Engineering Cellulases for Biorefinery

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Global New Business Development
White Biotechnology
Royal DSM

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DSM: Company Profile 2009

Life & Material Science 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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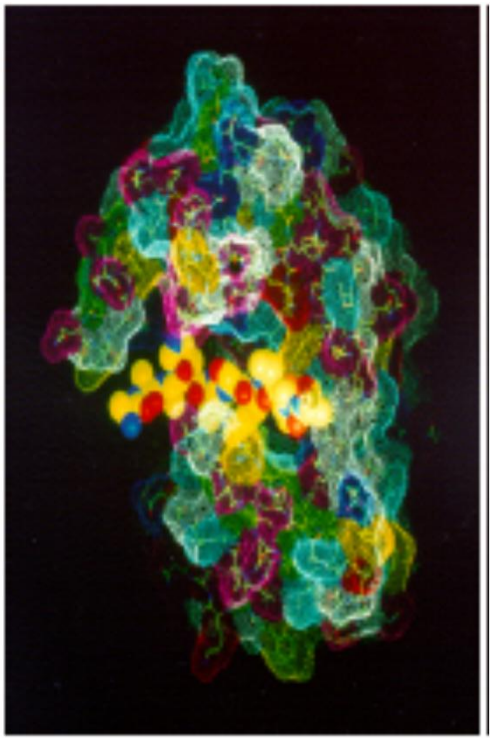
Enzymes: *A Core Bio-based Building Block Platform for DSM*



- 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, antibiotics, flavors, and biocatalysis
- US DoE Partnership with DSM for commercial development of enzymes for lignocellulosic feed-stocks saccharification.
- DSM has a global enzyme business

DSM Engineered Cellulases Solution

- Differentiated and Tailor made for Biomass saccharification
  - Peers 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 efficient for SSF, SHF, SHCF
- Fast viscosity reduction allowing higher DS via fed-batch
- No interference with yeast growth
- Insignificant inhibition (glucose) up to 6% w/w
- No Inhibition (ethanol) up to 8% w/w
- On-site manufacturing/ whole broth: provides enough nutrients for yeast growth

Thermostable Cellulases: *Why ?*

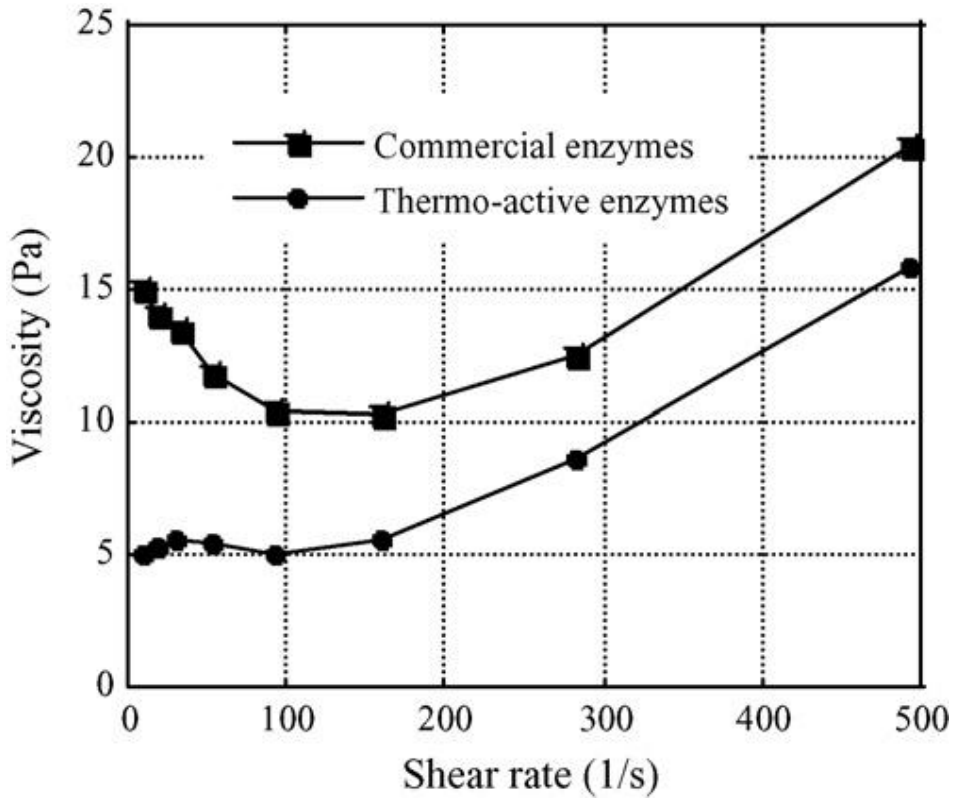
- Increased rate of cellulase activity, less energy cost for cooling, higher DS loading, and decreased risk of contamination.
- Can be cloned and over-expressed at high levels in *fungal hosts*.
- Ease of DSP/Formulation since one can introduce heat step to precipitate *fungal host* proteins.
- Biotransformation 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 liquifaction 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 Pascal.sec
    - 65 °C: 60 Pascal.sec
- Higher throughput in all process steps
- Reduction in $/gallon capital installed having
  - lower capital and fixed costs, less evaporation energy per gallon produced.
- DSM enzyme's effective cellulase concentration with its low absorption features to the substrate provides more available enzyme with high dosage of substrate resulting in faster generation of glucose.

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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Prevention of Contamination

- Operating at higher temperature during hydrolysis will result in a *'more sterile'* hydrolysate.
  - Hydrolysate obtained at 65°C will have a **significantly** 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

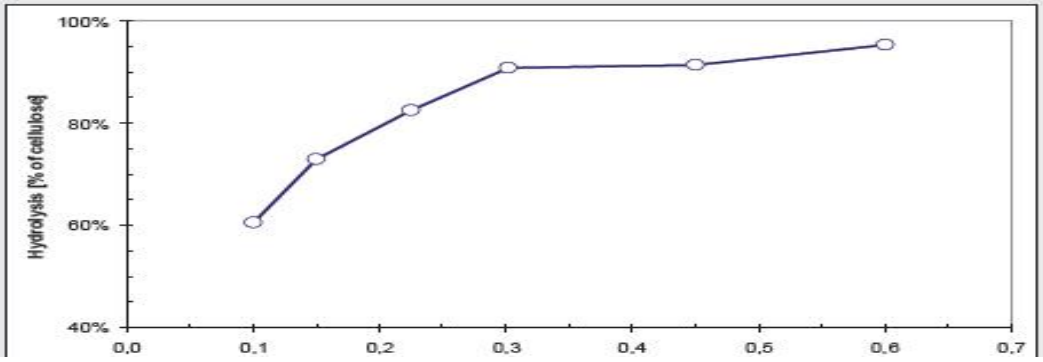
- The energy cost savings are related to the delta temperature between the unit operations during the whole process.
  - When fermentation is done at a higher temperature, cooling costs can be significantly lower.
- The advantage of thermostable enzymes
  - can be added in an earlier stage (higher temperature) 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 capacity:
    - 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

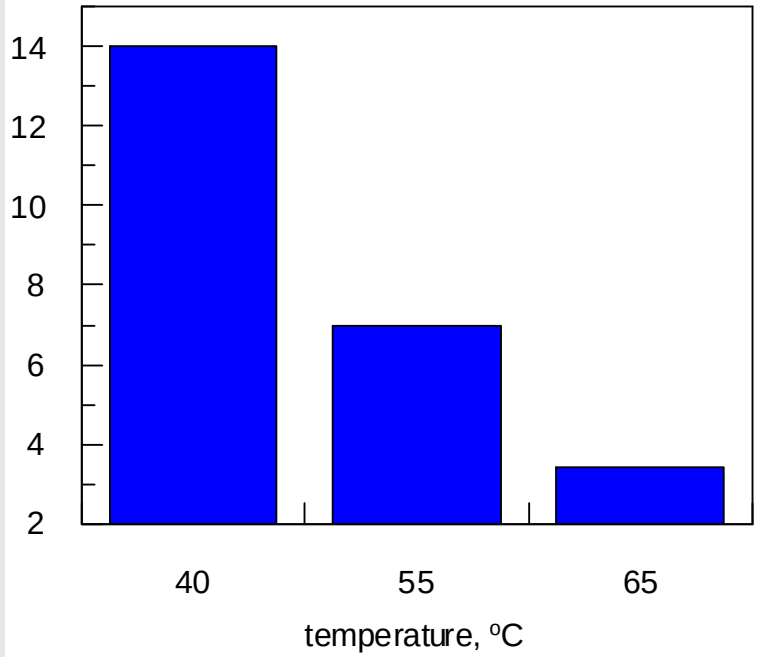
- DSM whole broth enzyme cocktail has a long storage stability of so far minimum of 3 months at RT, potentially related to the thermo-stability.
- Whole broth enzyme stability is essential for building the *on-site* manufacturing and supply chain reliability.
- Cost of shelf, warehousing, transport, 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.

DSM Cellulosic Enzyme System Performance

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



Enzyme Dosage



DSM enzymes work faster at higher temperatures

Lowering both Capital & Operating Cost

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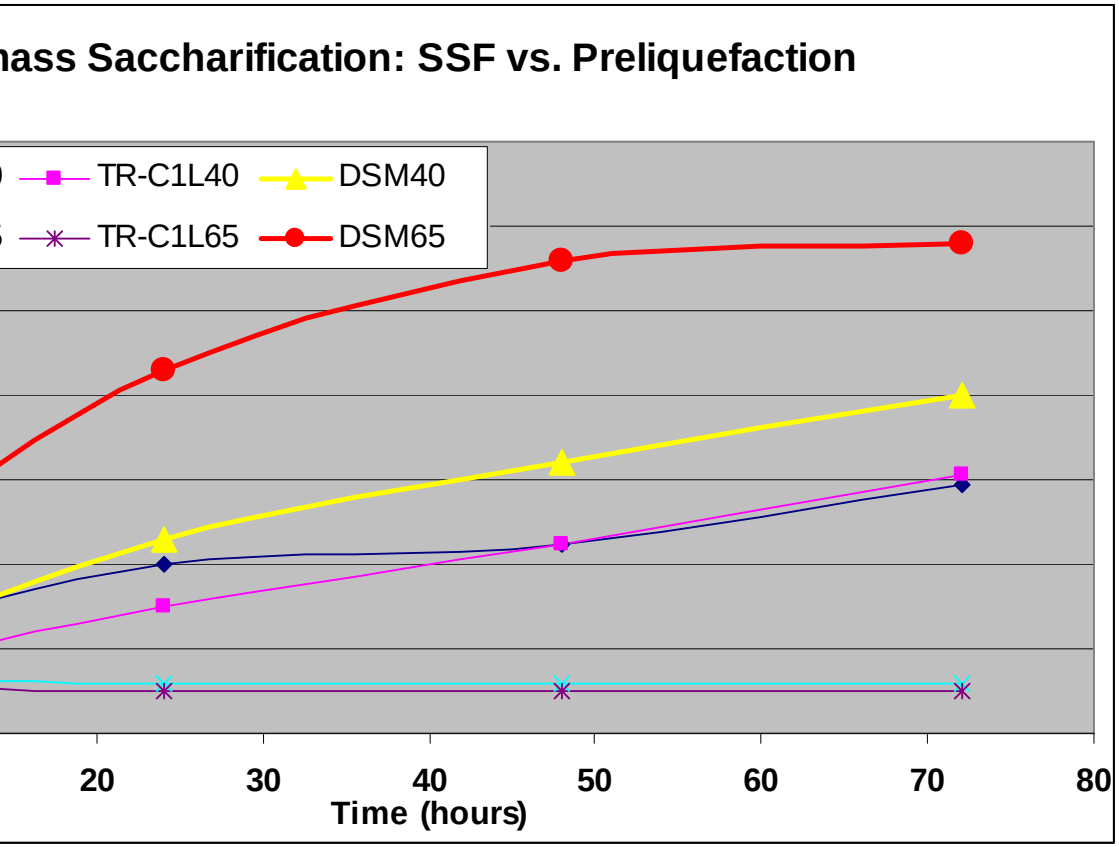
DSM Cellulase Enzyme Cocktail Performance



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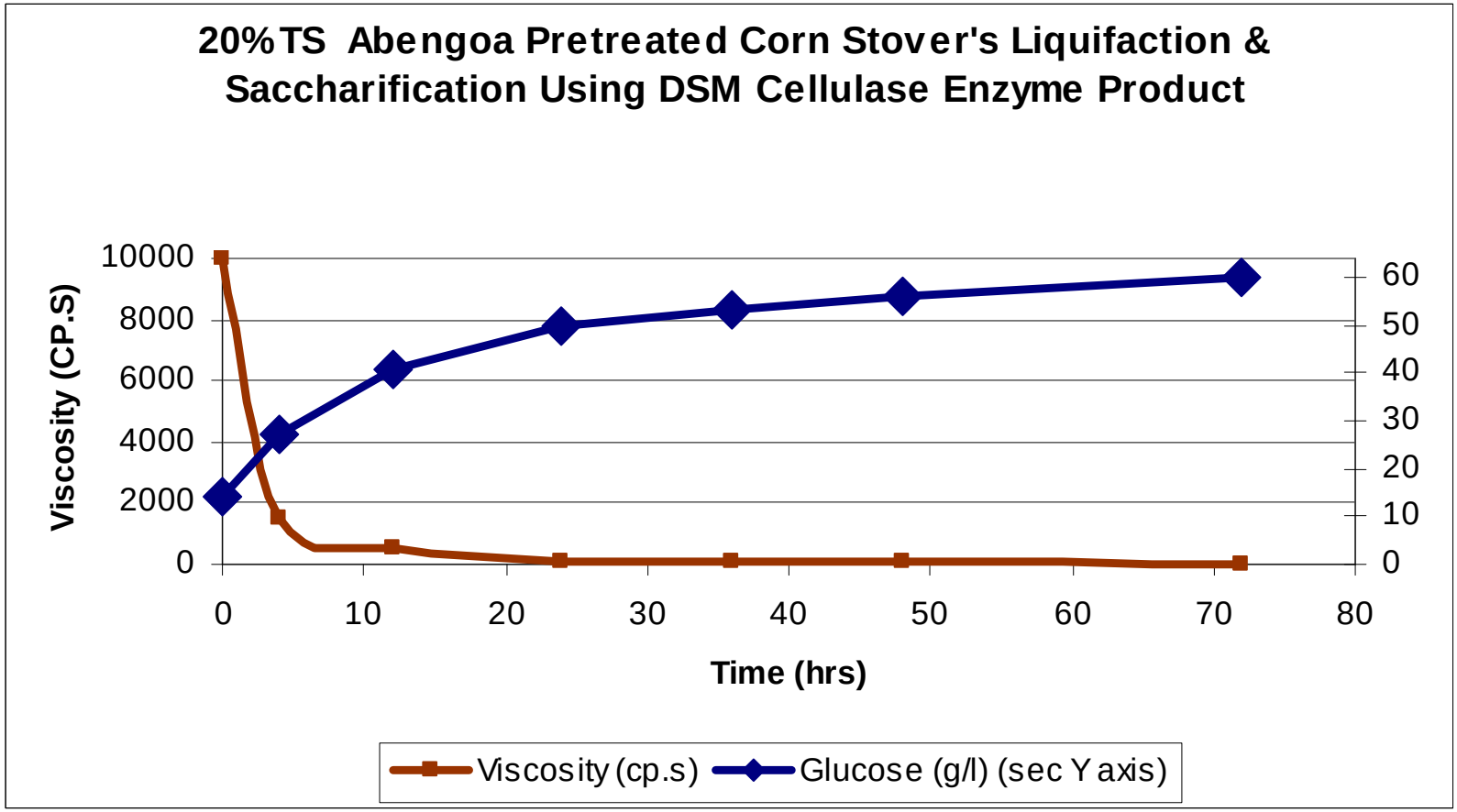
Pilot Scale (2000 liter) Test of DSM Enzyme @ ABNT York Site



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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)

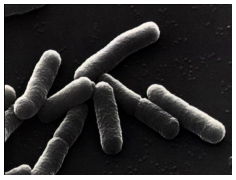
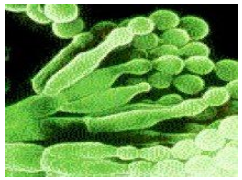
Thermostable Cellulases - *How?*

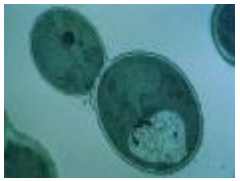
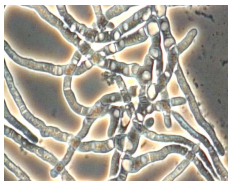
- Diversity
  - Large libraries of (bio)catalysts required
  - Access to genomes and meta-genomes
  - Directed evolution to optimize biocatalysts
- Screening Power
  - Automation and miniaturization
  - High through-put screening
  - Fast and reliable analysis
- Protein Engineering
  - Directed Evolution
  - 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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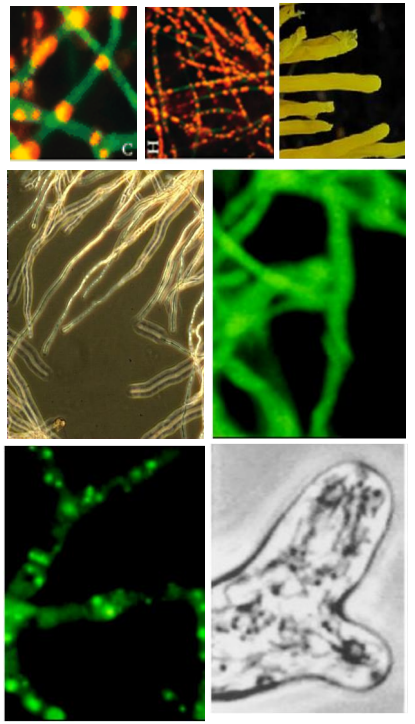
DSM Industrial Strain Development:
"Design & Build" concept

- **'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'**
  - (DSM Patents)
- **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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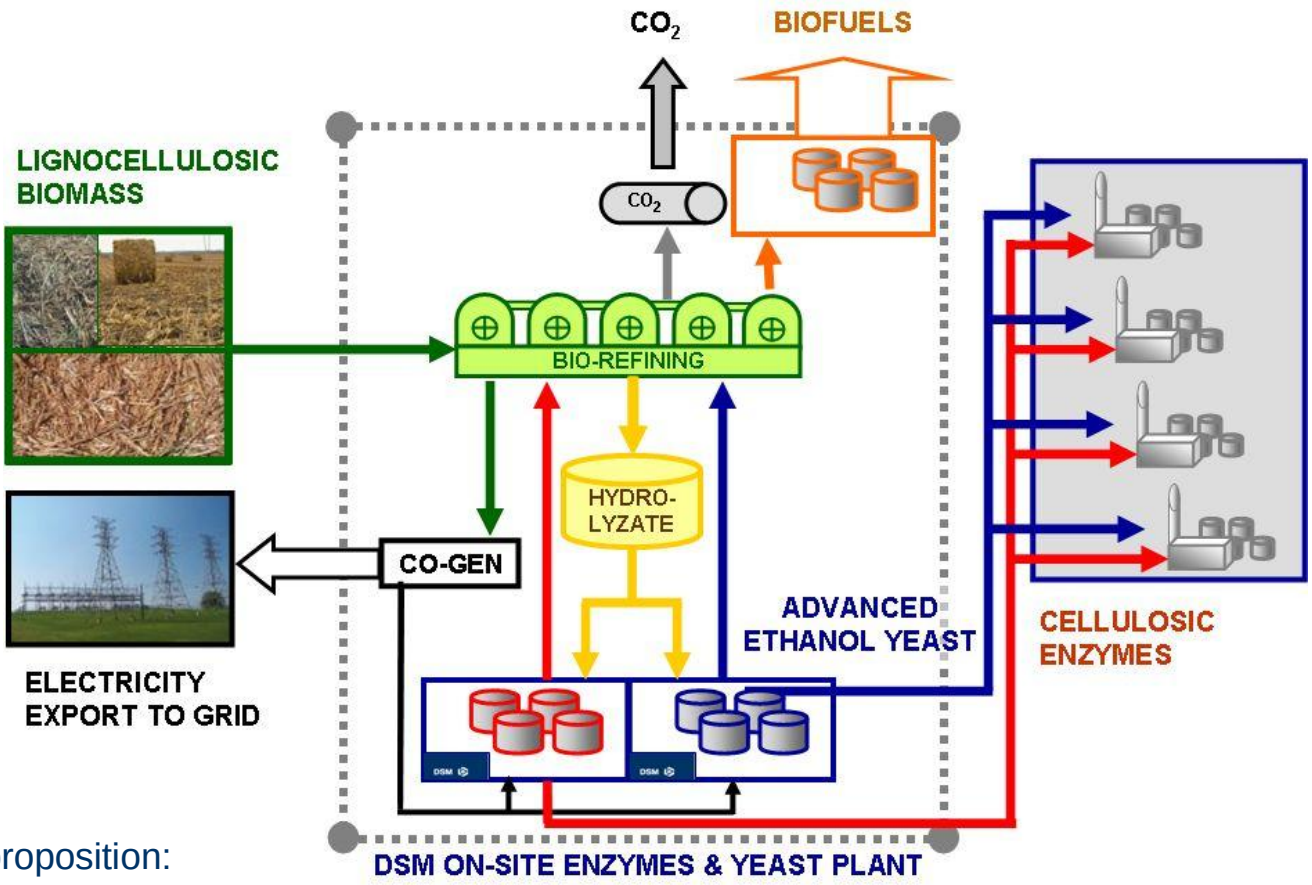
DSM *On-site* Enzyme & Yeast Production Model



- Value proposition:
  - cellulosic ethanol productivity and yield improvements,
  - a positive operational cost delta
  - capital expenditure savings
  - an improved eco-footprint of the cellulosic ethanol production process

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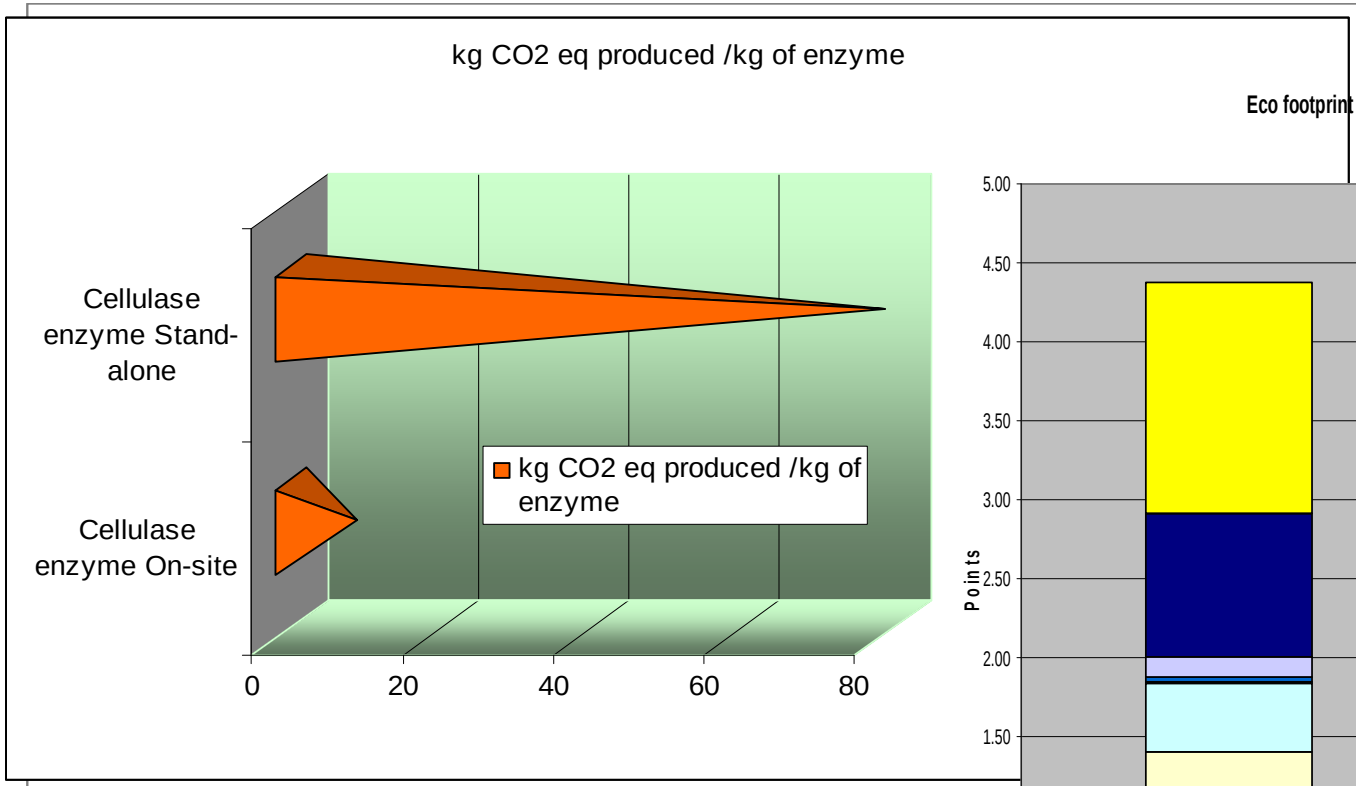
Integrated Biorefining: *On-site Enzyme Production*- partnering across the value chain

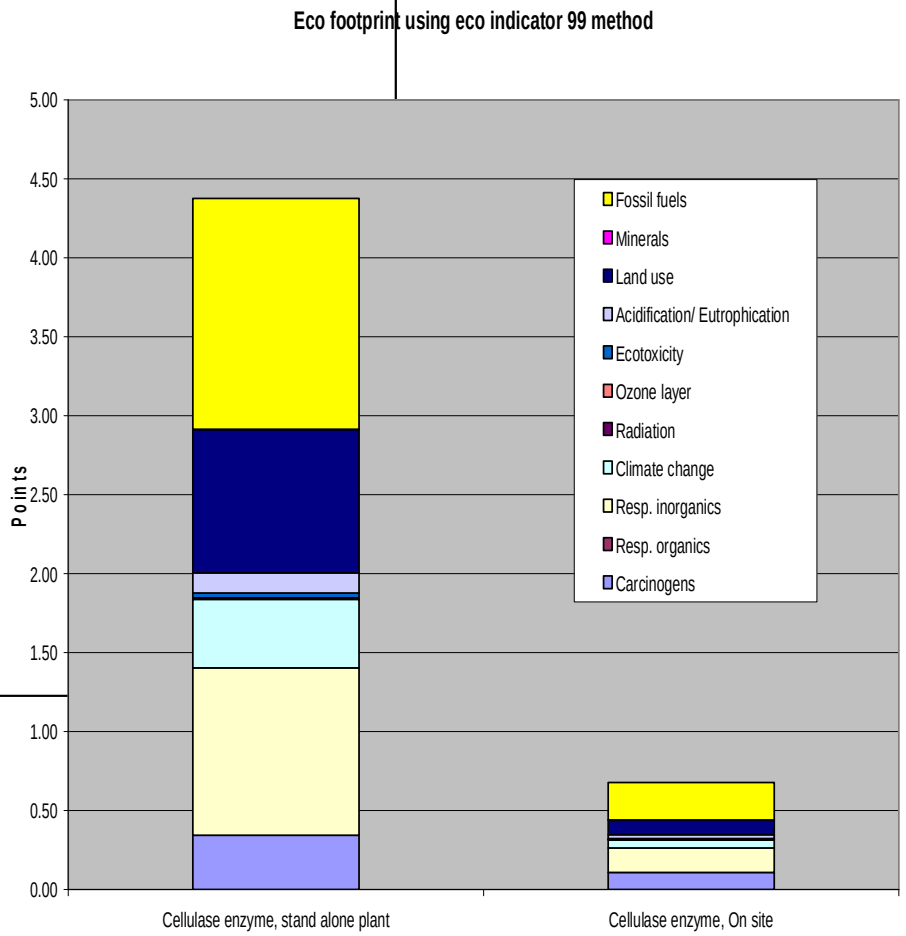
- ***Advantages for the biofuels and bio-based chemical producing partner:***
  - Overall lowest cost option for sourcing bio-conversion components
  - Enzyme mix is continuously tuned to the specific substrate and process conditions at hand
  - **Overall improved eco-footprint**
  - Increased total process stability
  - Increased versatility
  - Increased security of supply for bio-conversion components
  - Bio-conversion technologies are managed by a highly competent on-site biotechnology partner.

Integrated Biorefining: *On-site Enzyme & Yeast Production-* partnering across the value chain

- ***Advantages for DSM (the enzymes and yeast producing partner):***
  - Down-stream processing costs including water
  - Transportation and packaging costs
  - E&Y process losses via direct transfer to the ethanol process
  - Reduced capital expenditure costs vs. dedicated (remote) facilities
  - Lower cost carbon source for the E &Y production fermentations
  - Direct availability of induction and adaptation components for E &Y
  - Direct access to utilities and energy sources within the biorefinery
  - Continuous E &Y output tuning and improvement opportunities directly within the biorefinery

Sustainable Product Entrepreneurship:
On-Site Enzymes & Yeast Production





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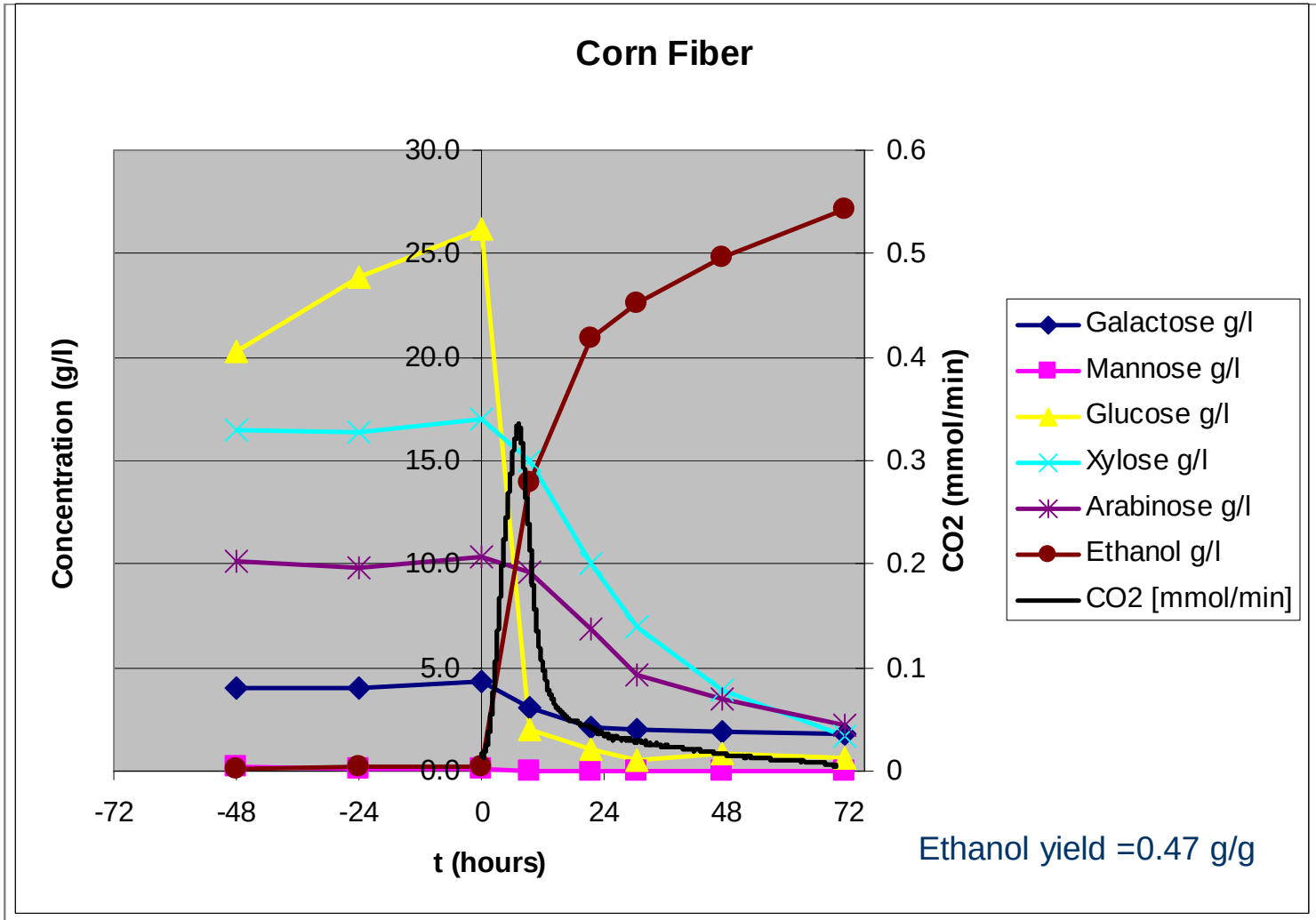
Full Technology Package: Example 13.8% TS Corn-fiber (low severity dilute acid PT)



Ethanol yield =0.47 g/g

Glucose utilization & use rate = 97 % used @ 2.8 gram glucose / gram yeast dry matter / hour

Xylose utilization & use rate = 90 % used @ , 0.22 g xylose / gram yeast dry matter / hour

Arabinose utilization & use rate = 79 % used @ 0.12 gram arabinose / gram yeast dry matter / hour

Galactose utilization and use rate = 58 % used @ 0.03 galactose / gram yeast dry matter / hour

Pretreatment conditions were150'C, 20 min residence time, acid loading of 1% v/v H2SO4 on total slurry.

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Recap: *DSM Cellulosic Enzyme Solution*

- **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- production process scaled and successfully piloted
- 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
- On site manufacturing of yeast and enzymes advantageous from an economic and LCA point of view.

Acknowledgements

Groups	All in One Yeast	Thermostable Cellulases	
Genetics	Paul Klaassen	Alrik Los	Ina Kerkhof
	Beate Wiedemann	Robbert Damveld	Aad Vollebregt
	Denise van Suylekom	Brenda Vonk	Ilja Westerlaken
	Bianca Gielesen	Ilse de Lange	Hilde Huininga
	Wilbert Heijne	Wilbert Heijne	Kim van Zijl
	Saskia Pellis	John Perkins	Bert Koekman
	Jan Roerig	Yvonne Arendsen	Ruta Keflon
Fermentation	Panos Sarantinopoulos	Panos Sarantinopoulos	Hein Stam
	Hein Stam	Yang Xiang	Pieter Nelisse
	Aldo Greeve	Derek Kane	Prabu Vijayan
	Wim de Laat	Wim de Laat	Michel Plachta
Down Stream Processing	Pieter Kerkhof	Gabrie Meesters	Pieter Kerkhof
Biochemistry	René de Jong	Margot Schooneveld	Fred Kattevilder
		Monica Vlasie	Joost van 't Hoff
		René de Jong	Denise Jacobs
		Joyce Heuvink	
Enzymology/Application Development	Michel Berkhout	Jan Smits	
	Liesbeth Gierveld	Liesbeth Gierveld	
	Fred van der Hor	Fred van der Hor	
	Ben Kepper	Ben Kepper	
	Cees Visser	Cees Visser	
Analytical	Judith Vis	Jort Gerritsma	Andre Vente
	Coralie Selin	Joep van Rijn	
	Adriana Carvalho dS	Michiel Akeroyd	
	Jort Gerritsma	Marijke Misset	
IP	Paul Kleiborn	Paul Kleiborn	Wim de Boer
Regulatory	Jack Reuvers	Jack Reuvers	
	Lisettte Mohrmann	Lisettte Mohrmann	
	Piet van Dijck	Piet van Dijck	
Process Integration	Jarno Kuijvenhoven	Jarno Kuijvenhoven	Hans Koon
Project Management	Piet van Egmond	Peter Oldenhof	Piet van Egmond
		Hein Stam	Taryn Stettler
Business	Volkert Claassen	Volkert Claassen	
	John Monks	John Monks	
	Marcel Wubbolts	Marcel Wubbolts	
	Hans van der Sluijs	Hans van der Sluijs	
	Reinder Hanstra	Reinder Hamstra	
	Scott Hall	Scott Hall	
	Sjoerd Dijkstra	Sjoerd Dijkstra	
	Edwin Berends	Edwin Berends	
Funding		***US Department of Energy***	