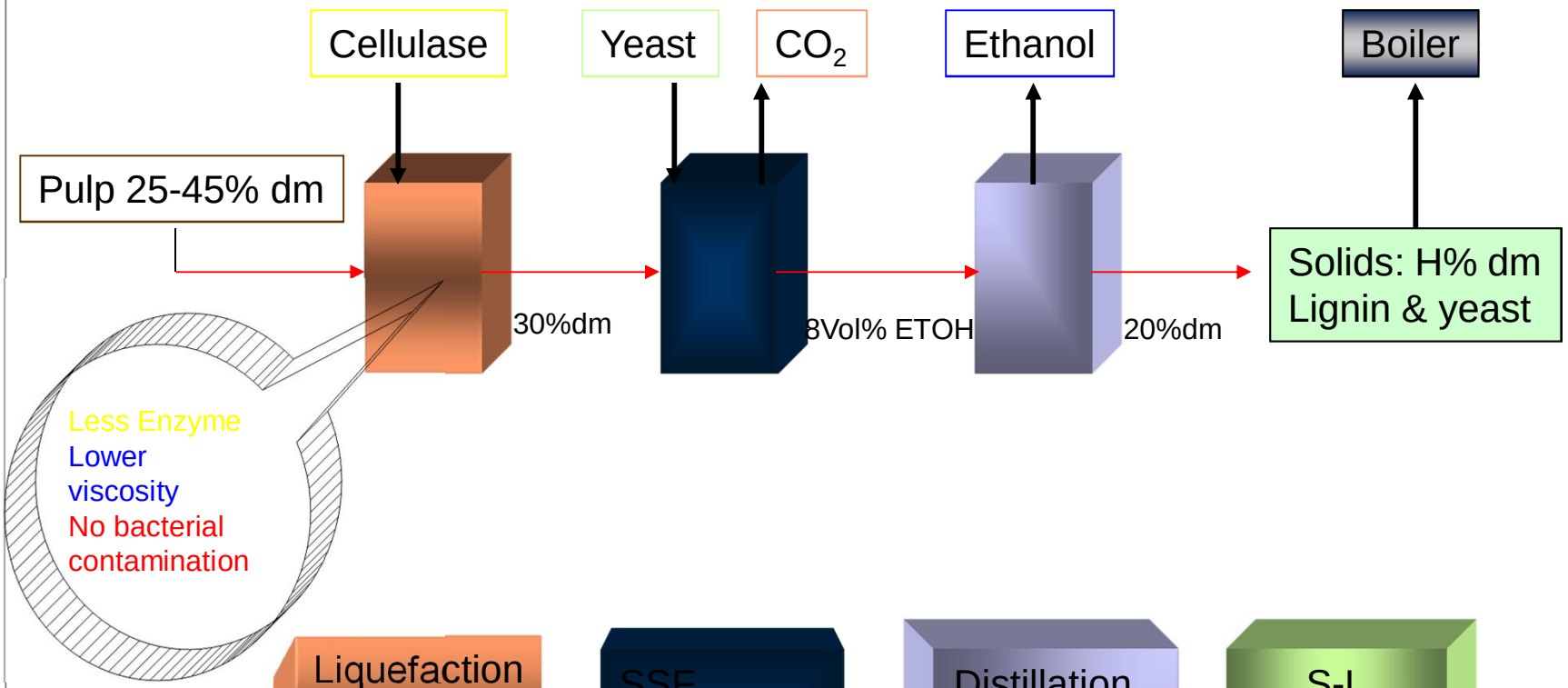
Energy costs saving

- The energy cost savings are related to the delta temperature between the unit operations during the whole process.
  - When fermentation is done at a higher temperature, cooling costs can be significantly lower.
- The advantage of thermostable enzymes
  - can be added in an earlier stage (higher temperature) after pretreatment thus reduced chilling cost to start liquefaction/saccharification/biomass hydrolysis
  - Reduction in overall process time.
  - Energy cost reduction significance depends on scale, process, and equipment.
- Reduction of energy cost on mixing during hydrolysis.
  - Adding enzymes in an earlier stage of the process also results in an earlier liquefaction which makes mixing easier
- Earlier liquefaction allows reduction in cooling capcity:
    - heat transfer is better in a mixed liquid than a static solid (or slurry).
- DSM thermostable cellulase cocktail is fully active between 60-65C.
  - cooling rate reduction during hydrolysis
  - reduction in cooling capacity

Stability, More Robust, Ease to Use

- DSM whole broth enzyme cocktail has a long storage stability of so far minimum of 3 months at RT, potentially related to the thermo-stability.
- Essential for building the *on-site* manufacturing and supply chain reliability.
- Cost of shelf, warehousing, transport, and inventory maintenance minimized
- DSM enzyme retained its full activity during SSF/SHF operation using 20% DS unwashed pCS (400h at 60 °C).
- Easy to use from an operation point of view.

High Temperature Advantages



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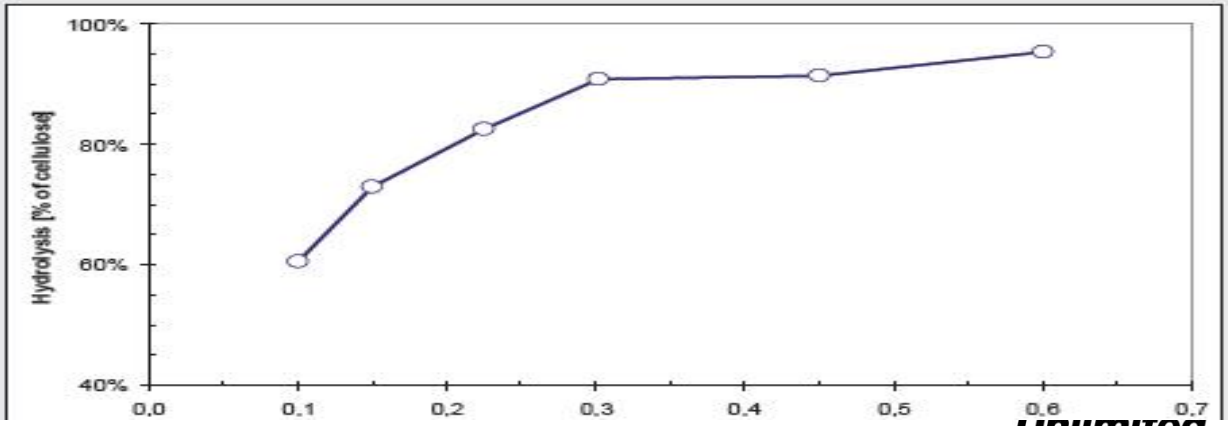
DSM Cellulosic Enzyme System Performance

Washed pWS before (left) and after (right) hydrolysis.



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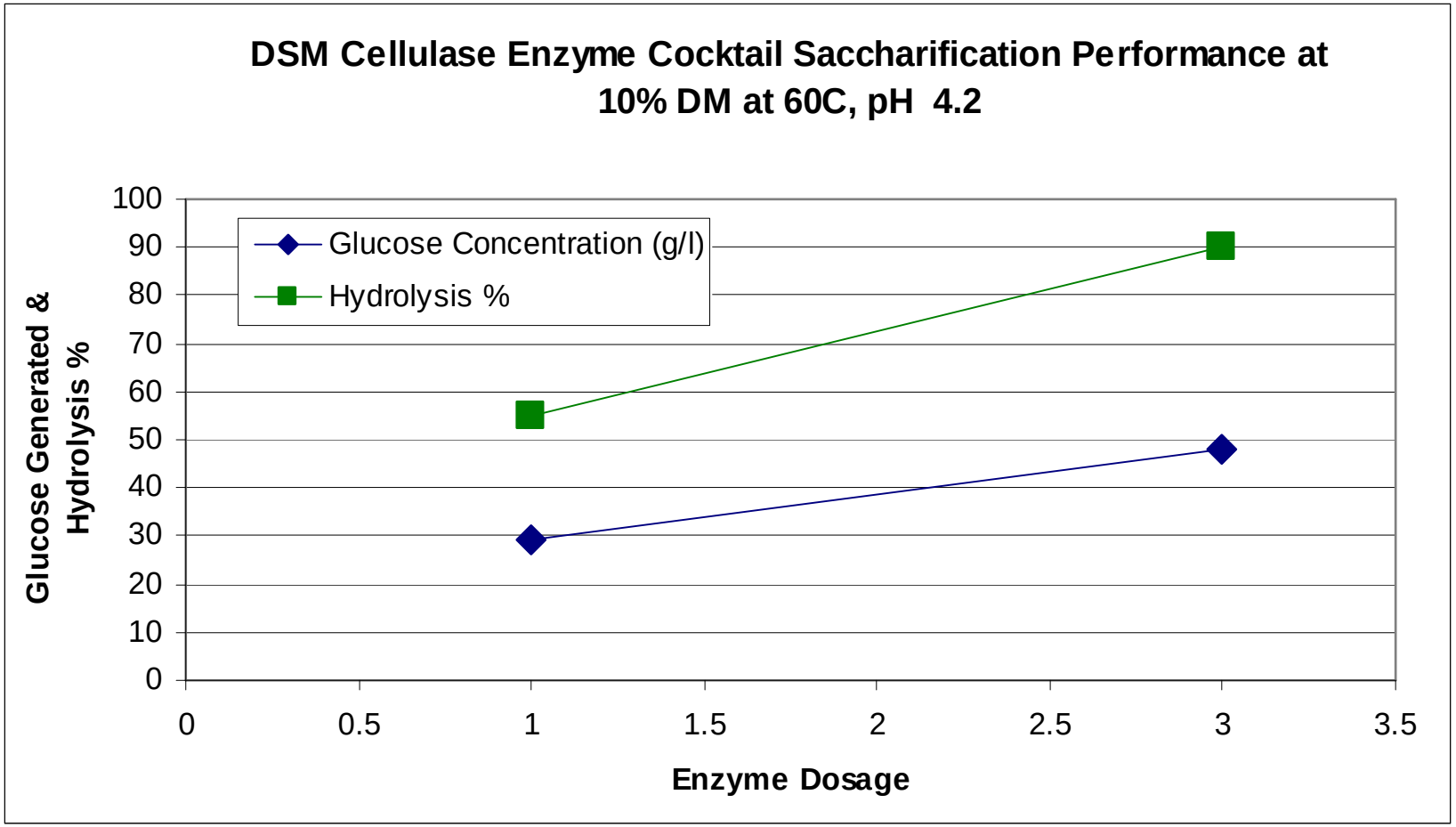
Saccharification at Low Enzyme Dose



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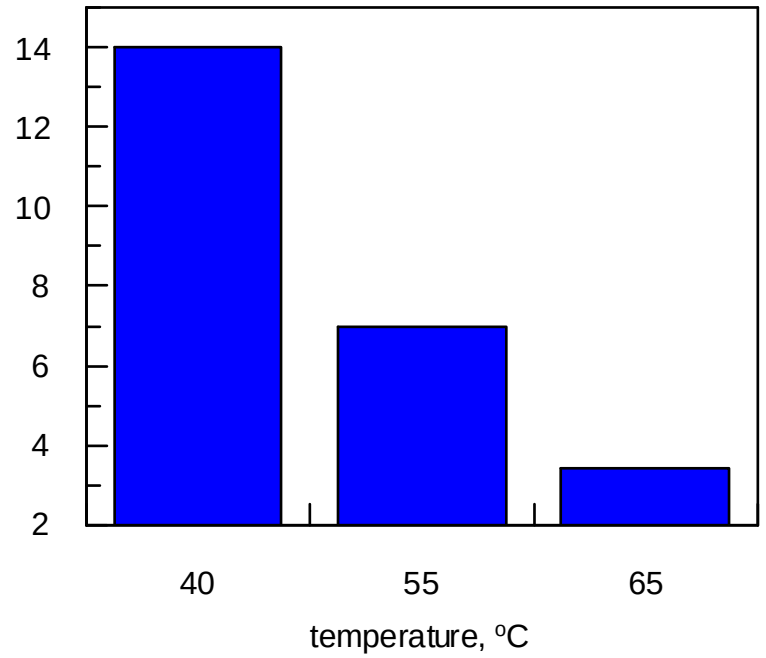
DSM Cellulase Enzyme Cocktail Performance

DSM enzymes work faster at higher temperatures

* Lowering both Capital & Operating Cost



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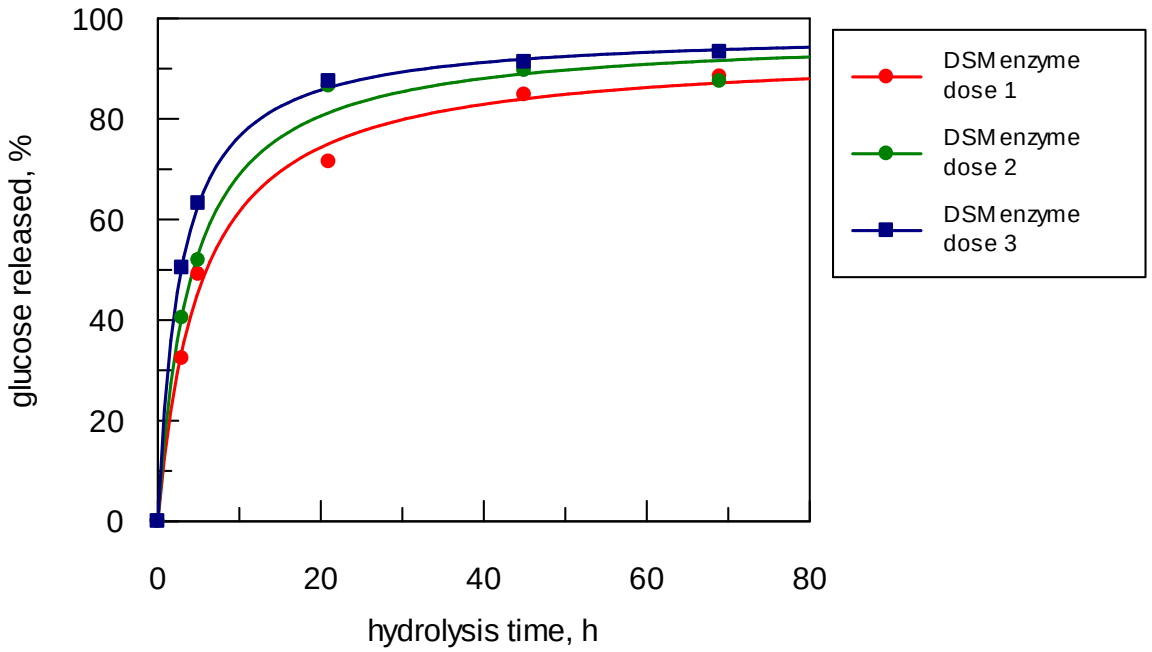
DSM Cellulase Enzyme Cocktail Performance

Glucose production at 65C from pretreated wheat straw using DSM enzyme system at different dosages



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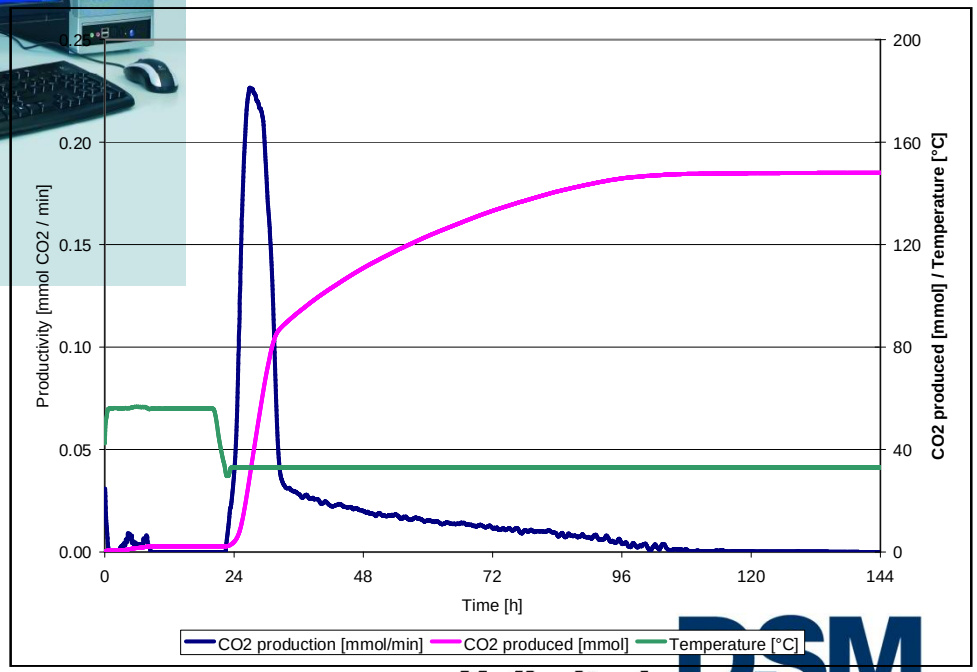
DSM Cellulase Enzyme Cocktail and Yeast: Partial Liquefaction and Fermentation to Ethanol



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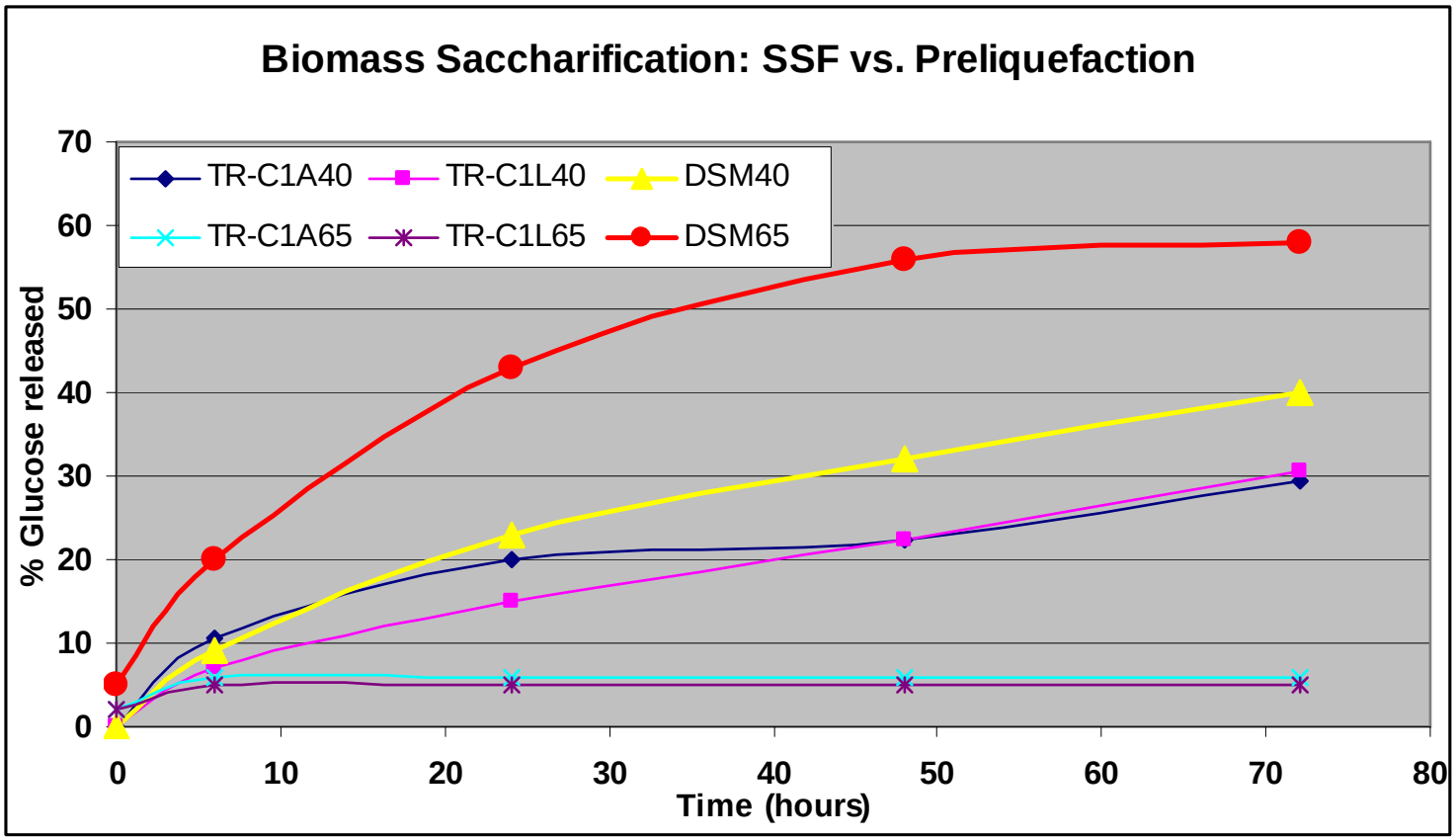
DSM White Biotechnology

DSM Cellulase Enzyme Cocktail Performance



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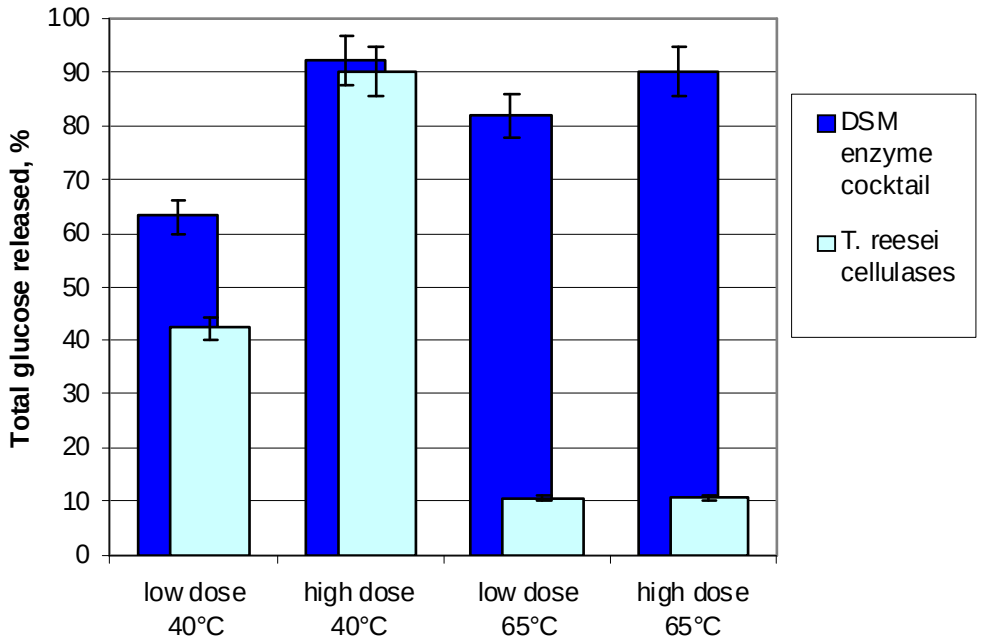
DSM Cellulase Enzyme Cocktail Performance

- Comparison of DSM enzyme performance on pretreated wheat straw with *T. reseei* derived cellulases at different temperatures



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DSM Cellulosic Enzyme System Product

- Differentiated and Tailor made for Biomass saccharification
  - Competitors enzymes based on *Trichoderma* technology basically originated for textile and paper industry
- Thermostable enzymes suited to work at 65C vs. 40-50C for *Trichoderma*
  - Lower dosage, no contamination, higher dry solid loading
- DSM enzyme system equally efficient for SSF, SHF, SHCF
- No interference with yeast growth
- Insignificant inhibition (glucose) up to 2% w/w
- No Inhibition (ethanol) up to 8% w/w
- Provides enough nutrients for yeast growth

Successful Feed-Stock Tests with DSM Enzyme Cocktail

- Corn fiber (dilute acid; hot water; steam explosion)
- Corn stover (dilute acid)
- Wheat straw (dilute acid; hot water; steam explosion)
- Spruce (SO_2 catalyzed steam explosion)
- Switch grass (dilute acid)
- Poplar (dilute acid)

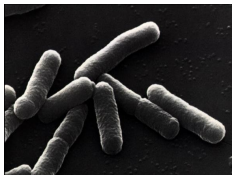
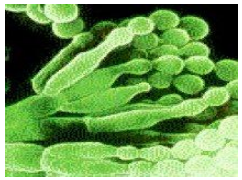
Thermostable Cellulases - *How?*

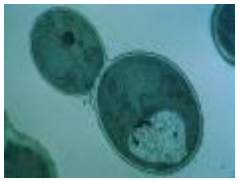
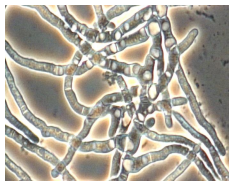
- **Key to success:**
- Diversity
  - Large libraries of (bio)catalysts required
  - Access to genomes and meta-genomes
  - Directed evolution to optimize biocatalysts
- Screening Power
  - Automation and miniaturization
  - High through-put screening
  - Fast and reliable analysis
- Protein Engineering
  - Directed Evolution
  - Rational Design
  - Molecular biology assisted variants generation

DSM plu*G*bug® : *Key to Industrial Process Development*

- Limited number of enzyme production organisms - focus
- Overexpression: higher activity per cell than natural isolates
- Higher cell density in less time as compared to isolates
- Higher volumetric productivity (activity / fermentation volume)
- Less interfering enzymatic activities (known background activities)

- Lower *Enzyme* costs & shorter development times

- plu*G*bug® technology for rapid and cost effective *Biocat* development
- *In-house* production with plu*G*bugs® provides maximum flexibility

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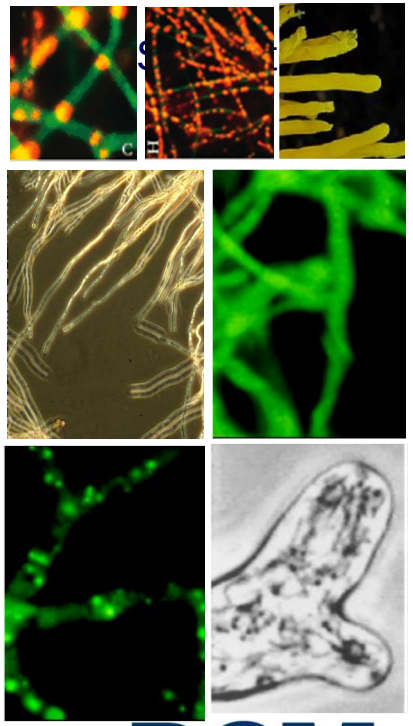
DSM Industrial Strain Development: *"Design & Build" concept*

- **'Developing genetic tools and procedures to generate genetically pre-defined and stable rDNA production strains, improved for protein production, which possess finally no selection marker-gene at all'**

- **Selection marker-gene free system**
  - genetic tool for repeated modification of host properties
  - important legislation issue

- **Modified *A.niger* pluGBug™ host**
  - (Key Enzyme negative / protease poor / etc…..)

- **Genetically entirely (pre-)defined using amplicons**
  - all expression cassettes targeted in 'empty' *key enzyme* loci – controlled construction
  - prevents change in fermentation behavior – defined protocol
  - higher enzyme expression levels
  - early registration



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Synopsis

- Thermo-stability and lower pH optimum properties of DSM enzyme cocktail provides multiple economical, technical, and functional advantages
- DSM cellulase enzyme is ready to be used for various pretreatments and feed-stocks
- Full saccharification >90% glucan hydrolysis in both SHF and SSF (lower enzyme load) batch process possible
- Fed batch application (feeding fibers to enzyme) at higher dry matter possible
- Use of DSM's cellulase enzyme cocktail for producing feed-stocks for its industrial biotechnology enterprise
  - ***A company with fermentation-based products revenue of 1.5 billion Euro***