

Thermostable Cellulases: Why & How?

Manoj Kumar, PhD
Director Science & Technology
Global New Business Development
White Biotechnology
Royal DSM

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
 - DSM in 2009 again No. 1
- **Strong technological toolbox:**
Integrated use of biotechnology, biocatalysis, organic chemistry, chemical and polymer technology materials sciences



World Business Council for Sustainable Development



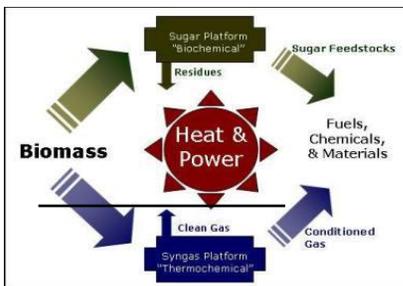
Oil-Refineries and Bio-Refineries



- **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



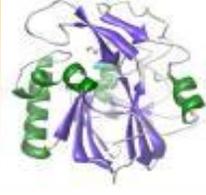
- **Bio-refinery (2nd generation):**
 - Biomass (renewable) as input
 - Logistics?
 - Technology very much in development (Enzymes & FO)
 - Byproducts?
 - Less efficient use of feedstock

Replenishing Chemistry by Biotechnology

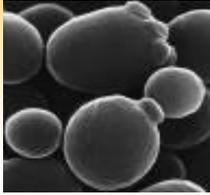
Carbo hydrates



Enzymes



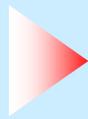
Yeast



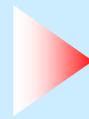
Bio-fuels



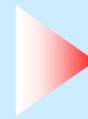
Oil



Chemistry



Polymerization



Materials

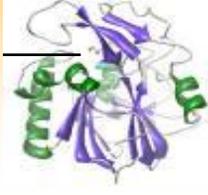


New Opportunities from the Combination

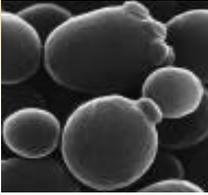
Carbo hydrates



Enzymes



Fermentation organism



Bio-fuels



Oil



Chemistry/
Enzymology



Polymerization

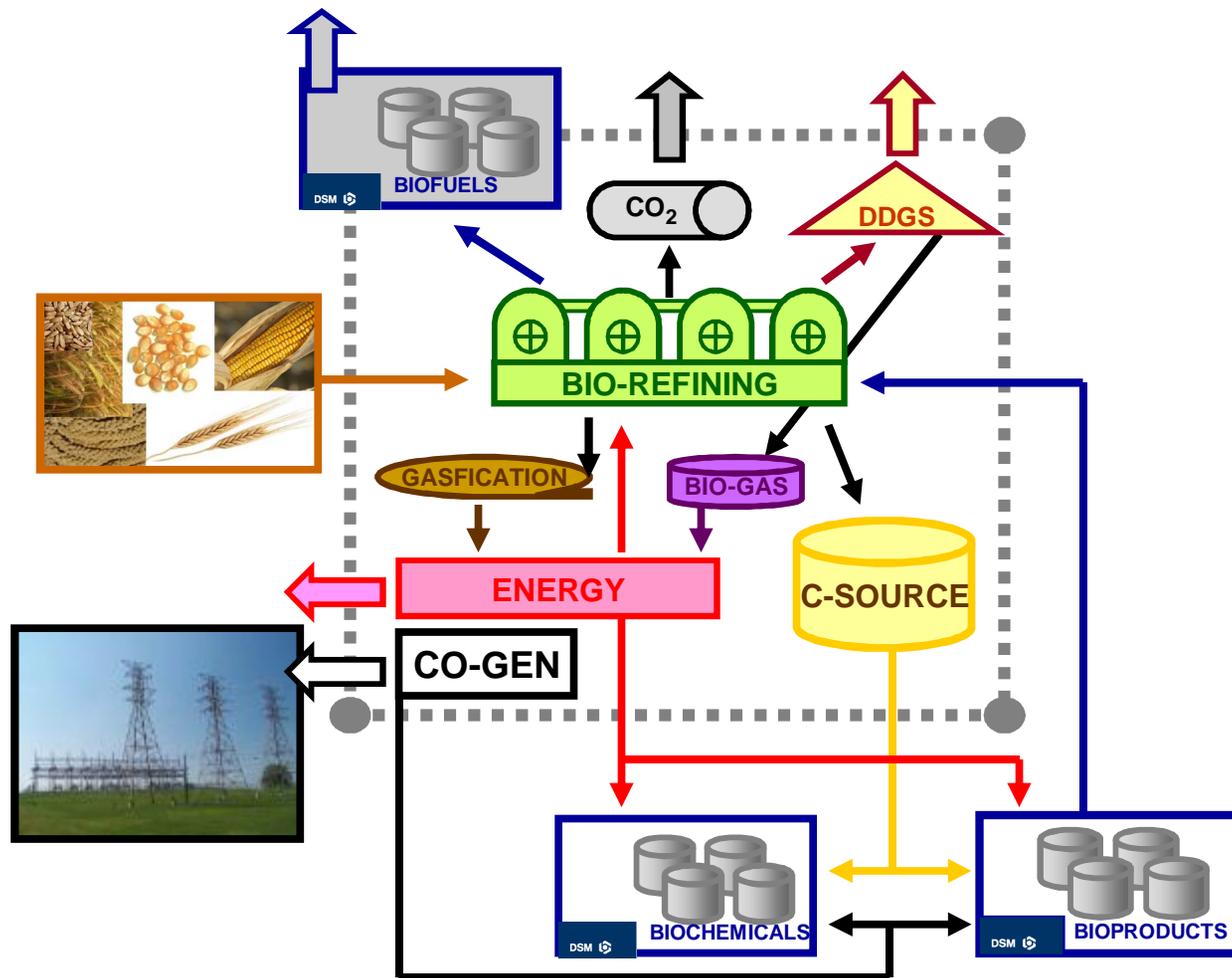


BioChemicals
BioPolymers
BioMaterials

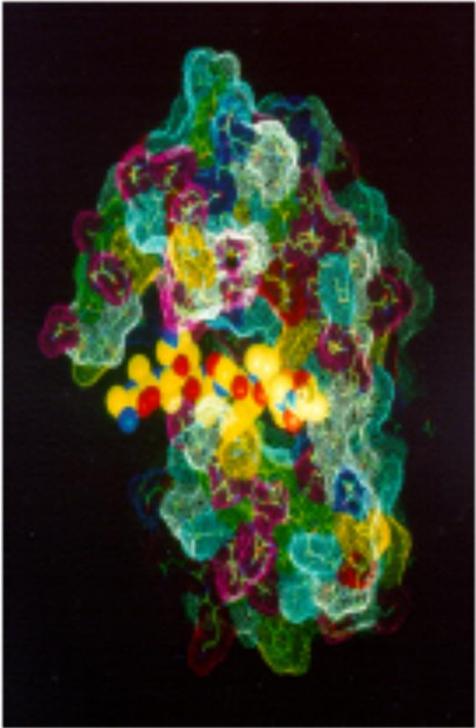


Resulting in a broad range of innovative bio-based businesses

Biorefinery for Bio-based Building Blocks (Sugars)



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



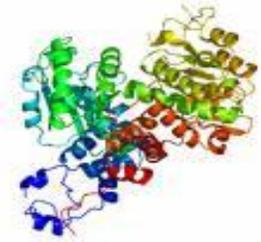
- **DSM possesses a strong knowledge base within enzyme research-, application and manufacturing**
- **DSM holds hundreds of patents based on enzyme technology including biomass deconstruction enzymes**
- **DSM sells \$300MM worth of enzymes in baking, food & fruit processing, brewing, wine, dairy, animal 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**

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*

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

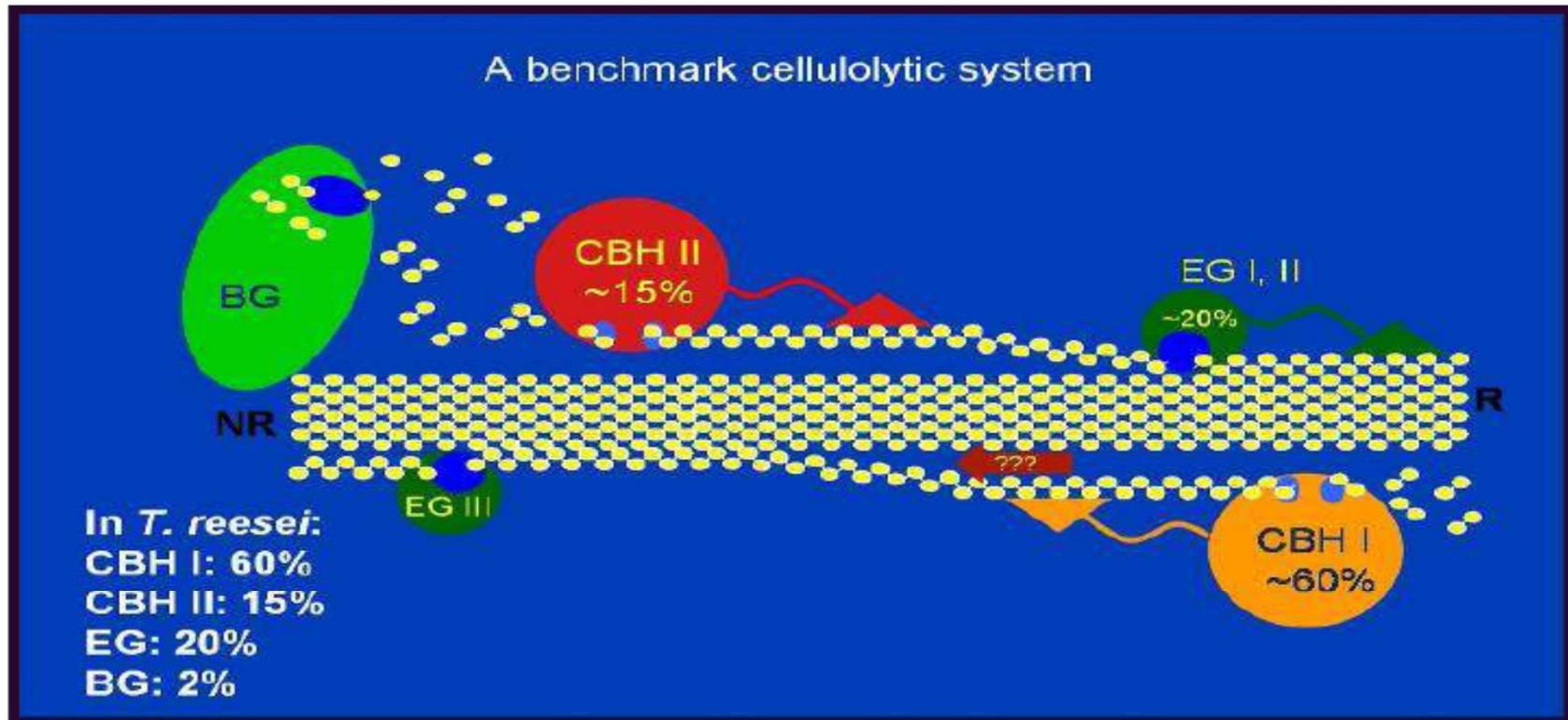
P. chrysogenum sequence: van den Berg *et al.* (2008) Nature Biotech. **26**, 1161



Cellulase
Enzymes

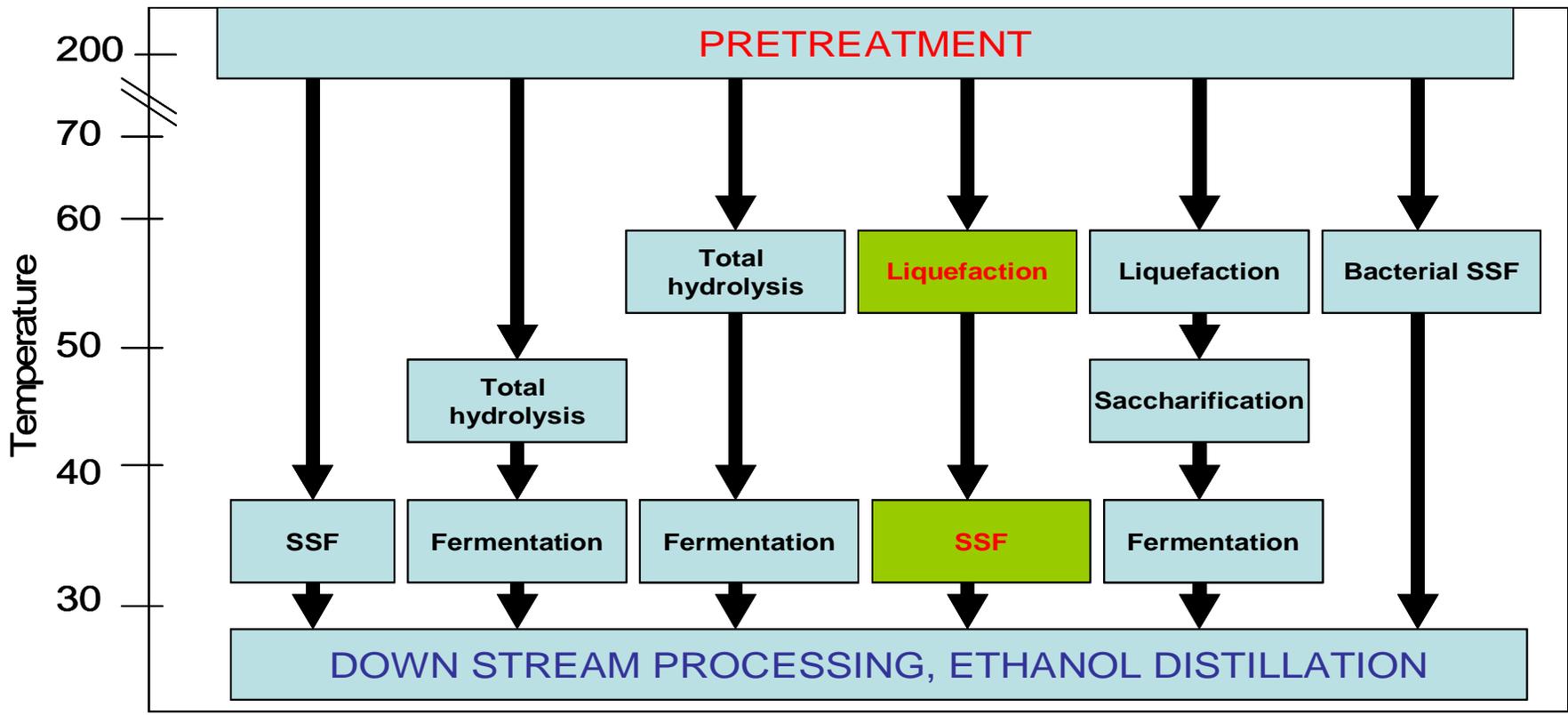


Cellulases: *Enzymes need to cooperate*



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

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



Thermostable Cellulases: *Why ?*

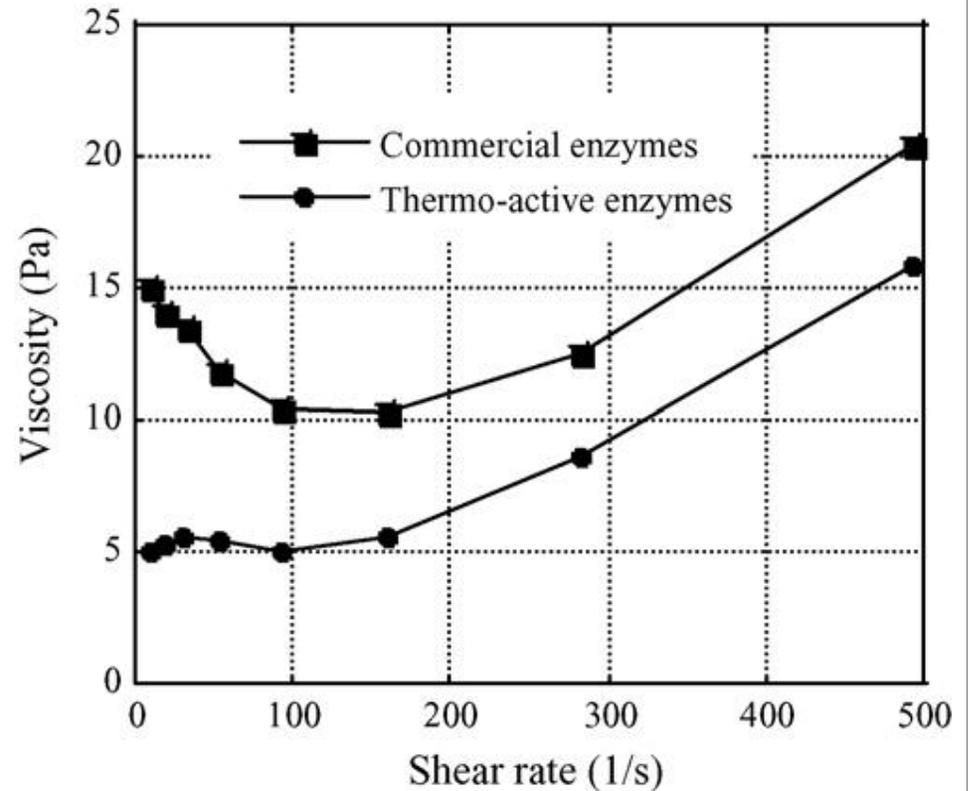
- Increased rate of cellulase activity, less energy cost for cooling, higher DS loading, and decreased risk of contamination.
- Can be cloned and over-expressed at high levels in *fungus* hosts.
- Ease of DSP/Formulation since one can introduce heat step to precipitate *fungus* host proteins.
- Biotransformation 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 Pa
 - 65 °C: 60 Pa
- 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 enzyme 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.

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.

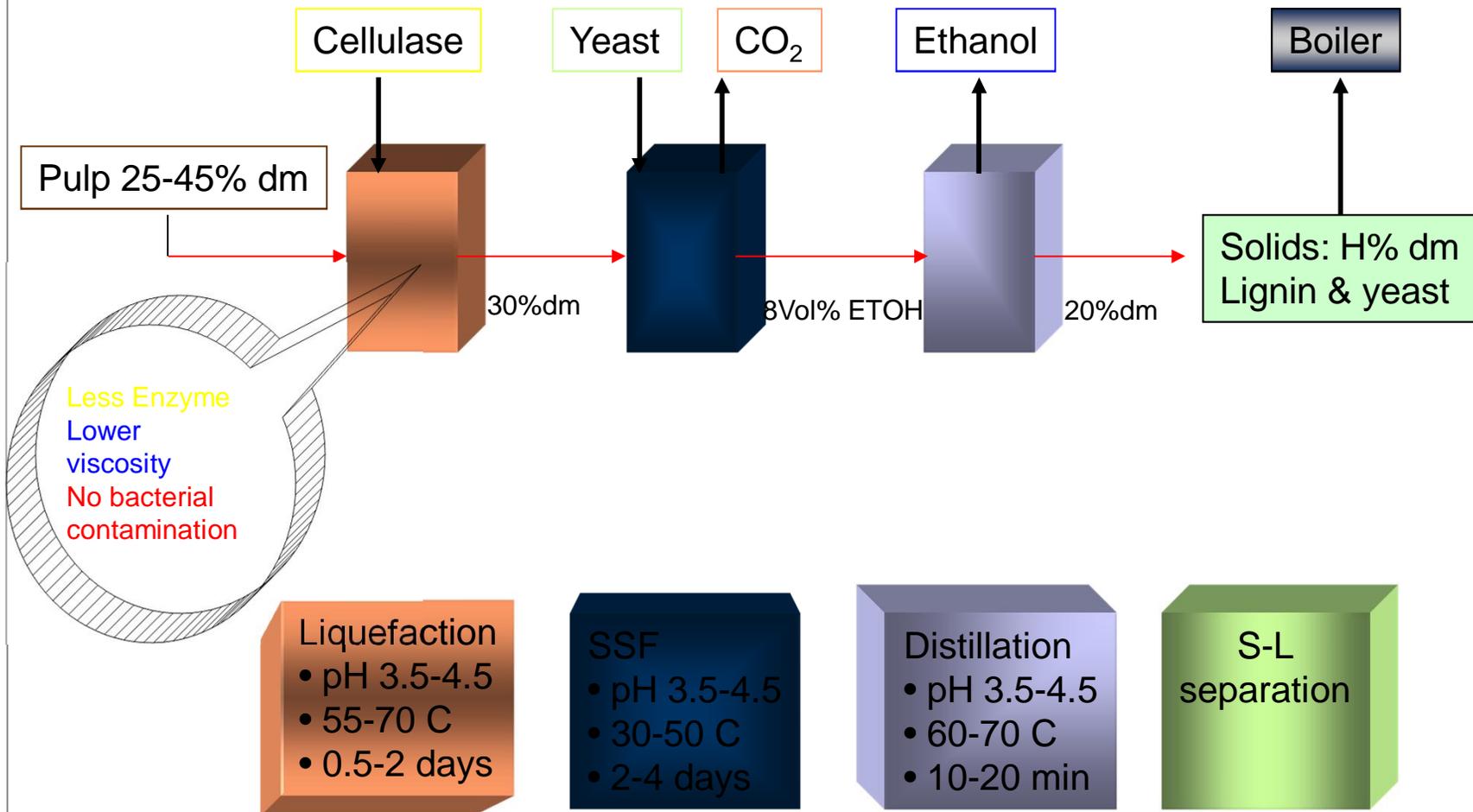
Energy costs saving

- 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.

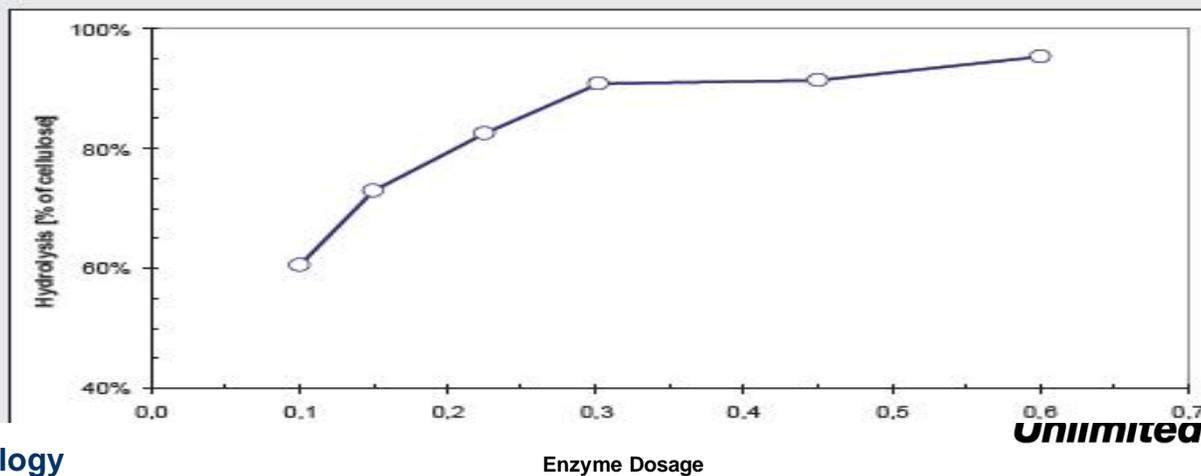
High Temperature Advantages



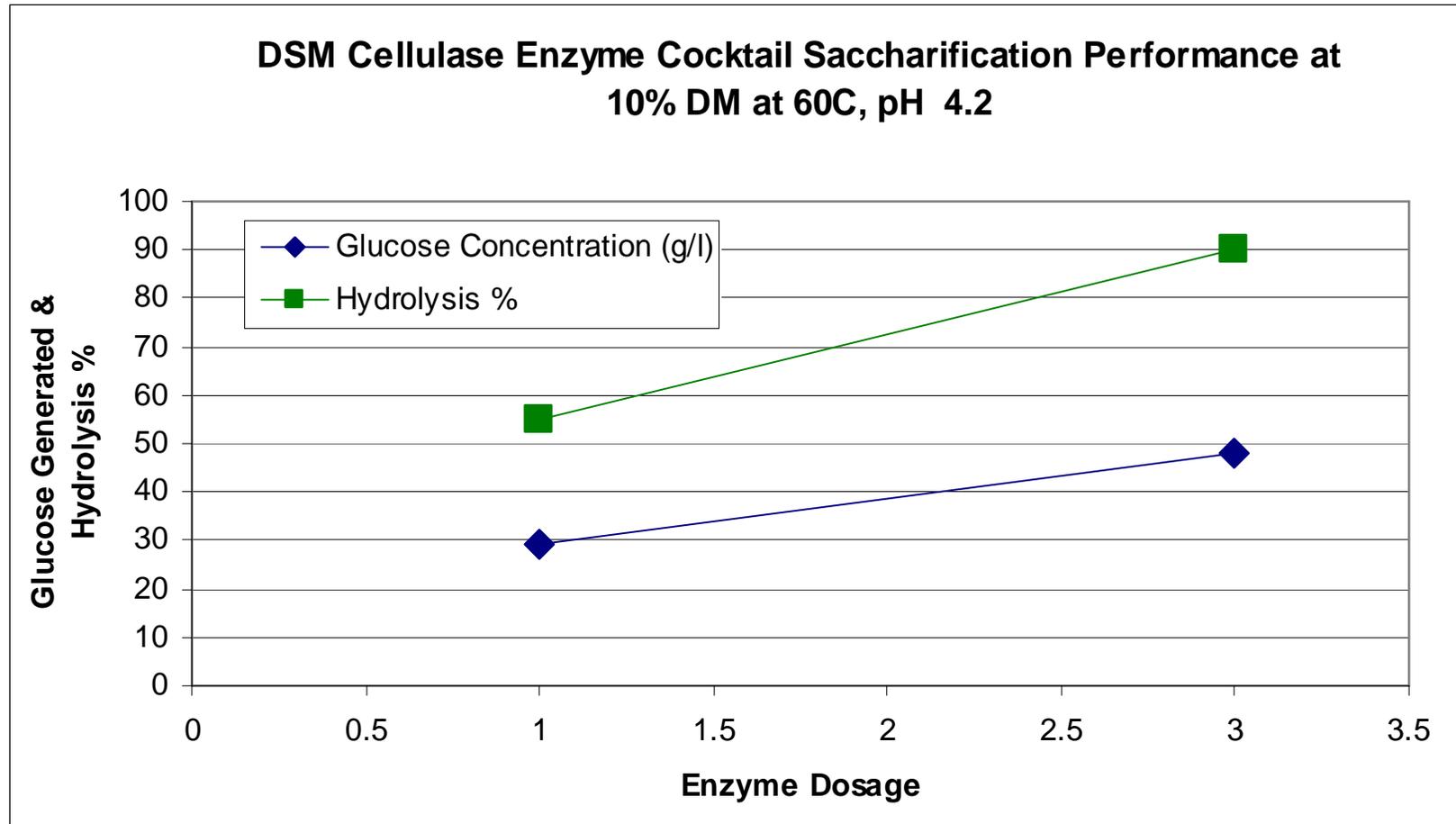
DSM Cellulosic Enzyme System Performance



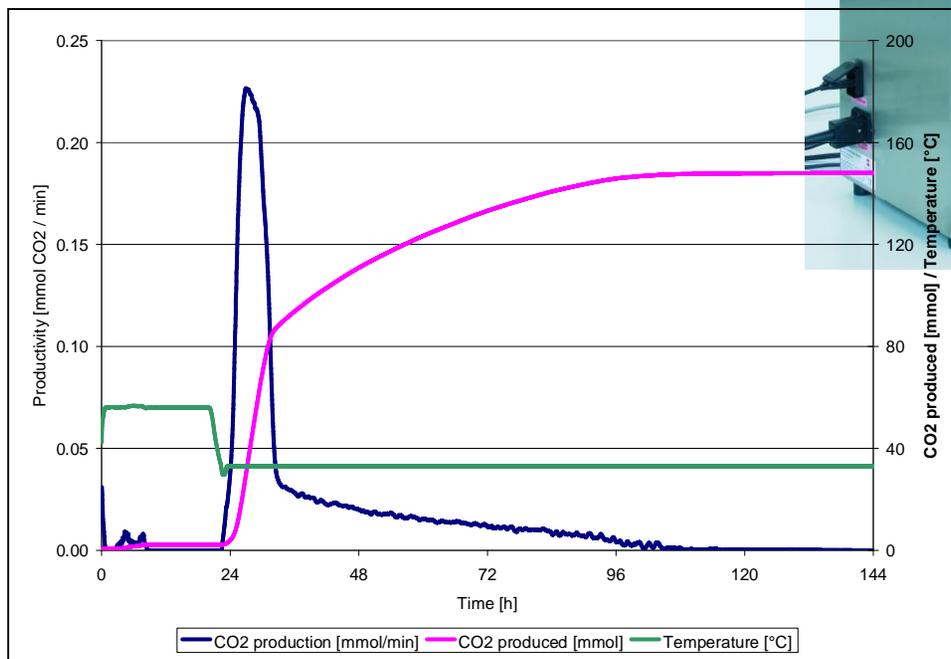
Washed pWS before (left) and after (right) hydrolysis.



Saccharification at Low Enzyme Dose



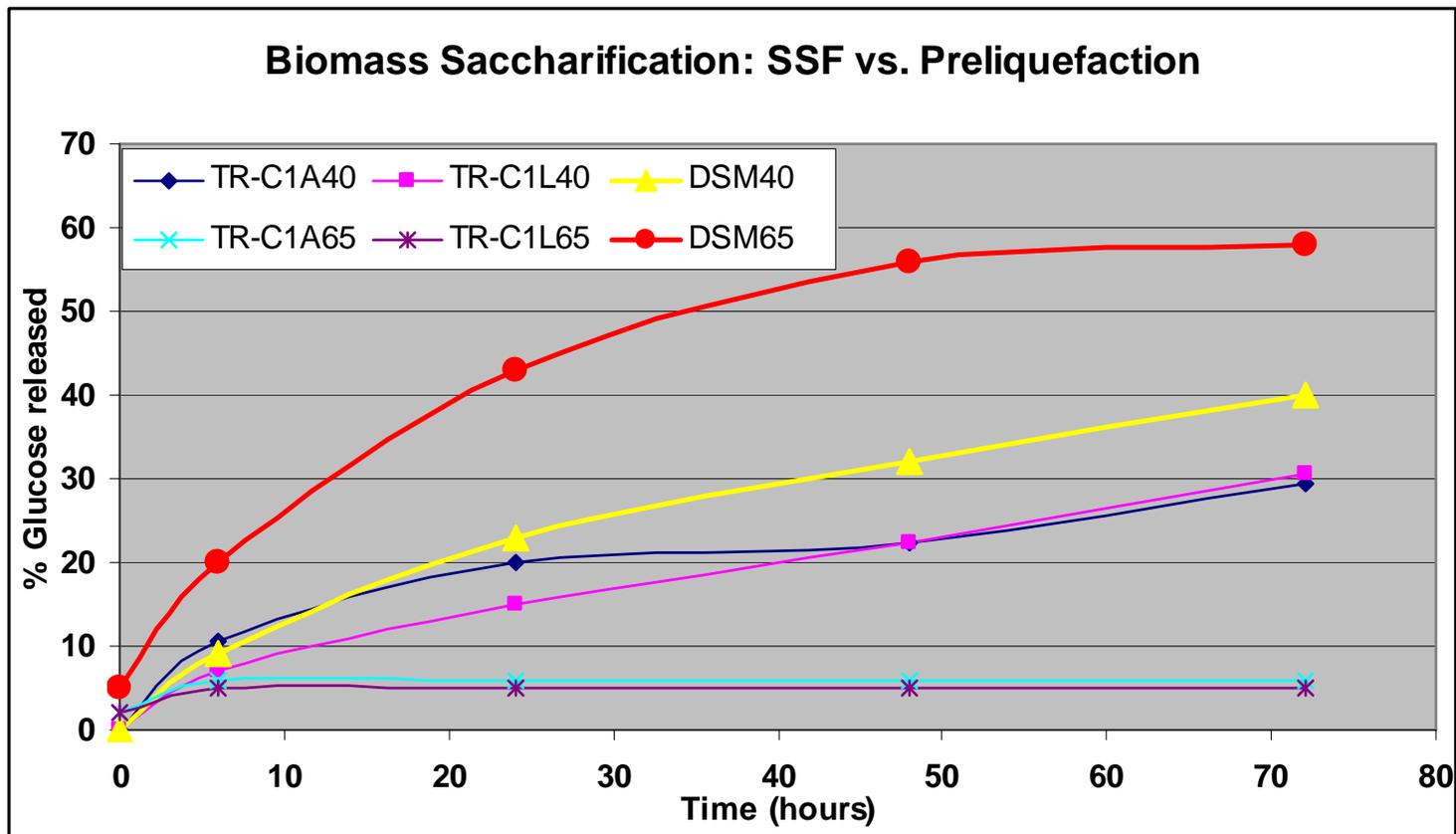
DSM Cellulase Enzyme Cocktail and Yeast: Partial Liquefaction and Fermentation to Ethanol



DSM White Biotechnology

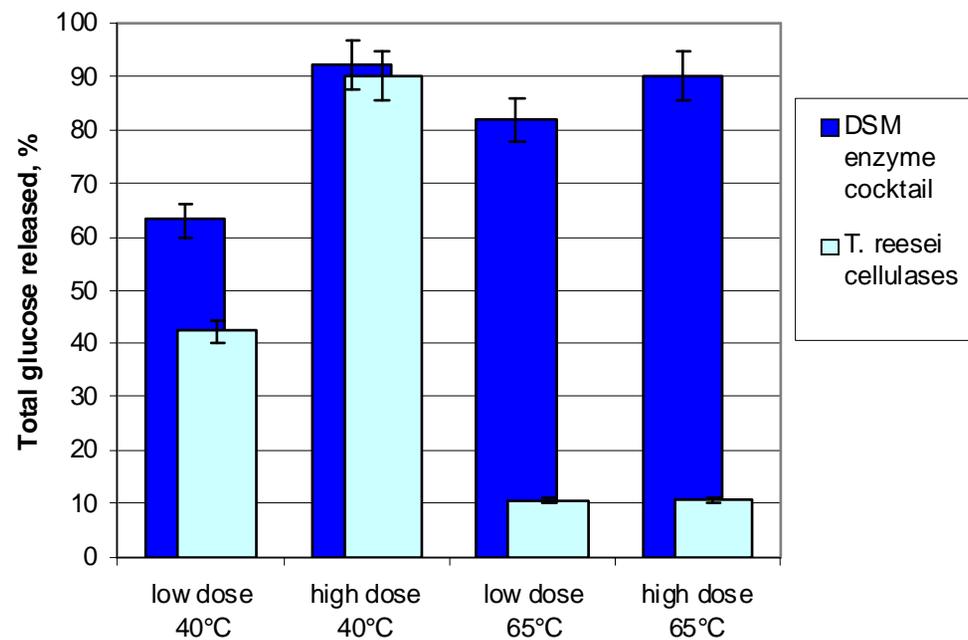
Unlimited. **DSM**

DSM Cellulase Enzyme Cocktail Performance



DSM Cellulase Enzyme Cocktail Performance

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



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 (SO₂ 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

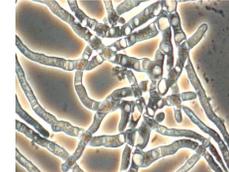
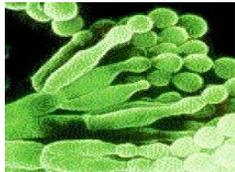
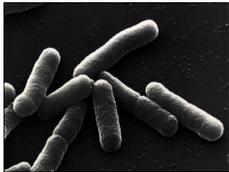
● Protein Engineering

- Directed Evolution
- Rational Design
- Molecular biology assisted variants generation

DSM pluGbug[®] : *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



- pluGbug[®] technology for rapid and cost effective *Biocat* development
- *In-house* production with pluGbugs[®] provides maximum flexibility

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**

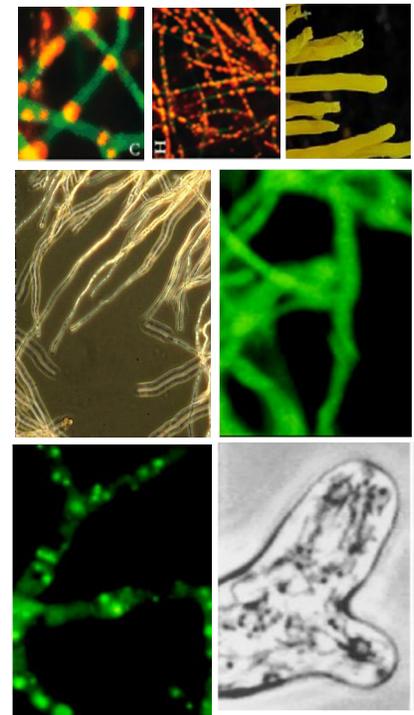
- genetic tool for repeated modification of host properties
- important legislation issue

- **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



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*

DSM White Biotechnology

Unlimited. DSM