NRC·CNRC Biotechnology Research Institute

CNRC·NRC Institut de recherche en biotechnologie

2008 INTERNATIONAL CONFERENCE ON FLAX & OTHER BAST PLANTS
JULY 21-23, 2008 SASKATOON, CANADA

Processing of flax fibres for biocomposites - using of a thermostable pectate lyase

Denis Rho, J. Yang, M.-J. Lorrain, and P. C.K. Lau
BRI-NRC, Montréal, Qc, Canada
M.-T. Ton-That, K. Cole, W. Hu, and J. Denault
IMI-NRC, Boucherville, Qc, Canada

FAO / ESCORENA 2008 - Saskatoon, SK, Canada
July 21-23, 2008

National Research Council Canada
Conseil national de recherches Canada

Canada

NRC·CNRC
Biotechnology Research Institute
Institut de recherche en biotechnologie

Outline

- Introduction
  - From flax straw → cellulosic fibres → short-fibre polypropylene (PP) composites
  - Objectives and Challenges
- Results
  - The biocatalyst: Pectate lyase (pectinase)
  - The bioscouring process (treatment of flax fibre bundles)
  - The product, the fibres
    - Fibre quality and Fibre properties
  - Biocomposites
    - Fabrication
    - Mechanical properties
- Discussion and Conclusion

Introduction

- Separation of the bast fibres from the non-fibre components of the flax straw is one of the bottlenecks on the path that leads from straw to fibres
  - Separation of the bast fibres by mechanical means is feasible, but only after retting, good to excellent (high quality) fibres can be obtained
  - In other words, w/o retting the desired level of cleanliness and fineness can't be achieved
- The fibre properties and other characteristics are closely related to the mechanical properties of the biocomposites
  - Cellulosic fibres are hydrophilic; PP is very hydrophobic, thus to make a biocomposite, it is necessary to use of a coupling agent (CA) that is grafted onto PP «interacts » with other (non grafted) PP molecules via co-crystallisation or molecular entanglement
  - Aspect ratio of the fibre (i.e. L/D) relates to optimum performance (i.e. strength of the composites)

Introduction

- The utilization of a pure pectinase offers advantages over some commercial enzyme formulations, especially those that contain cellulases, which are known to attack fibre nodes and the fibre's cellulose structure, which consequently reduce the strength of the fibres, as well as the mechanical properties of the composite.

- **This investigation focuses on**
  - the utilization of a new (engineered) alkaline PL_{XC},
  - the development of a bioscouring process, and
  - the mechanical properties of short-flax fibre PP composites

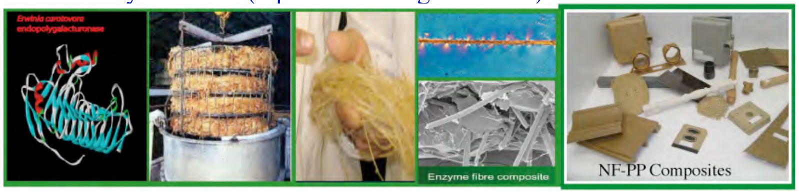
- **CBIN program** - project Natural Fibres Initiative for Biofibres and Biochemicals (lead by Adrien Pilon): The activities of two out of five scientific / engineering modules of this 3-y project were conducted at IMI-NRC (Dr. Johanne Denault) and BRI-NRC (Dr. Denis Rho).

Objectives and Challenges

- design and improve

OBJECTIVES

- Design of new enzymes and development of an ezyme-based process for the 'production' of fibres with better properites;
- Development of flax-fibre PP composites with optimized mechanical properties;
- Manufacturing of eco-friendly materials (replacement of glass fibres)



CHALLENGES

Enzymes and Bioprocess

- Activity ; Stability ; optimal pH and temp.
- Minimize enzyme loading

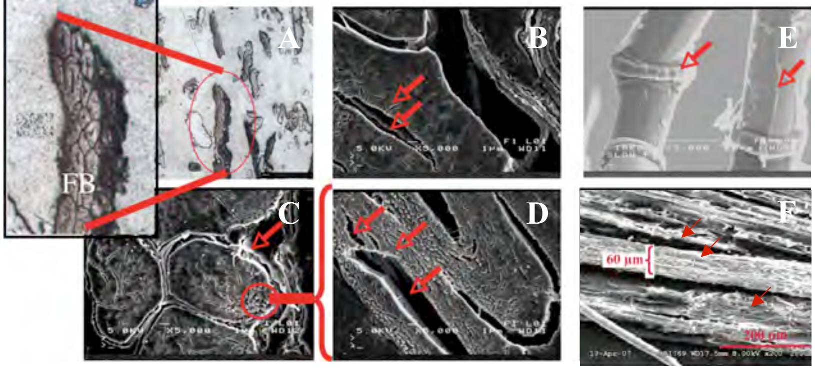
Composites

- Fibre properties (cleanliness, fibre's aspect ratio)
- Optimization of fibre-polymer compatibility // Fibre humidity sensitivity
- Stability during processing // Flammability resistance

Flax fibre (micro) structures

- Challenges and technological barriers

SEM MICROGRAPHS OF FLAX FIBRES



- In an epoxy matrix (A), flax fibres looks very heterogeneous: single fibre cells and multiple fibre-cell bundles (FB) of different sizes can be observed;
- Cross-sections B, C, D: fibre cells are not regular circle, hollow and porous structure can also be observed, and defects inside the fibre cell wall structure;
- Presence of fibre nodes (E) and debris (F) at the surface of non or partially retted fibres.

NRC·CNRC
Biotechnology Research Institute
Institut de recherche en biotechnologie

Retting

- a post-harvest treatment

separation of cellulosic (bast) fibres
from non-fibre tissues

Water retting

Field (dew) retting

Retting of flax straw

Water-retting: best method (fibre quality), but pollution, cost;

Dew-retting: current method of choice lower fibre quality, land use, geographic limitations, dirty 'contamination';

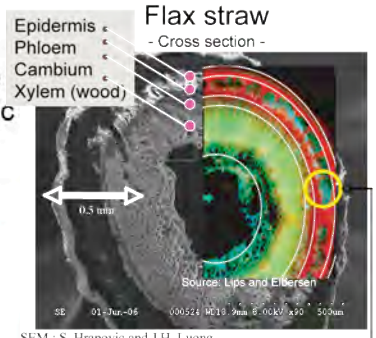
"**Snow-retting**" in Canada, colder climates;

Chemical-retting: explored but no commercial method;

Enzyme-retting: explored in Europe (1980's) and US (1990's) but no commercial method – continued R&D effort.

van Sumere, The biology and processing of flax, 1992
Akin et al., J. Nat. Fibres vol. 1, 2004
Foulk et al. BioResources vol 3, 2008

Flax straw
- Cross section -



SEM : S. Hrapovic and J.H. Luong,
Natotechnology Group, BRI

FB: Fibre bundles embedded in a pectin-rich structure

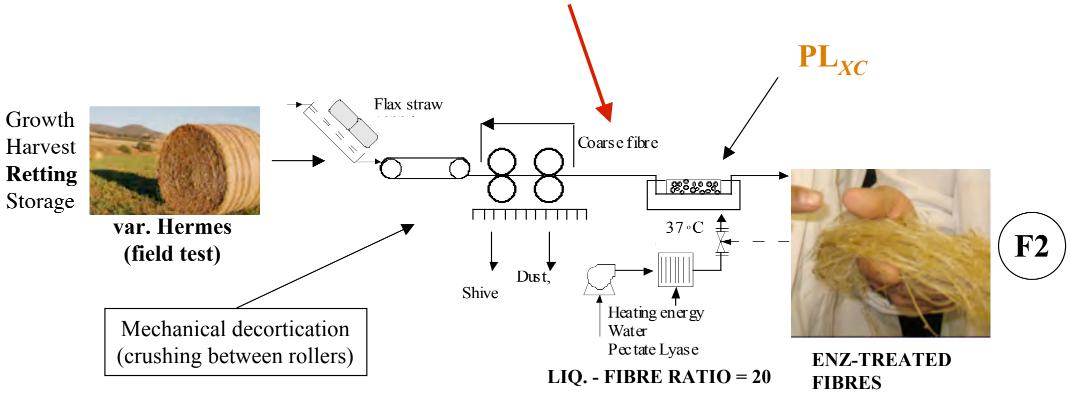
Fibre content ranging from 8 % to 40 %

NRC·CNRC
Biotechnology Research Institute
Institut de recherche en biotechnologie

Our retting strategy

- bioscouring

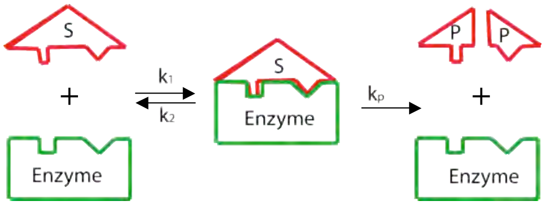
- Bioscouring process of flax fibre bundles (overview)



Biotechnology Research Institute
Institut de recherche en biotechnologie

An enzyme-based process
- overview

- The substrate (S) → **The straw**
- The enzyme (biocatalyst) → **The pectinase**
- The product (P) → **The fibre/sugars**
- The process (op. conditions) → **The bioreactor**



Biotechnology Research Institute
Institut de recherche en biotechnologie

Novel and engineered pectinases and cutinases

Effective (new) pectinases / cutinases could be obtained using molecular biology techniques

Directed evolution

Screening and gene cloning of bacterial and / or fungal pectinase / cutinase genes

Expression in *E. coil* or *P. pastoris* (selection of the proper host - vector system)

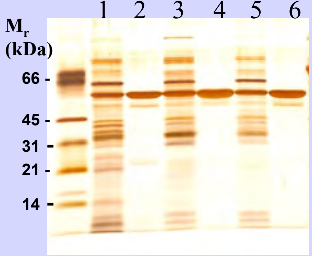
Characterization of the expressed enzymes

Key targets :
pH stability
Thermal stability
Substrate affinity

Lab-scale production of the engineered enzymes

Proof of concept - Technology 1 : ER of flax straw
- Technology 2 : Bioscouring of flax fibre bundles

Pectate Lyases



1) PL (wt) crude extract
2) PL (wt) purified

3) PL (A30G) crude extract
4) PL (A30G) purified

5) PL (R236F) crude extract
6) PL (R236F) purified

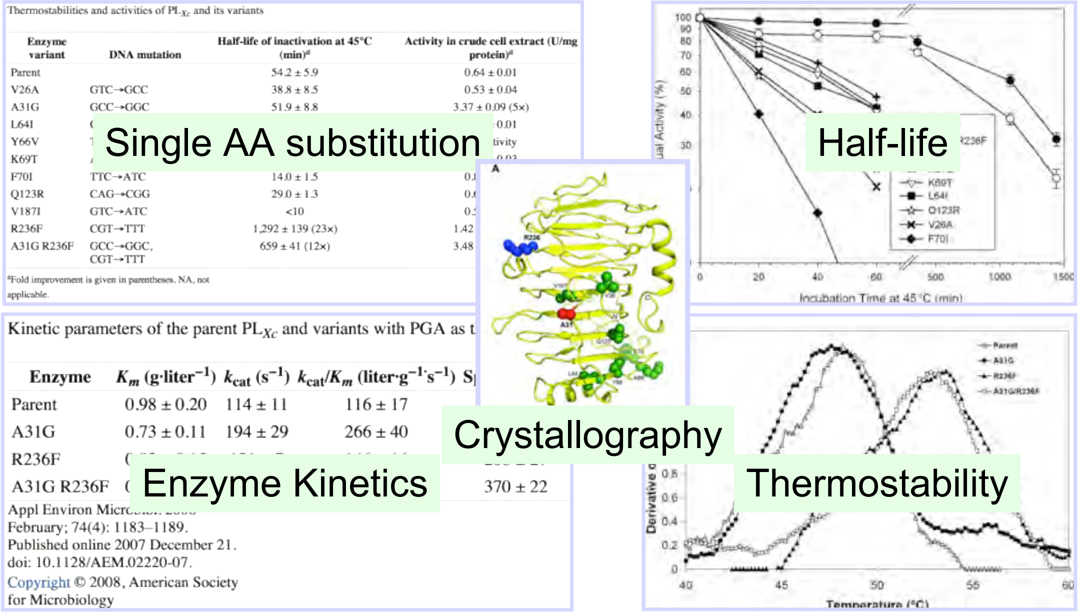
Purification

Enzyme	Total Prot. (g)	Total Act. (U)
PL (A30G, R236F)	4.3	1,500,000

Pectate lyases (PL_{XC})

- Improvement of the thermostability

Z. Xiao, P.C.K. Lau, M. Cygler and coll.
Appl. Environ. Microbiol. (2008) 74(4) 1183-1189



Single AA substitution

Thermostabilities and activities of PL_{Xc} and its variants

Enzyme variant	DNA mutation	Half-life of inactivation at 45°C (min)[a]	Activity in crude cell extract (U/mg protein)[a]
Parent		54.2 ± 5.9	0.64 ± 0.01
V26A	GTC→GCC	38.8 ± 8.5	0.53 ± 0.04
A31G	GCC→GGC	51.9 ± 8.8	3.37 ± 0.09 (5×)
L64I	C		0.01
Y66V	T		tivity
K69T	A		0.03
F70I	TTC→ATC	14.0 ± 1.5	0.
Q123R	CAG→CGG	29.0 ± 1.3	0.
V187I	GTC→ATC	<10	0.
R236F	CGT→TTT	1,292 ± 139 (23×)	1.42
A31G R236F	GCC→GGC, CGT→TTT	659 ± 41 (12×)	3.48

[a]Fold improvement is given in parentheses. NA, not applicable.

Enzyme Kinetics

Kinetic parameters of the parent PL_{Xc} and variants with PGA as t

Enzyme	K_m (g·liter^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (liter·g^{-1}·s^{-1})	Sp
Parent	0.98 ± 0.20	114 ± 11	116 ± 17	
A31G	0.73 ± 0.11	194 ± 29	266 ± 40	
R236F	0			
A31G R236F	0			370 ± 22

Appl Environ Micro
February; 74(4): 1183–1189.

doi: 10.1128/AEM.02220-07.

Crystallography

Half-life

Thermostability

Bioscouring process

- Process conditions

- Results (SEM, NIR)

Mechanical decortication

1 kg

Flax (bast) fibre bundles (1 kg)

2 U/mL
New engineered PLase

20 L

37 °C, pH 8.5

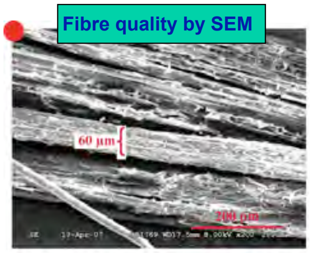
0.2 kg

F2
Enz.-treated fibres

High quality fibre (210 g)

Fabrication Biocomposites

Fibre quality by SEM

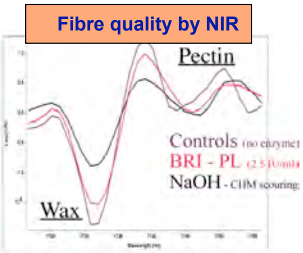


Untreated fibre bundles from flax stems. Diameter : 60 - 100 µm.

SEM Micrographs
Hitachi model 2600N

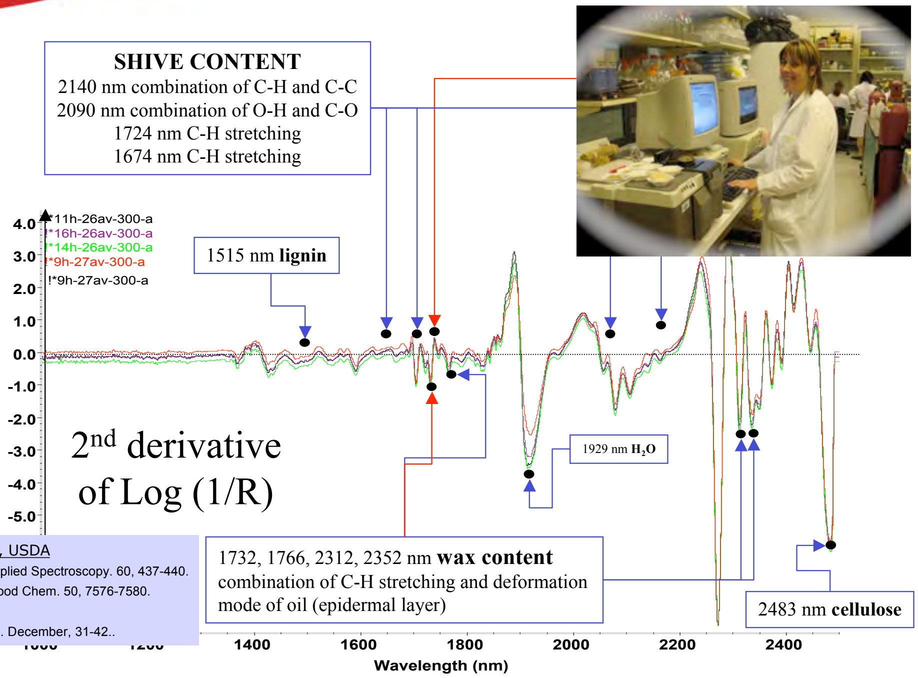
Enzyme treated fibres, separated from bundles; more single fibres. Diameter : 6 -20 µm.

Fibre quality by NIR



Near Infra-red (NIR) technique for analyzing rapidly several fibre samples.

NIR
- Spectral Analysis



SHIVE CONTENT
2140 nm combination of C-H and C-C
2090 nm combination of O-H and C-O
1724 nm C-H stretching
1674 nm C-H stretching

1515 nm **lignin**

1929 nm $\mathbf{H_2O}$

2nd derivative of Log (1/R)

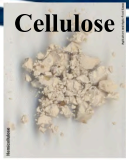
1732, 1766, 2312, 2352 nm **wax content**
combination of C-H stretching and deformation mode of oil (epidermal layer)

2483 nm **cellulose**

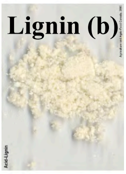
Agriculture Research Service, USDA
Sohn, Himmelsbach, *et al.* (2006) Applied Spectroscopy. 60, 437-440.
Barton, Akin, *et al.* (2002) J. Agric. Food Chem. 50, 7576-7580.

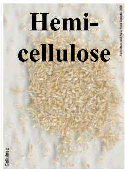
Kessler and Kohler (1990) Chemtech. December, 31-42..

Fibre quality by NIR
- Similarity Index


Cellulose


Lignin (a)


Lignin (b)


Hemi-cellulose

FLAX FIBRES
Cellulose relative content (%), based on NIR - SI

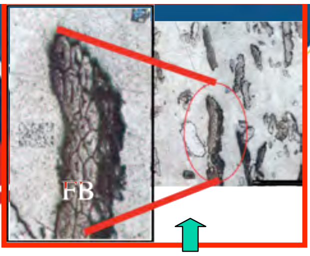
Target macromolecules	Prior to the treatment	Enzyme scouring	Chemical scouring	No treatment
	Step A⇒	Steps B, C	Steps B, C	Step C
Cellulose	34% ⇒	→ 58%	→ 68%	→ 40%
Lignin	20% ⇒	→ 16%	→ 8%	→ 20 %
Pectin	7.5% ⇒	→ 4.5%	→ 1.6%	→ 7.3%
Epidermal layer	++ ⇒	→ +	→ -	→ +

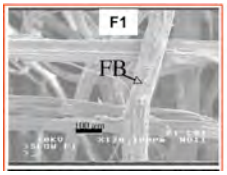
Enz. Scouring: Pectate lyase (double mutant) (2.5 U/mL) in 50 mM Tris buffer (pH 8.5) 24 h, 37 °C
Chm. Scouring: NaOH (0.2 N) 1 h, 121 °C, 1.5 psig
No treatment: Soaking in 50 mM Tris buffer (pH 8.5) 24 h, 37 °C

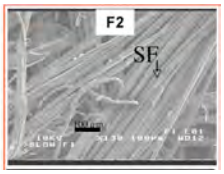
Step A: Mechanical decortication (straw -> fibre bundles)
Step B: Rinsing and Drying
Step C: Carding

Biopolymers separated from flax shive using a
Pressurized Low Polarity Water Extraction (PLPW) system
Dr. Giuseppe (Joe) Mazza, AAFC, Summerland, BC, Canada

Flax fibre properties





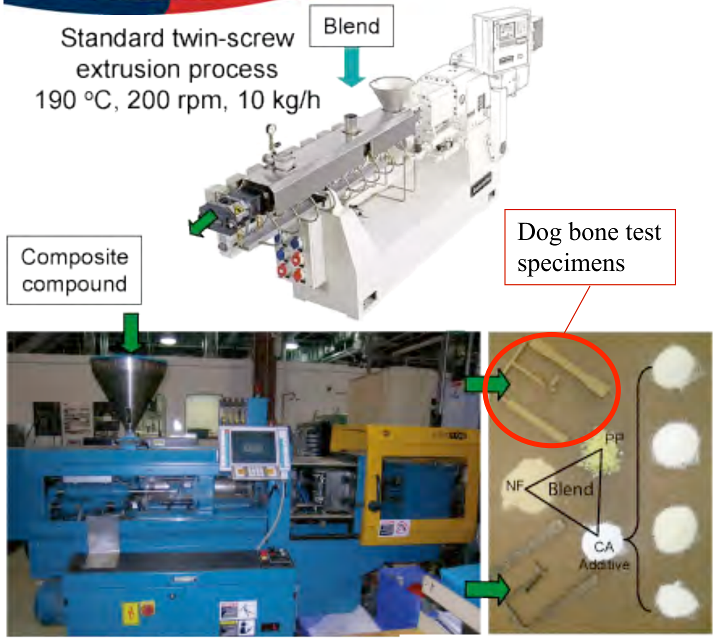


Sample	Elementary Fibres (#)	Diameter (µm)	Strength (MPa)	Modulus (GPa)	Strain (%)
F1	13.5 ± 0.2	42.6 ± 6.7 ^{c}	400.6 ± 40.8	48.2 ± 4.9	2.03 ± 0.84
F2	34.2 ± 9.0	54.6 ± 12.8 ^{c}	456.5 ± 33.9	57.1 ± 7.2	2.08 ± 0.68

- ☐ Bundles can be observed (F1), still attached with pectin, lignin, hemicellulose…
- ☐ Large cuticle material present (F1), but not shown in this SEM
- ☐ Obvious separation of fibre bundles to elementary fibres (F2)
- ☐ Cleaner surface (F2), much less non-fibre stem tissue materials
- ☐ F2: Mechanically decorticated flax straws combined with the bio-scouring of the flax fibre bundles
  - ➢ Greater strength and modulus: high cellulose content (58%) and elimination of impurities (hemicelluloses, lignin, pectin, etc.);
  - ➢ Fineness (diameter) is less than F1 (non-enzyme treated fibres)

Flax fibre-PP composites

- fabrication
- formulation



Standard twin-screw extrusion process
190 °C, 200 rpm, 10 kg/h

Blend

Composite compound

Dog bone test specimens

Injection molding process 200 °C

Flax straws: Fibres, Shives, Blend of shives and fibres, or Dust

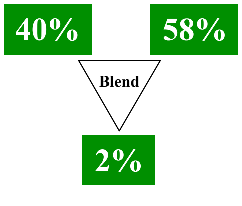
Fibre length: approx. 5 mm

Polymer matrix: Virgin PP6100 SM (Montell)

Coupling agent: maleic acid anhydride (MA) grafted polypropylenes (Eastman Chemical)

- Epolene-43 (Mn = 9,100; ~4.81 wt% of MA)
- Epolene-3015 (Mn = 47,000; ~1.31 wt% of MA)
- Polybond 3150

Additives: CaO



40%

58%

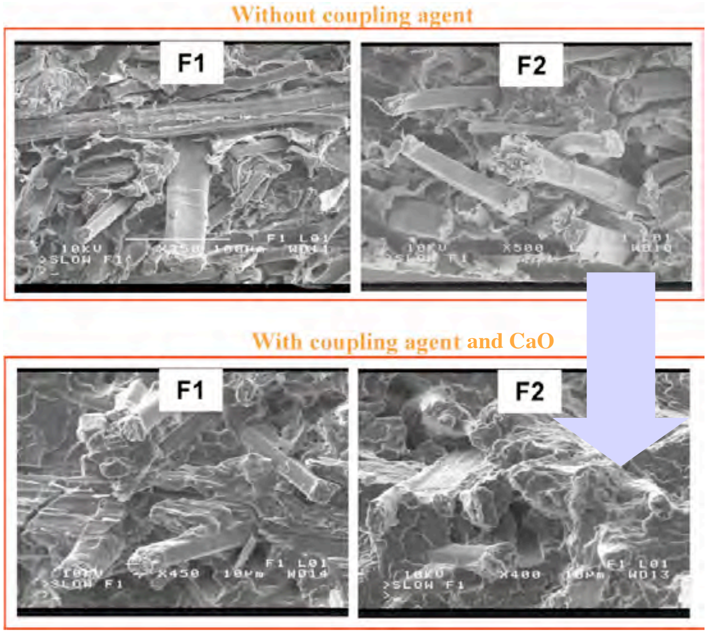
Blend

2%

Flax fibre-PP composites

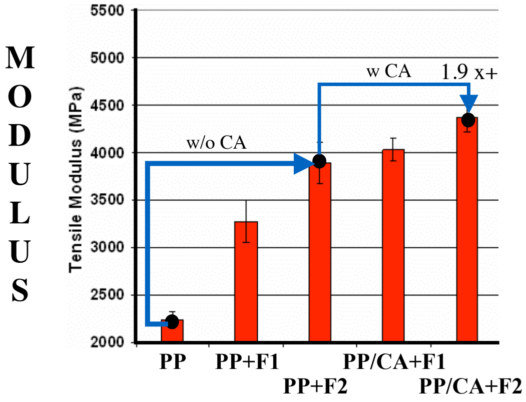
- Interface

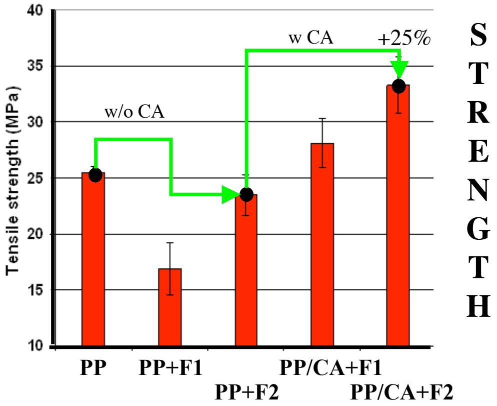
- Very poor interface between the PP matrix and the fibres regardless of fibre type (F1, F2); since the hydrophobic PP matrix is incompatible with the hydrophilic fibres.
- Good interface between the PP matrix and the fibres is due to the presence of a coupling agent
-



Mechanical properties

F1 - non Enz-treated flax fibres
F2 - Enz-treated flax fibres





- Without coupling agent
  - Modulus: improved significantly
  - Strength: reduced strength (PP-F1), because of very poor interface
- With coupling agent (maleic acid anhydride)
  - Both modulus and strength improved significantly
  - F2 composite appears superior due to better surface properties and better interface with the PP matrix

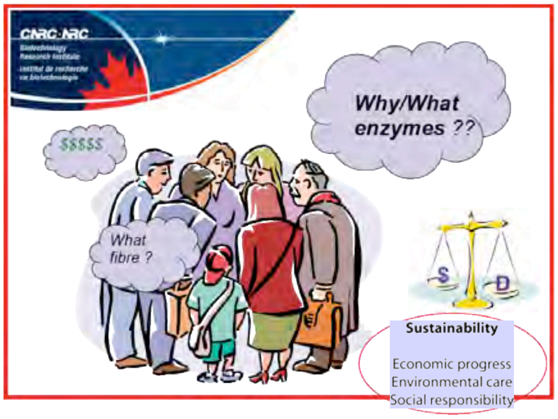
Discussion

- Advances in microbiology, molecular biology and enzymology have brought new lines of research and have accelerated the development of new alternatives for the design of new biocatalysts. (Z. Xiao et al. Appl. Microbiol. Environ. 74: 1183-1189, 2008)
  - **Half-life** of the engineered **alkaline PL_{XC}** at 45 °C was increased 12x
  - **Pectinase activity** was increased 5x
  - A new approach to **enzyme thermostabilization** was conceived, which make possible the thermostabilization of mesophilic enzymes
- Fibre properties and Fibre quality
  - **SEM and NIR** methods were used to monitor fibre quality and other characteristics. Surface cleanliness was improved and better subdivision of the fibre bundle into ultimate fibre was observed by SEM. As a result, the (some) mechanical properties of the biocomposites were superior.
  - **Near-IR** is an elegant approach to determine (rapidly and efficiently) the quality of the fibres; it can be used to measure cellulose content as well as the content of other polymers,
    - but it is not yet a method that could be used on a routine basis (e.g. some inter-laboratory experiments are still required to prove its practicability in the field or at the pilot-plant).

Discussion

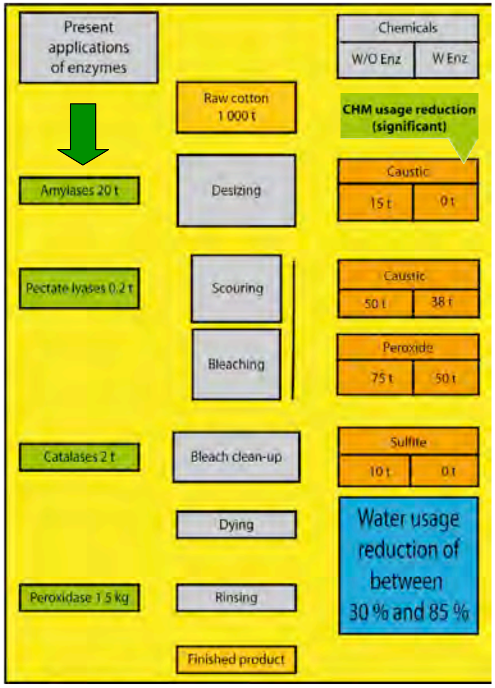
- Several attempts were made in the lab. to develop an efficient enzyme scouring process to clean the fibre's surface, but as importantly to individualize the « ultimate » fibres.
  - **BIOSCOURING PROCESS** :
    - Duration: 90 % completed after 2 h ; Dosage: 2 U / mL (I.e. **40 U / g Fibre**) ; Conditions: pH 8.5 and T = 37 °C ;
    - Specific activity alkaline PL_{XC} : 238 IU/ mg protein → 0.17 mg PL_{XC} / g Fibre
    - At the USDA Research Center, Akin and Foulk are currently investigating another retting strategy (SER), with great success,… interestingly, the dosage is similar: **120 U/g straw**.
- Short-flax fibre PP composites
  - Clean fibres (with less impurities)… proper aspect ratio (L/D)… are two key factors that contribute to the improvement of the mechanical properties
  - Proper formulation (fibres, coupling agent, basic oxides)… significantly improves the mechanical performance and fire resistance.
  - Recycling is possible : NF-PP composites (with maleic anhydride and CaO) can be regrinded, extruded and injection molded 3 times, without important loss of performance (results not shown).

Conclusion



Enzymes are known for their specificity, high efficiency and ability to work under mild conditions and provide a promising solution to these challenges. It is clear that enzyme technology can be used to develop a usable, more environmental friendly, economical competitive scouring process.

Ecological footprint



Source: Novozymes

NRC·CNRC
Biotechnology Research Institute
Institut de recherche en biotechnologie

Future Works

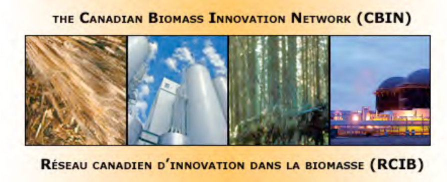
- **Bioscouring / Biocomposites** **(short term)**
  - *New* Enzymes / Enzyme cocktails => Tailor made biocatatlysts
  - Optimization of the enzyme scouring process
  - Surface compatibilization
    - Removal of all hydrophobic non-cellulose components (wax)… cutinases
  - Study blends of natural (flax) fibres with other polymers
  - NIR as a tool for monitoring fibre quality and the fibre content (straw)
- **Other lines of research for *materials of the future*** **(long term)**
  - Structural materials w high mechanical performances
    - Fabrication of MCC (microcrystaline cellulose) from flax fibre or flax straw
  - Advanced technical textiles
  - Fonctionalizaiton of cellulose-fibres (super-absorbent, radicalization of fibres, … bactericidal activities)
  - **Intelligent materials** (e.g. intelligent textiles and biopolymers)
- Natural Fibres for the Green Economy Network (NAFGEN)… ABIP

NRC·CNRC
Biotechnology Research Institute
Institut de recherche en biotechnologie

Acknowledgements

- Marie-Josée Lorrain
- Manon Beauchemin
- Zhizhuang Xiao (molecular biologist)
- Stephen Grosse (enzymologist)
- Giuseppe (Joe) Mazza, AAFC, Summerland, BC, Canada
- Linda Braun, Executive Director, SaskFlax
- Alvin Ulrich, Biolin Research Inc.
- Joe Hogue, Schweitzer-Mauduit Canada Inc.
- Nicole Richer, CBIN program coordinator

The project was financed by National Research Council Canada (NRC);
The CBIN project was financed (2006-2008) by Natural Resources Canada.



Questions ?

We are ALL responsible for the state of OUR environment!

Nous sommes TOUS responsables de l'état de NOTRE environnement !