Improved Cellulases for the BioRefinery:

A review of Genencor's progress in the DOE subcontract for cellulase cost reduction for bioethanol.

Stanford GCEP Biomass Energy Workshop, April 2004

Dr. Colin Mitchinson
Genencor International, Inc.

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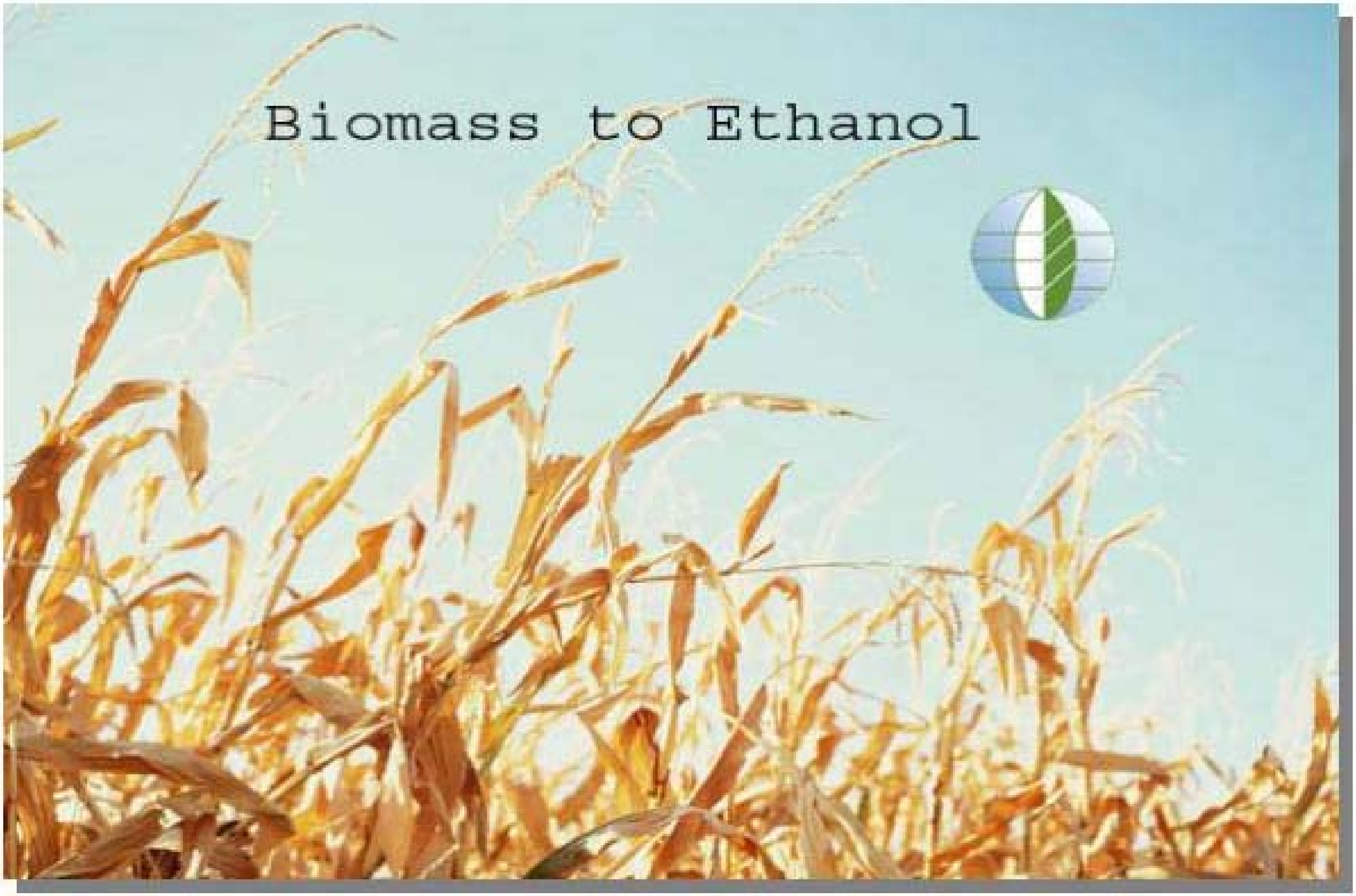
PROJECT BACKGROUND & STRATEGY



Sources of CELLULOSIC Biomass

Courtesy of Rod Bothast

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One man's waste is another's treasure

Corn stover has the potential for conversion to 4-5 billion gal/yr of ethanol, tripling US production

-field recovered corn stover

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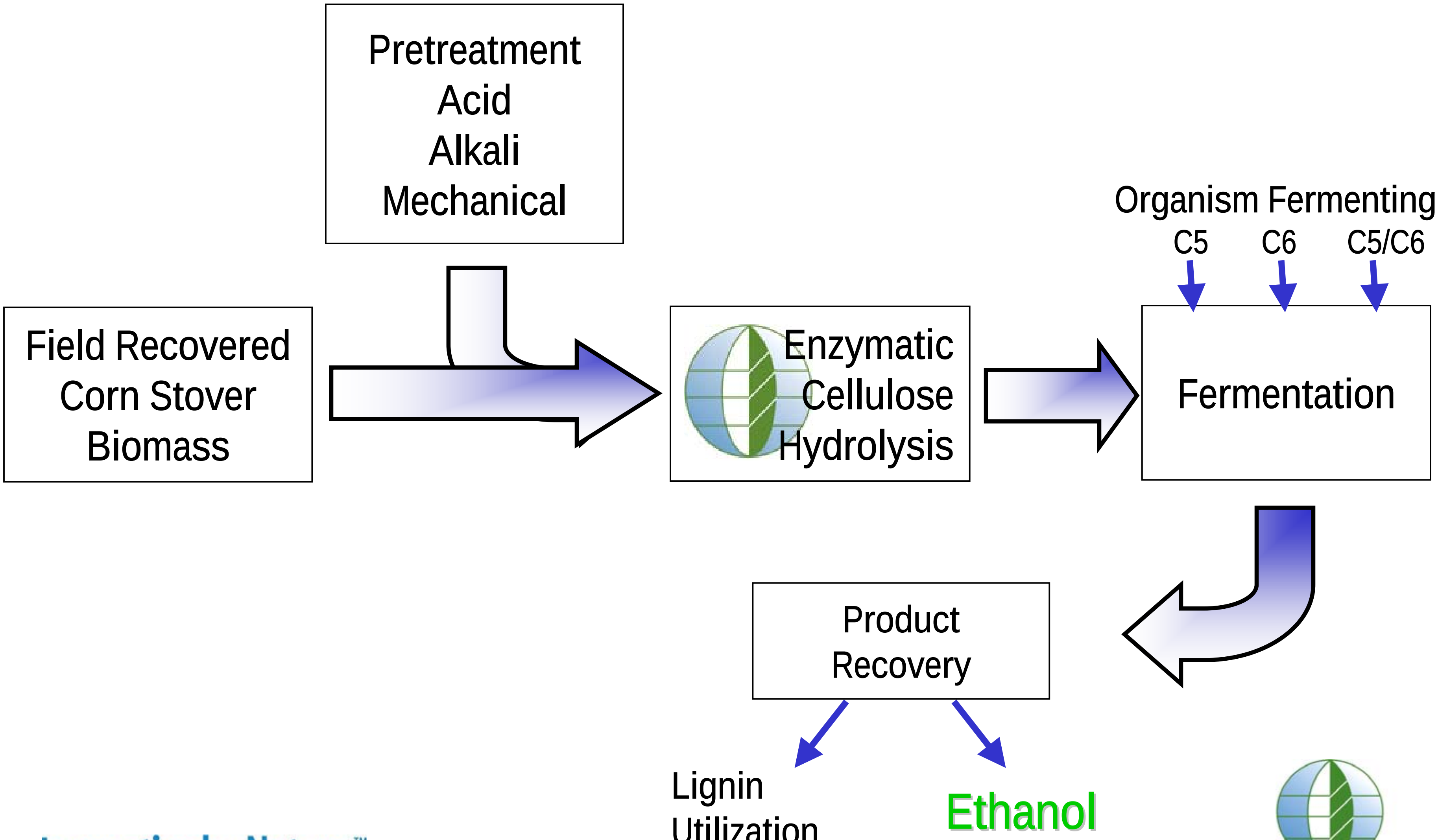
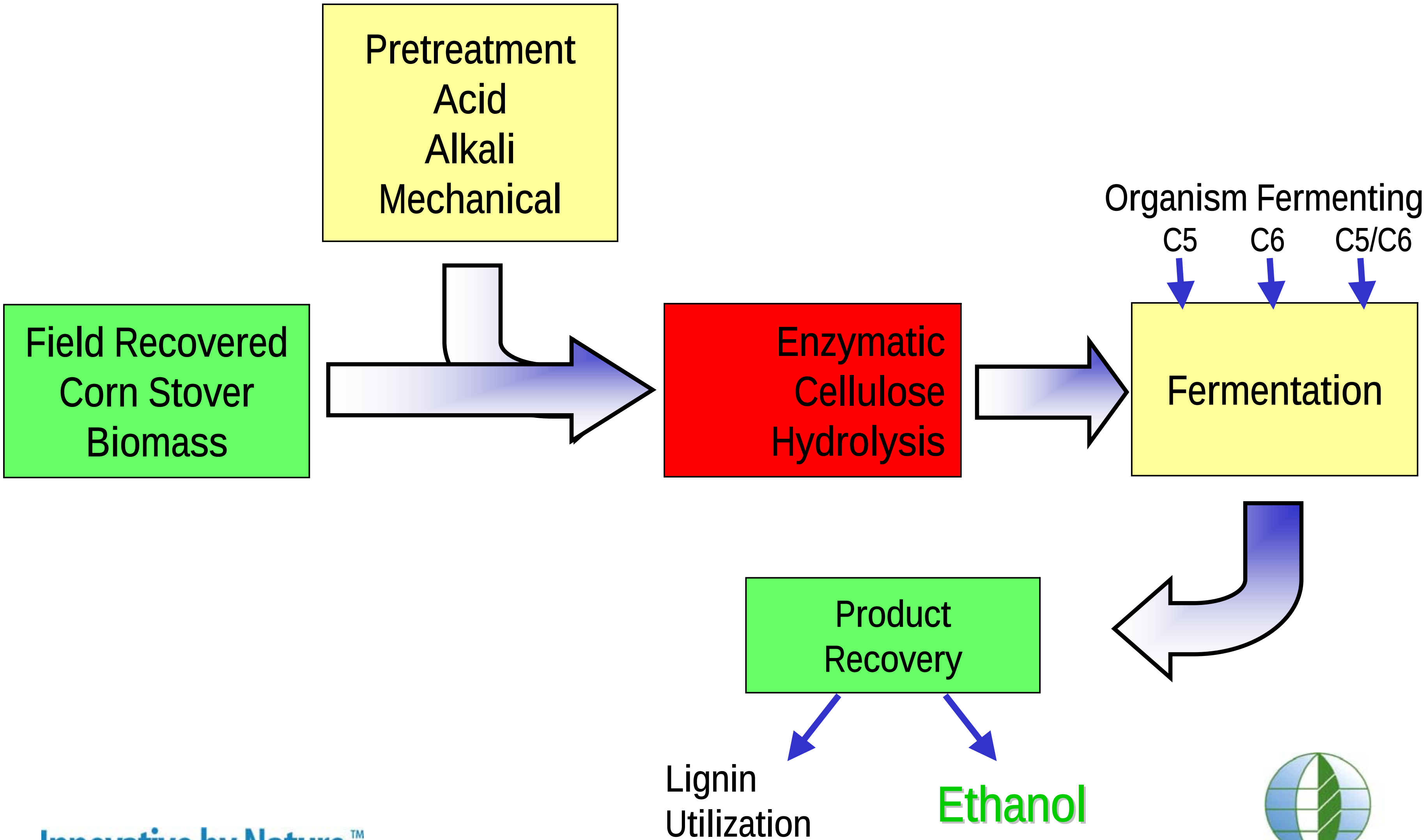
The Bioprocess Challenge



Status Pre DOE / NREL Program



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Project Background.

- DOE Subcontract with Genencor for "Cellulase Cost Reduction for Bioethanol" administered by National Renewable Energy Laboratory (NREL)

- Focus: Economically Acceptable Enzymatic Conversion of Cellulosic Biomass to Glucose for Fermentation to Ethanol or Other Products.
  Renewable fuel & chemical alternative to petroleum.

- Project Goal: 10-Fold Reduction in Cost of Enzymes for Biomass Conversion.

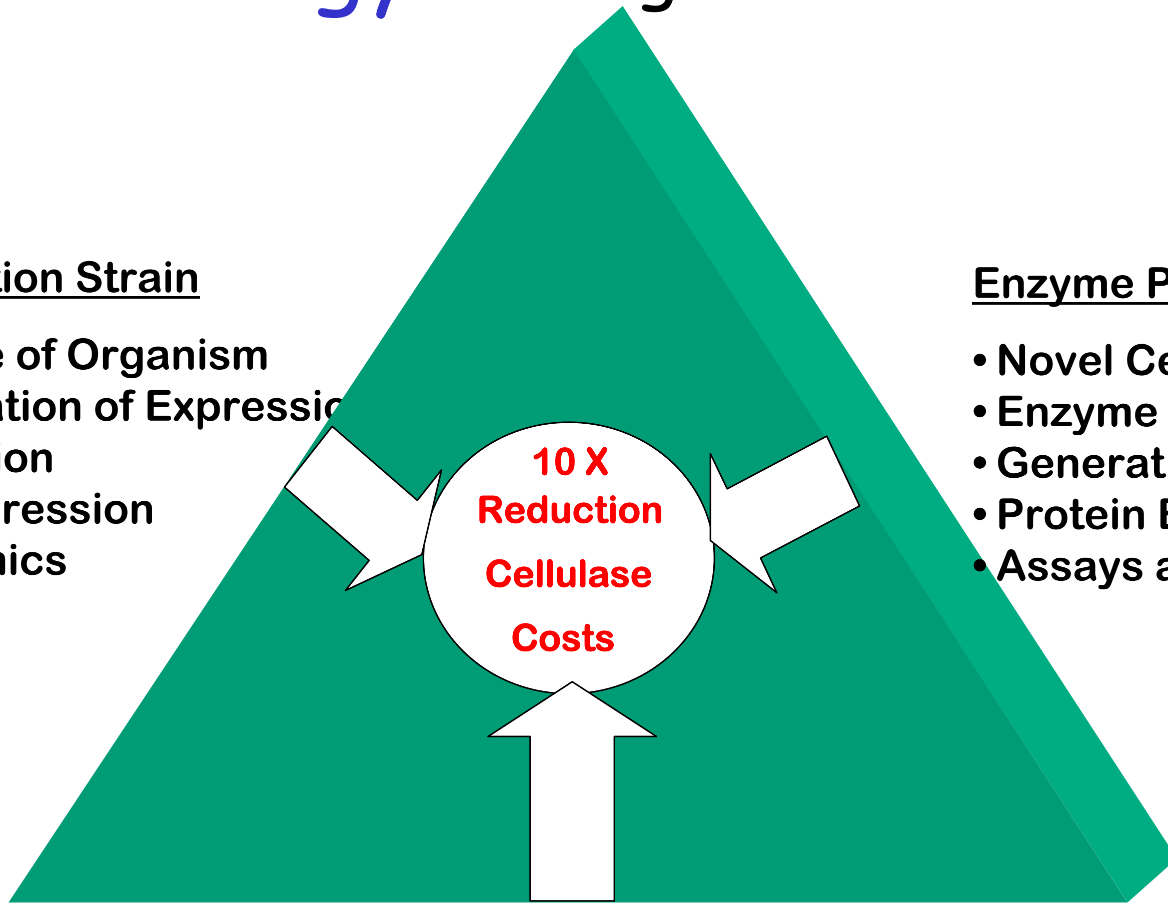
  - Project start date: April 27, 2000.

Strategy

- 10X cost reduction goal can only be achieved by BOTH:
  - Improved production economics (reduced $/gm enzyme)
  - Improved cellulase performance (reduced gm enzyme/gal EtOH)

$$\text{Effective cellulase cost}\left(\frac{\$}{\text{gal EtOH}}\right)=\left(\frac{\$}{\text{gm enzyme}}\right)\cdot\left(\frac{\text{gm enzyme}}{\text{gal EtOH}}\right)$$

Strategy: Integrated Plan of Action



Production Strain

- Choice of Organism
- Regulation of Expressio
- Induction
- De-repression
- Genomics

Enzyme Performance

- Novel Cellulolytic Activities
- Enzyme Discovery
- Generation of Diversity
- Protein Engineering
- Assays and Screens

Production Process

- Host Engineering
- Fermentation Process Development
- Breakthrough Production Economics
- Product Recovery Manufacturing Economics of Scale

PROJECT BACKGROUND & STRATEGY

IMPROVED PRODUCTION ECONOMICS

- Process Improvement
- Strain Improvement
  - Functional Genomics

IMPROVED CELLULASE PERFORMANCE

- Known Cellulases
- New Cellulases
- Improved Cellulases

IMPROVED CELLULASE STRAIN

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IMPROVED CELLULASE STRAIN

Improved production economics

NOTE: Whole cellulase production by *T. reesei* is arguably the best protein production economics existing today.

Production Process Improvement

- **Elimination of post-fermentation steps.**
- **Lower cost carbon /energy source for fermentation.**
- **Continual fermentation process optimization. (guided partly by Functional Genomics)**

Production Strain Improvement

- **Random Mutagenesis**
- **Targeted Mutagenesis**

Elimination of post-fermentation recovery and formulation.

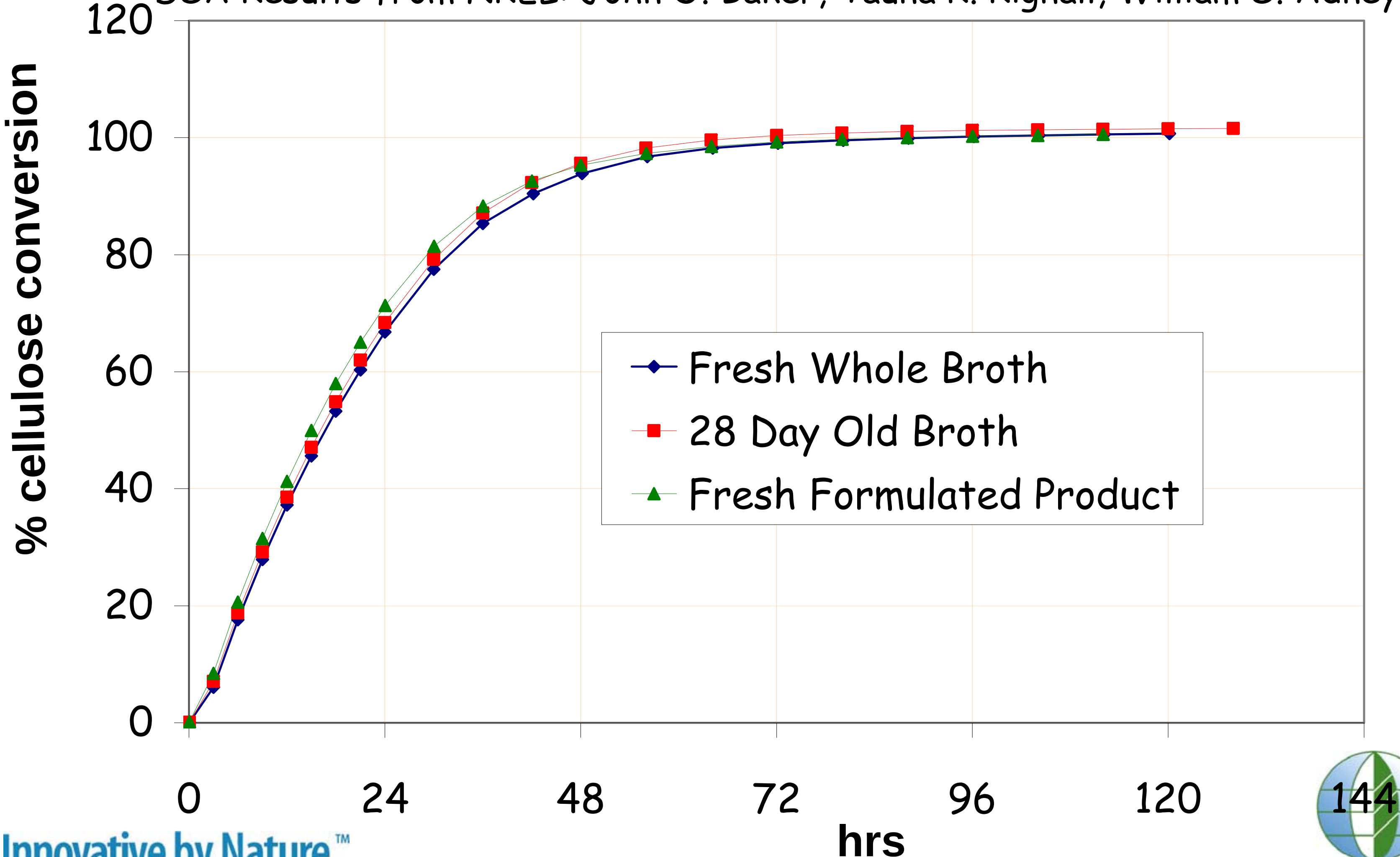
- Current commercial cellulase applications require cell-free product formulated for extended shelf-life at high temperature
- Possibility: Elimination of downstream processing

  No cell separation + No formulation
- Reduced cost Whole Broth, unformulated product acceptable for BioRefinery market, *if it performs*:

Biomass saccharification with whole broth

Effect of 28-Day Storage at 15°C upon Cellulolytic Performance of Unformulated Whole Broth

DSA Results from NREL: John O. Baker, Tauna R. Rignall, William S. Adney



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Lower cost carbon source for fermentation.

- Normal *T. reesei* fermentation requires lactose for cellulase induction and as a carbon / energy source

- Lactose is ca. 2X the cost of glucose, but glucose represses cellulase expression

- Sophorose is the most potent inducer of cellulase, but

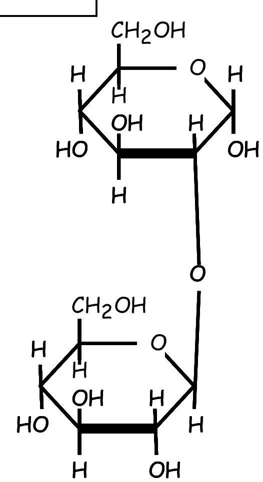
- Sophorose is (orders of magnitude) X the cost of glucose

Lower cost carbon source for fermentation.

Replacing Lactose feed with treated Glucose, without Glucose Repression:

In situ treatment with cellulase

Glucose + Cellulase → **[** Glucose + Sophorose **]** → Trichoderma fermentation → Cellulase



Sophorose

Lower cost carbon source for fermentation.

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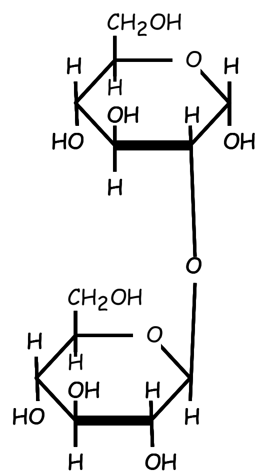
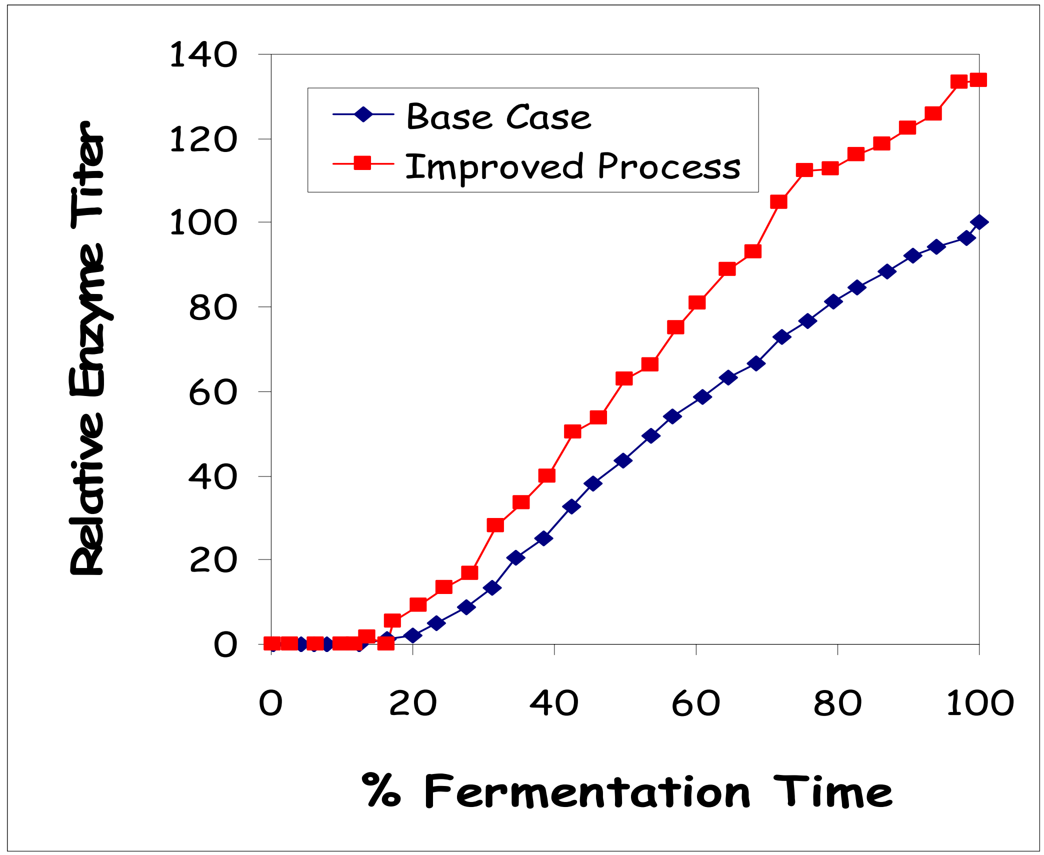


Sophorose

Metric	% of Lactose Control
Volumetric productivity	117
Yield (of cellulase on sugar)	112

Fermentation process optimization
with improved cellulase production strains

- Media optimization
  - C/N ratio
  - Salt ratios
- Process parameters
  - Temperature
  - pH
  - Substrate feed rate
- Functional Genomics:



Monitoring, Control and Improvement of fermentation.

Production Strain Improvement

Random Mutagenesis of *T.reesei* production strain.

Screen	**Target**
Cellulose clearing zones	High cellulase expression Catabolite de-repression (+ glucose)
X-Glu (β-glucosidase substrate) zones	High cellulase expression Catabolite repression
"Toyama"	High cellulase expression
2-deoxyglucose	Catabolite repression
Chemical resistance	Secretion

Random Mutagenesis of *T.reesei* production strain.

Evolution of improved strain 41G

Production Strain

NTG ↓ Screen 1

Mutant 1

NTG ↓ Screen 2

Mutant 2

NTG ↓ Screen 3

41G

Strain	Yield gm E/ gm C	Productivity gm E/ L/hr
Production Strain	100 %	100 %
Mutant 1	132 %	119 %
Mutant 2	136 %	119 %
41G	146 %	125 %

Targeted Mutagenesis of *T.reesei* production strain.

Transcript Profiling used to Identify genes for manipulation in targeted strain improvement:

- New genes co-regulated with cellulases, therefore potentially involved in cellulase expression:
  - -Up-regulated in conditions known to produce cellulolytic activity: eg in the presence of cellulase inducers.
  - -Up-regulated in strains known to over-express cellulolytic activity: eg production strains vs wild type.
- New genes for proteins involved in cellulose degradation.

Functional Genomics: Conclusions

- **Documented response of known & new Glycosyl Hydrolases to induction by sophorose,and by lactose.**
- **Expression of many known genes correlated with high levels of cellulase expression.**
- **Discovery of many unknown genes co-regulated with these Glycosyl Hydrolases.**

- **Several new Glycosyl Hydrolases found.**

- **Many genes encoding previously unknown *T. reesei* proteins found: glucanases, chitinases, proteases, etc.**

Foreman *et al*. (2003) J. Biol. Chem. 278, 31988-31997.

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Improved production economics

SUMMARY

- Elimination of downstream processing
- Lower cost carbon source: Glucose/Sophorose
- Improved strains by Random Mutagenesis e.g. 41G
- Fermentation improvement around new process & new strains.
- Functional Genomics identified many genes for Targeted strain improvement.

Improvements in cellulase production resulted in a cost reduction of 5 X.

Improved Cellulase Performance

- 10X cost reduction goal can only be achieved by BOTH:
  - Improved production economics (reduced \$/gm enzyme)
  - Improved cellulase performance (reduced gm enzyme/gal EtOH)

$$\text{Effective cellulase cost}\left(\frac{\$}{\text{gal EtOH}}\right) = \left(\frac{\$}{\text{gm enzyme}}\right)\cdot\left(\frac{\text{gm enzyme}}{\text{gal EtOH}}\right)$$

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IMPROVED PRODUCTION ECONOMICS
- Process Improvement
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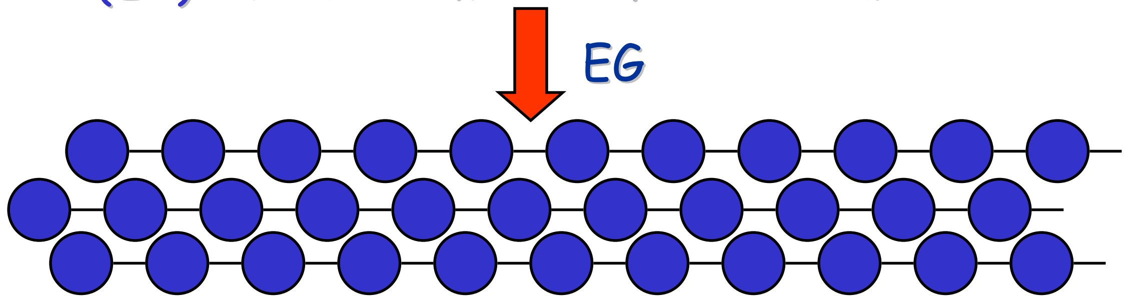
IMPROVED CELLULASE PERFORMANCE
- Known Cellulases
- New Cellulases: Functional Genomics
- Improved Cellulases

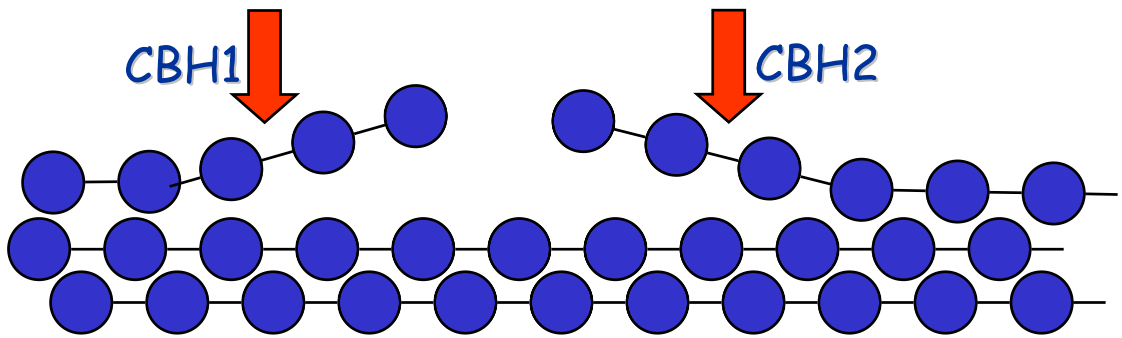
IMPROVED CELLULASE STRAIN

T. reesei Cellulase Components

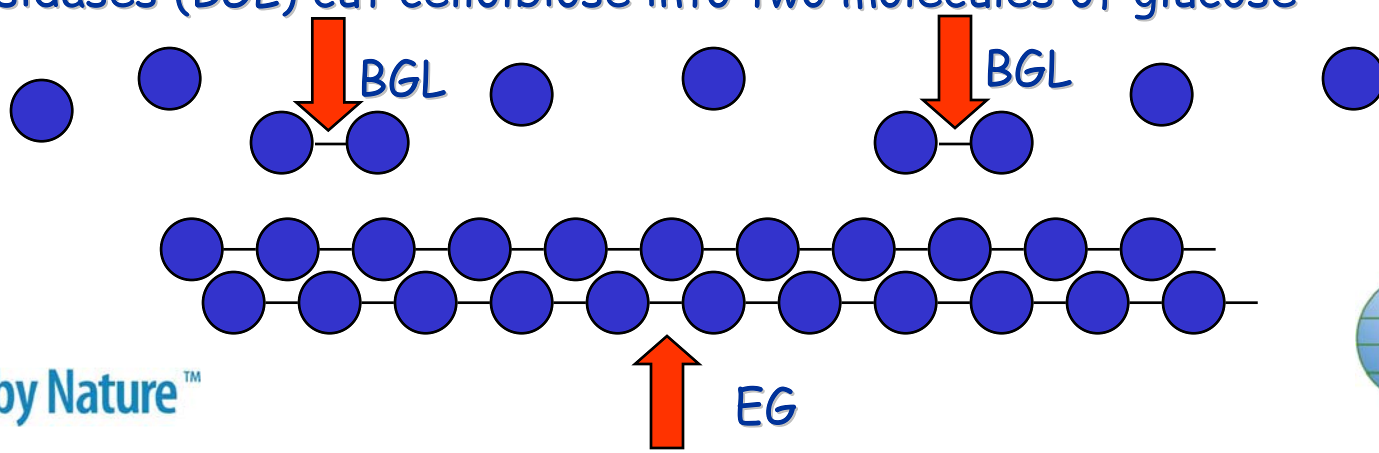
- Endoglucanases (EG) cut in the middle of the chain.



- Cellobiohydrolases (CBH) cut cellobiose (DP_2) from either end



- ß-glucosidases (BGL) cut cellolbiose into two molecules of glucose



Improved Cellulase Performance

- KNOWN portfolio of enzymes for testing
  Express, purify and assess performance of cellulases from *T.reesei* and other organisms.

- NEW products of *T. reesei* genomics

- KNOWN & NEW products of homologous cloning: e.g. CBH I

- IMPROVED products of protein engineering e.g. CBH I

Known Cellulase Components in *T. reesei*

Enzyme	% of Total Protein
CBHI (Cel7A)	**50**
CBHII (Cel 6A)	14
EGI (Cel7B)	12
EGII (Cel5A)	9
EGIII (Cel 12A)	<2

EGIV (Cel61A), EGV (Cel45A)
Bgl1 (GH family 3), Bgl2 (GH family 1)
Swollenin: may aid in cellulose hydrolysis
Hemi-cellulases aid in biomass hydrolysis.

NEW Cellulases Identified from Functional Genomics

Previously identified : CBHI, CBHII, EGI, EGII, EGIII, EGIV, EGV
BGL1, BGL2

•EGVI (Cel74A)	C-terminal CBD	MWt approx 100 kDa.
•EGVII (Cel61B)	No CBD	Predicted MWt 26 kDa.
•EGVIII (Cel5B)	Cell surface ?	Predicted MWt 46 kDa.
•BGL3 (Cel3b)	Secreted	Predicted MWt 92 kDa.
•BGL4 (Cel3c)	Intracellular	Predicted MWt 91 kDa
•BGL5 (Cel1b)	Intracellular	Predicted MWt 75 kDa
•BGL6 (Cel3d)	Intracellular	Predicted MWt 92 kDa
•BGL7 (Cel3e)	Secreted	Predicted MWt 83 kDa

Foreman *et al*. (2003) J. Biol. Chem. 278, 31988-31997.

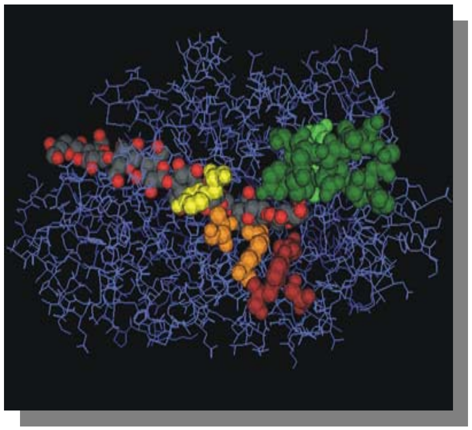
Need for a better CBHI

CBH1: ExoCellulase (one of two)

The major cellulase component in *T. reesei,* critical for performance.

Targets for improvement:

- Improved thermal stability to raise the cellulose conversion process temperature
- Higher specific activity



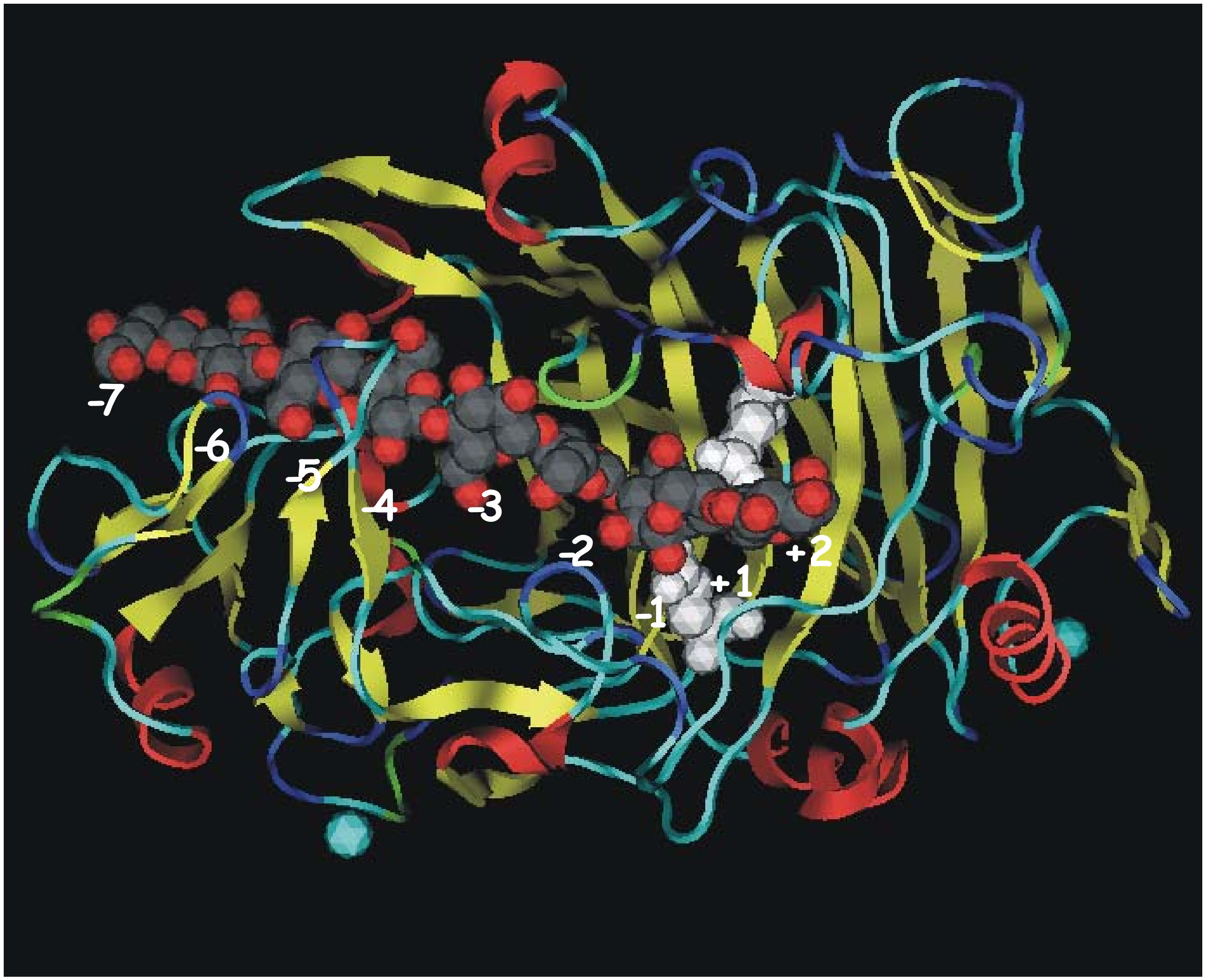
to drive the dose and cost down.

Products of Homologous Cloning: CBHI's cloned from Natural Diversity.

strains	% identity	Expression	Tm (°C)	ΔTm (°C)
Trichoderma reesei	100	Yes	62.5	
Hypocrea orientalis	97	Yes	62.8	0.3
Hypocrea schweinitzii	96	Yes	61.4	-1.1
Trichoderma viride	95	Yes	nd	
Trichoderma pseudokoningii	95	Yes	57.5	-5.0
Trichoderma citrinoviride	94	Yes	62.6	0.1
Trichoderma koninlangbra	93	Yes	59.4	-3.1
Trichoderma harzianum	81	Yes	nd	
Aspergillus niger	58	Yes	59.3	-3.2
Fusarium oxysporum	57	No		
Neurospora crassa	57	No		
Aspergillus aculeatus	57	Yes	63.7	1.2
Penicillium janthinellum	57	Yes	63.3	0.8
Humicola grisea var thermoidea	56	Yes	72.5	10.0
Agaricus bisporus	53	No		
Irpex lacteus	52	No		
Phanerochaete chrysosporium	46	No		

IMPROVED *T. reesei* Cel7A (CBH I)

Substrate binding sites are numbered



Divne et al., 1998. J. Mol. Biol. 274, 309-325.

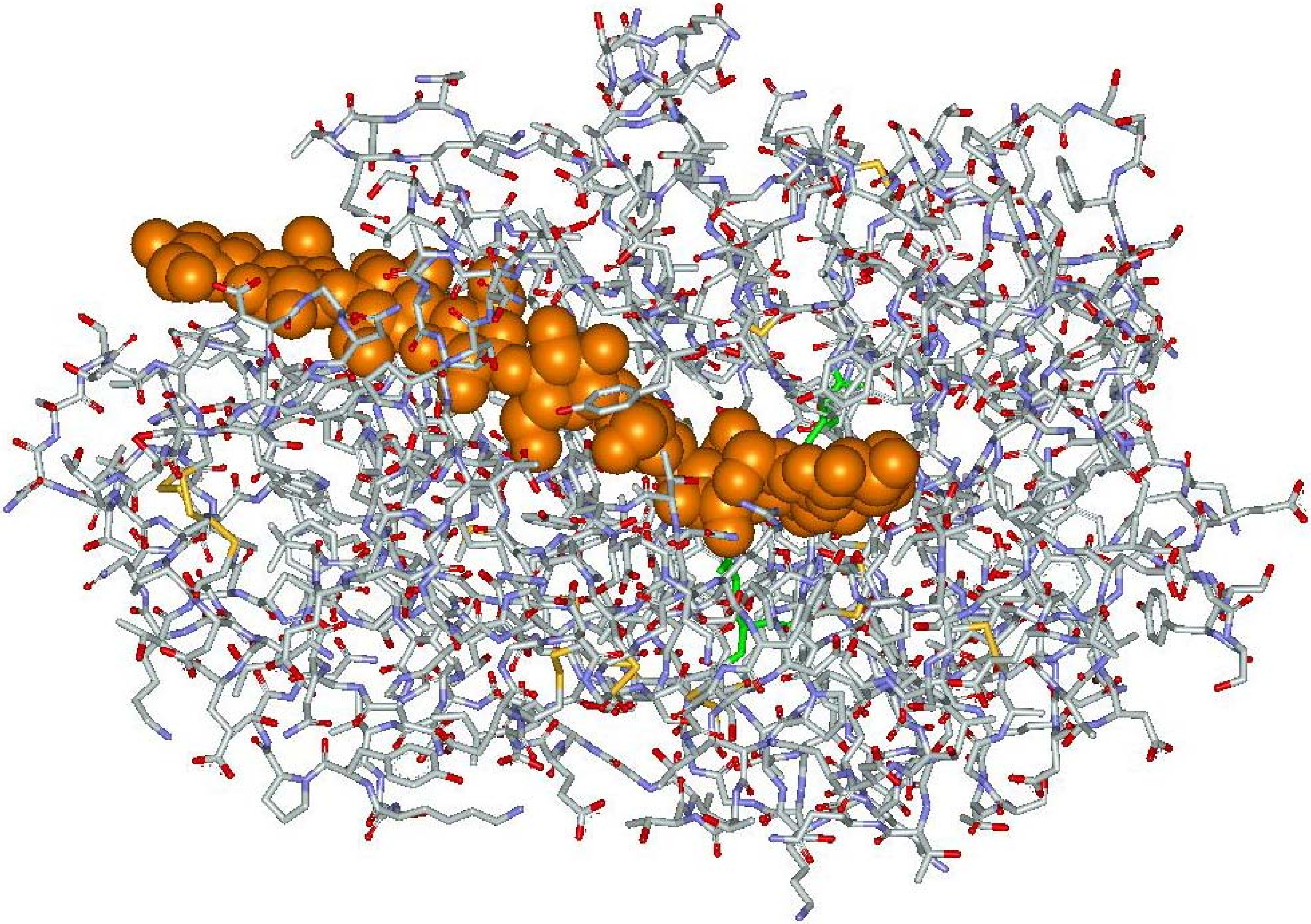
+1 & +2 = product binding Sites, for Cellobiose

436 amino acids in the catalytic module

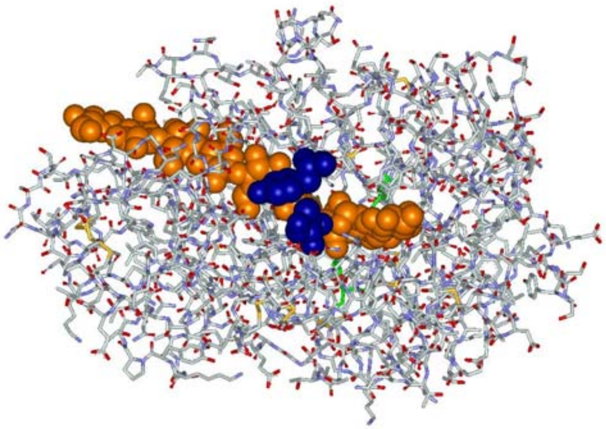
active site residues E212 and E217 (in white double displacement mechanism, retaining

10 disulfide bonds in the catalytic module

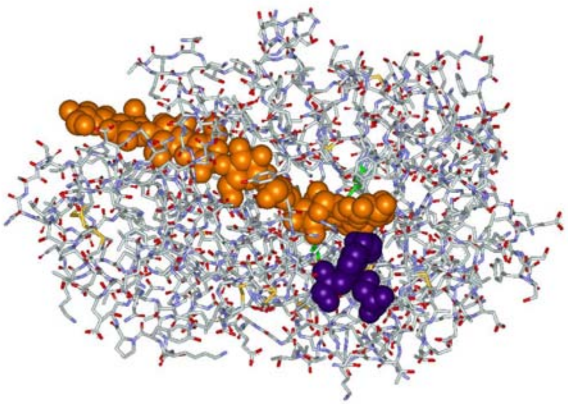
Modification of binding cleft of CBHI



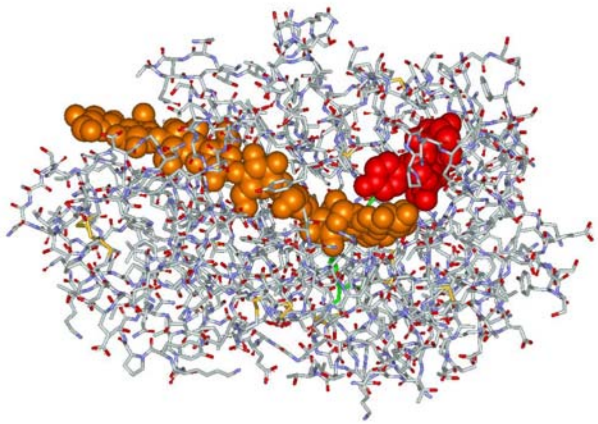
Modification of binding cleft of CBHI



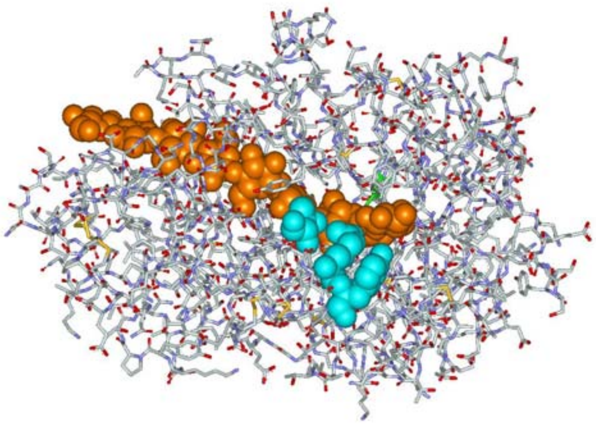
T246C/Y371C



Y252Q/D259W/S342Y



T380G/Y381D/R394A



T246A/R251A/Y252A

CBH I Product Binding Constructs

Enzyme	K_m (uM)	k_{cat} (1/minute)	ΔT_m ° C
Wild type	45	1.5	
T246C/Y371C	130	0.27	+ 2.5
Y252Q/D259W/S342Y	21	0.38	- 4.4
T246A/R251A/Y252A	310	10.5	+ 1.0
T380G/Y381D/R394A	4550	63	- 2.6

The kinetic parameters, K_m and k_{cat}, for the hydrolysis of CNPCellobiose at 40 °C derived by a single Michaelis-Menten model

Apparent T_m were measured for irreversible thermal denaturation by Circular Dichroism and a Delta T_m calculated relative to Cel7A.

In progress: Ki determinations & activities on celluloses.

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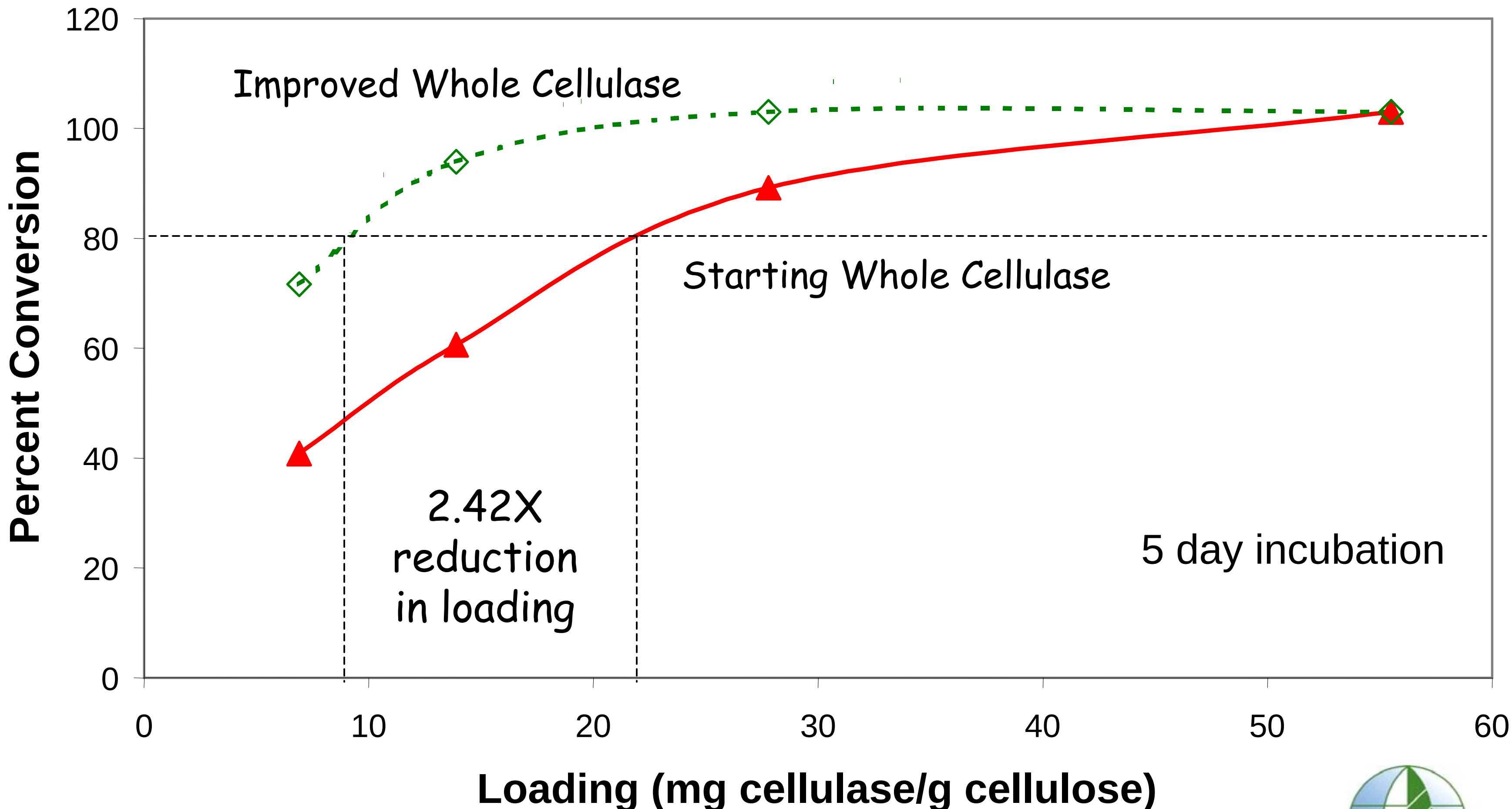
Improved Cellulase Strain

Improved cellulases produced in improved strain and process.

Conversion of 13% Pre-treated Corn Stover in a closed system assay.



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Improved Cellulase Strain

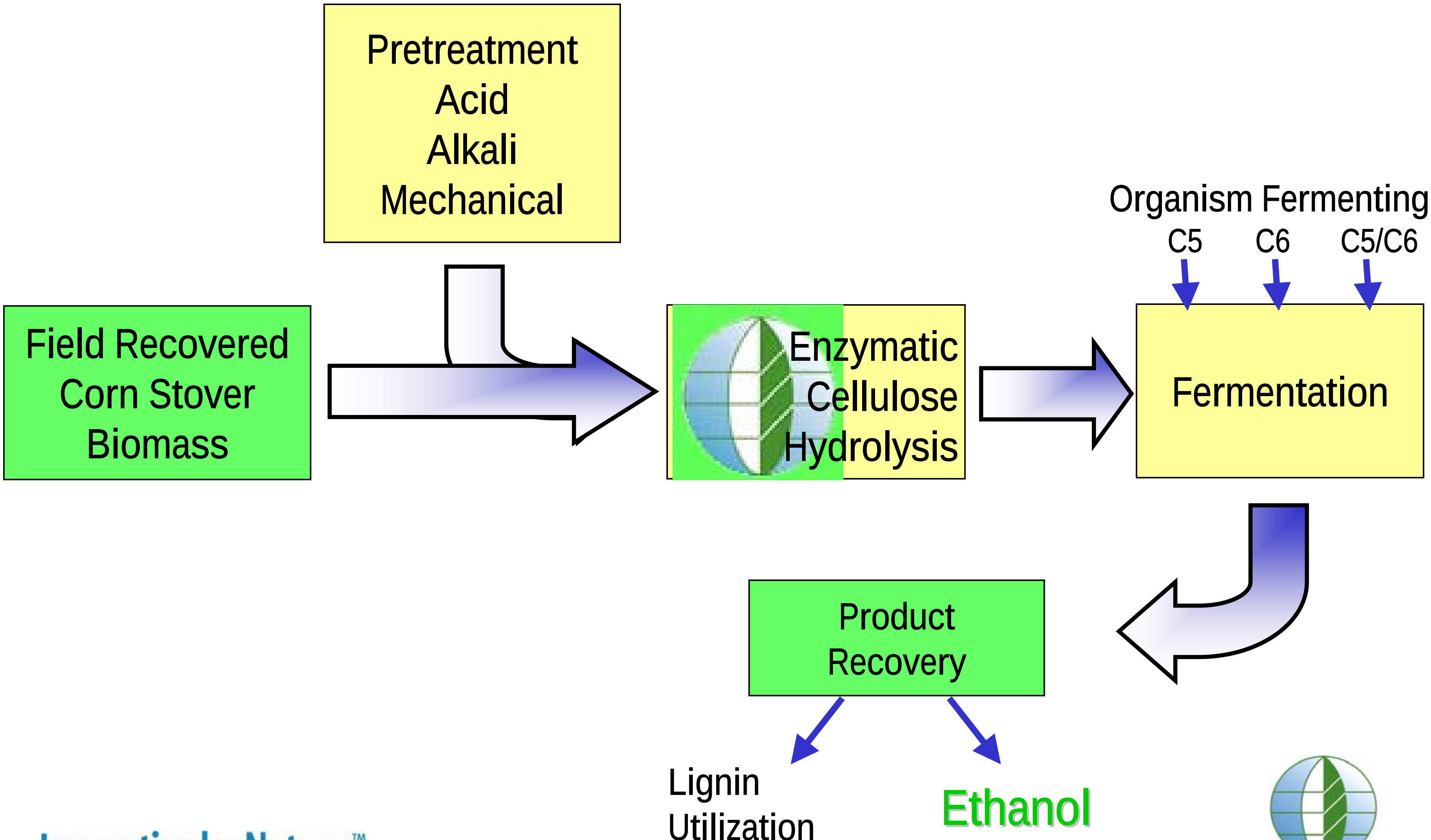
Improved cellulases produced in improved strain and process.

Improvement	Factor
Improved protein productivity - Elimination of post-fermentation - Media improvements - Carbon source - Strain Improvement 41G	4.9X
Improved hydrolysis process - 41G enzyme mix - Recruited cellulases into 41G	2.4X
Total	12X

Audited by NREL

Validated by NREL

Status Post GCOR-NREL Program



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