Surface Mapping Of Fibers As A Screening Mechanism For Fiber Modifying Enzymes

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ABSTRACT

The use of cellulase enzymes to condition fibers prior to refining is a proven science. The benefits of this approach are well known: reduction in refiner energy which gives the following benefits: increased bulk, improved softness, better drainage and so on. The search for new and better enzymes for this application can be lengthy and tedious. There are numerous techniques that have been used in this area with varying degrees of success. Buckman has begun using high throughput, automated screening techniques that yield as much if not more information than previous techniques. These techniques are more predictive than existing test methods. Furthermore, a novel characterization technique provides a better understanding of what types of cellulose and hemi cellulose are present on the surface of the fiber. This information, combined with the known activities of the enzymes, is critical to enzyme selection. This new technique has also sped product development efforts resulting in new products in this area. A description of the technique used, products developed, and case histories detailing the benefits to the tissue maker will be disclosed hereafter.

INTRODUCTION

Enzymatic technologies are well established in the papermaking process. As catalysts, enzymes can have a significant impact on surface of the fiber at relatively low working concentrations and provide a more environmentally friendly use profile (Torres 2012, Buzala 2015). Cellulases and xylanases have been shown to provide improvements in fiber strength, freeness, softness and bulk (Efrati 2013, Buzala 2015). In looking to appropriately apply enzymes, it is important to understand the composition of the fiber and to ensure that the enzymes being used match the substrates that are to be found at the point of interaction. This maximizes the benefit of the enzymatic technology and minimizes the opportunity for wasted activity and potential deterioration of the fiber (Buzala 2015). There are multiple methods of analyzing the composition of the fiber, including compositional analysis, surface imaging, index of crystallinity and degree of polymerization techniques. However, these methods can be limited by having a lower sample through-put, focusing on bulk versus surface characteristics, and requiring highly specialized equipment or expertise (Burkhardt 2013, Karimi 2016). The ability to better characterize the biopolymer surface of the fiber allows interesting insights regarding how enzymatic technologies can be tailored to more optimally interact with the fiber to obtain the desired final characteristics. Utilizing a technology that combines carbohydrate binding modules with fluorescence protein labels, it becomes possible to extract information regarding the relative abundance of crystalline cellulose, amorphous cellulose, xylan and mannan at the fiber surface (Hebert-Ouellet et al. 2017, see Figure 1). Previous reports have demonstrated that the technique can be used to demonstrate activity of enzymes on the fiber, and can also be used as a predictive tool for strength properties when the fiber is subjected to refining (Khatri et al. 2016, Hebert-Ouellet et al. 2017). This method allows for interrogation of a fiber sample in around 4 hours and allows for a more rapid assessment of the impact of numerous inputs such as furnish changes, process changes (e.g. refining), treatment changes, etc.

The data provided herein shows how this method can be used to compare different fiber sources and how this can provide exciting insight into the selection and subsequent impact of enzyme technologies.

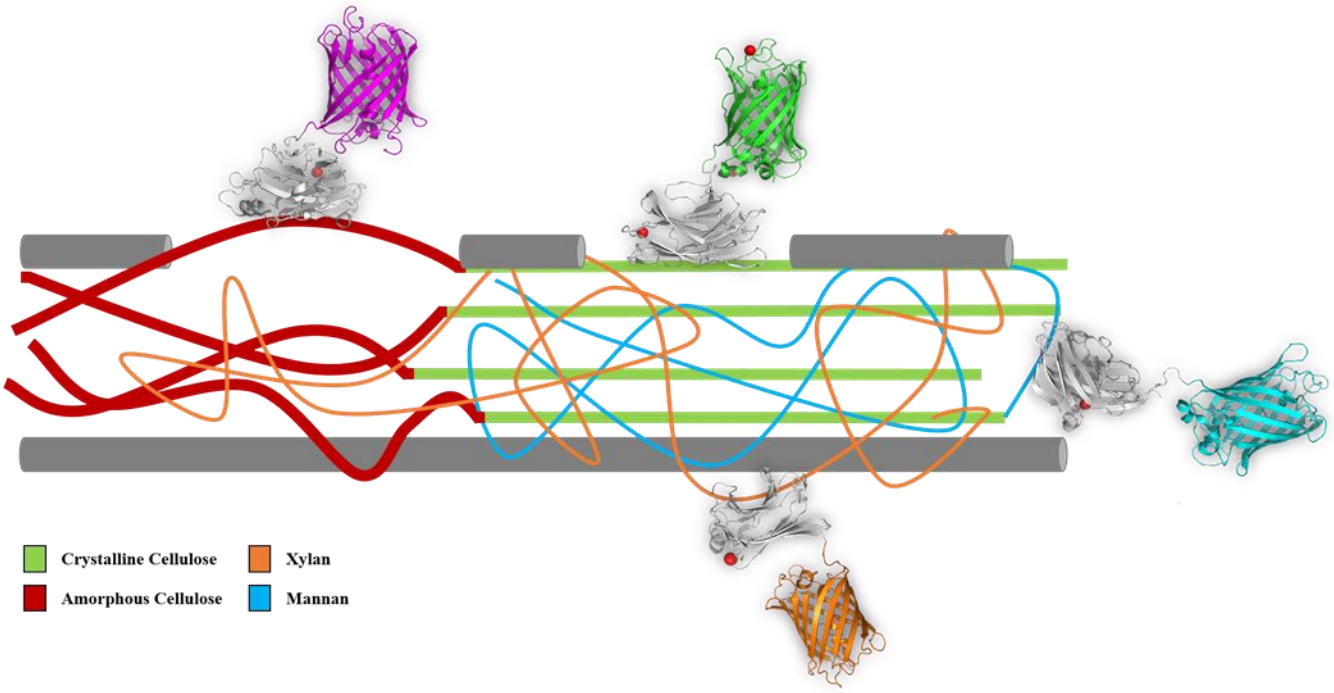

Figure 1. Schematic representation of probe binding to a wood fiber. The left side of the fiber shows a lignin free fiber, where amorphous cellulose dominates (cherry strings). On the right side, the straight green bars represent crystalline cellulose. Hemicelluloses such as xylan (orange) and mannan (blue) are shown as polymers that help keep the fiber together. The probes designed here attach specifically to their respective target polymer, as indicated by the matching color of their fluorescent module (Hebert-Ouellet et al. 2017).

METHODS

Probe Expression and Purification. The production and purification of the fluorescent CBM probes is described in detail elsewhere (Khatri et al. 2016, Hebert-Ouellet et al. 2017).

Pulp and handsheet formation. Pulp samples were obtained from various locations for comparison. Paper sheets of 60 ± 2 g m-2 in density were prepared as per the TAPPI T205 sp-02 methodology.

Enzyme treatment. Hardwood and softwood furnish samples were treated with an enzyme product at a dose reflecting 1 kg of enzyme product per ton of pulp. Pulp samples with and without enzyme were incubated at 50 ˚C at pH 7.0 for 60 minutes and subsequently dosed with 4kg/ton of a PAE wet strength additive. Treated softwood and hardwood samples were refined using a PFI refiner for 2500 and 1500 revs, respectively. Handsheets were generated as described above and wet tensile strength improvement measured relative to the untreated control.

CBM Probe assay. All fluorescence readings were acquired at 23 ˚C with a Cytation 5 microplate reader (BioTek) using the area scanning feature (3 x 3) with the top detection height set at 4.5 mm and the filters bandwidth at 9 mm. The excitation and emission wavelengths were set at 488 and 510 nm for eGFP-CBM3a, at 587 and 610 nm for mCherry-CBM17, at 549 and 568 nm for mOrange2-CBM15 and 434 and 477 nm for eCFP-CBM27. Fluorescence measurements were recorded after each step of the assay. Preparation of the microplate was carried out by affixing

handsheet punches on the bottom of 96-wells, black microplate (Costar) using a transparent nail polish. The surface characterization assay started by incubating the handsheet punches 1h, at 23 ˚C with agitation in a 20 Tris-HCl pH 7.5 buffer containing 20 mM NaCl, 5 mM $CaCl_2$ and 3% milk. Unbound milk constituents were removed by washing three times with the 20 Tris-HCl pH 7.5 buffer containing 20 mM NaCl, 5 mM $CaCl_2$. The blocked punches were incubated 1h, at 23 ˚C with agitation in a 20 Tris-HCl pH 7.5 buffer containing 20 mM NaCl, 5 mM $CaCl_2$, 3% milk and of 0.5 µg/µl of either eGFP-CBM3a (crystalline cellulose), mCherry-CBM17 (amorphous cellulose), mOrange2-CBM15 (xylan) or eCFP-CBM27 (mannan). Unbound probe was first removed with three buffer washes followed by three 0.05 % Tween 20 washes. Calculations started by subtracting the mean residual fluorescence values from the blocked ones (Hebert-Ouellet et al. 2017).

RESULTS AND DISCUSSION

In order to demonstrate the potential of the fiber surface characterization method, we compared samples of bleached virgin softwood, bleached virgin hardwood and bleached bamboo fibers for four different substrates using the fluorescent probes: amorphous cellulose, crystalline cellulose, mannan and xylan. The mean fluorescence intensity data was normalized by sample weight and the tested area was the same for all samples. The data shows that the method can readily detect differences in the different fiber sources. While xylan content was similar between the three furnishes, there were notable differences in the measured crystalline cellulose, amorphous cellulose and mannan content, with the bamboo having much higher crystalline cellulose content than the representative hardwood and softwood furnishes and lower amorphous cellulose and mannan content (Figure 2). Previous work has shown that the ratios of amorphous:crystalline cellulose and mannan:crystalline cellulose are predictive of strength properties such as tensile strength and internal bond (Hebert-Ouellet et al. 2017). While a direct comparison of strength was not undertaken with these samples, the data does illustrate that fiber substitution efforts should be mindful of differences in the fiber surface and that the selection of enzymes to drive physical properties should carefully consider what substrate is being targeted.

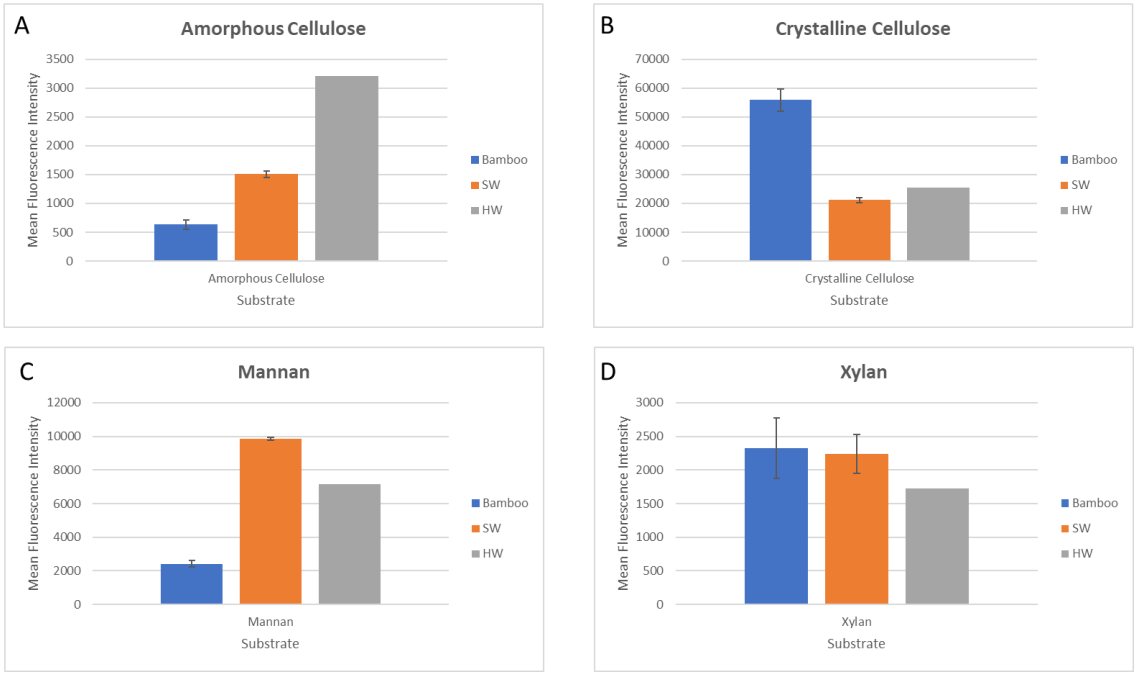

Figure 2: Comparison of four surface substrates using fiber surface characterization. A) Amorphous cellulose, B) Crystalline cellulose, C) Mannan, and D) Xylan. Values provided represent the mean of at least three samples.

To further illustrate the potential of the fiber surface characterization method, representative softwood ("SW") and hardwood ("HW") samples were treated with either wet strength resin alone ("WSR Only") or with a combination of wet strength resin and enzyme product ("Wet Strength + Enzyme") and compared to untreated fiber (Figure 3). In comparing the levels of amorphous cellulose in the samples, the data showed that levels increased with the addition of wet strength resin, but that a further increase was achieved in the presence of enzyme. The crystalline cellulose signal was seen to decrease between the control and the two treatment groups, with the lowest signal being observed in the enzyme treatment group. Similar levels of decrease were observed in the levels of mannan and xylan for the WSR Only and WSR + Enzyme groups. The data is interesting because it showed a change in substrate prevalence even when a non-enzymatic treatment was added. There will be further study made in this area to understand the mechanism for this, but as this is a surface area scan, it is possible that the combination of refining and polymer works to expose amorphous cellulose at the expense of some of the hemicelluloses and substrates that are not being measured directly by this method (e.g. lignin). The action of the enzyme is clearly observed in the concurrent increase in amorphous cellulose and decrease in crystalline cellulose. To further understand how these changes in signal relate to the strength characteristics of the sheet, a handsheet assessment of wet tensile strength was executed (Figure 4). The data shows that both treatment groups were able to improve the wet tensile strength significantly versus the untreated control and that the dual treatment of wet strength resin and enzyme could improve strength by 15.2% and 14.3% versus WSR Only treatment for softwood and hardwood furnish, respectively. This result supports the importance of the crystalline:amorphous cellulose ratio (Hebert-Ouellet et al. 2017), as both treatment groups showed a reduction in crystalline signal and an increase in the surface-available amorphous cellulose. Furthermore, the improvement in strength correlated with the trend in ratio between the two treatment groups, supporting that the surface characterization method has predictive power.

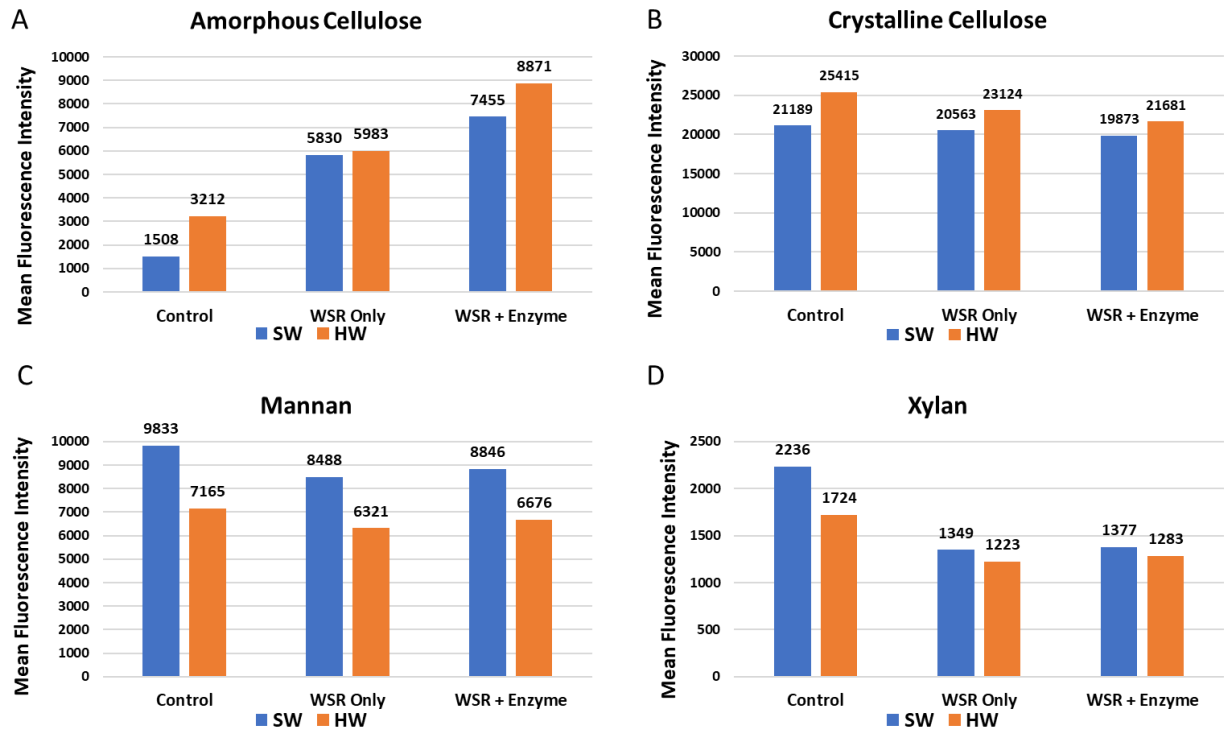

Figure 3: Surface characterization results for the four substrates across three treatment groups on hardwood (HW) and softwood (SW) furnish. A) Amorphous cellulose, B) Crystalline cellulose, C) Mannan, and D) Xylan. Values provided represent the mean of at least three samples.

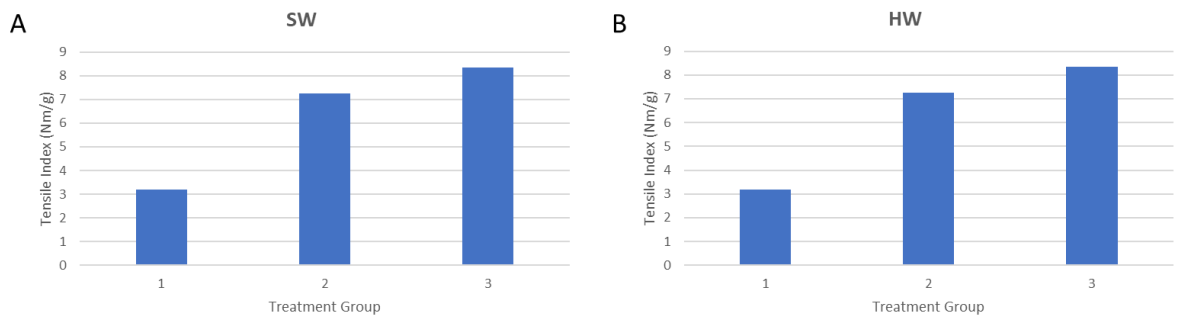

Figure 4: Summary of wet tensile data for furnish following treatment with either wet strength resin alone (WSR Only) or wet strength resin with a selected enzyme product (WSR +Enzyme). A) Softwood (SW), B) Hardwood (HW). Values provided are the mean of at least seven measurements.

By utilizing the fiber surface characterization method in the selection of enzymatic products, it becomes possible to select the ratio of enzyme activities to best match the ratio of substrates. In one practical case study example, the substrate ratio was determined from a furnish sample (data not shown) and six enzymatic formulations rapidly prototyped to provide a range of activities against the available substrates and taking into consideration the operating conditions present (temperature, pH, contact time, etc.). In matching the activity profile to the substrate profile of the fiber itself, it was possible to shortlist two products that were predicted to have the best impact on the final process in a short period of time. In running the selected product on a pilot asset, the results validated that the products could maintain strength targets while reducing energy use by 21-28% without impacting drainage or fiber length versus the untreated control (Table 1). In combining knowledge of the fiber surface with the activities of the enzymes under specific operating conditions, it now becomes possible to rapidly identify enzymatic technologies that deliver the specific benefit required (Figure 5).

		Treatment		
		Control	**Enzyme 1**	**Enzyme 2**
Refining Energy	% Improvement	-	28.1	22.3
Drainage	CSF (mL)	571	580	590
	vs. control		+9	+19
Fiber Length	mm, weighted	2.18	2.13	2.19

Table 1: Summary of results from a pilot trial utilizing two enzymatic technologies shortlisted using surface characterization.

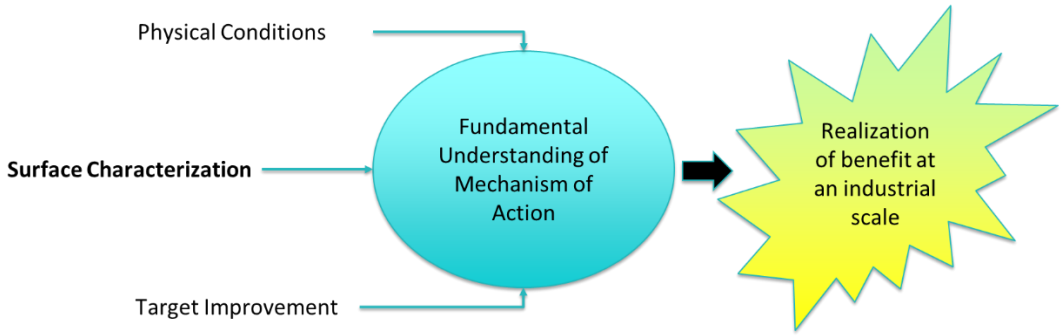

Figure 5: Overview showing how the combination of substrate, operating conditions, target benefit and enzyme can yield beneficial results.

In understanding the composition of the fiber surface with respect to substrate, the technique described herein provides a unique opportunity to understand the fiber at a level that is relevant to the action of enzymes. The data reinforces that fiber surface characterization is both a useful diagnostic tool to assist in understanding the impact of process change and enzyme selection but can also be used predictively in selecting new enzyme technologies for given applications.

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This work utilizes probes and methods based upon a previous collaborative project with Marc Beauregard, Yannick Hébert-Ouellet, Fatma Meddeb-Mouelhi, Vinay Khatri and Cui Li at the University of Quebec at Trois-Rivières.

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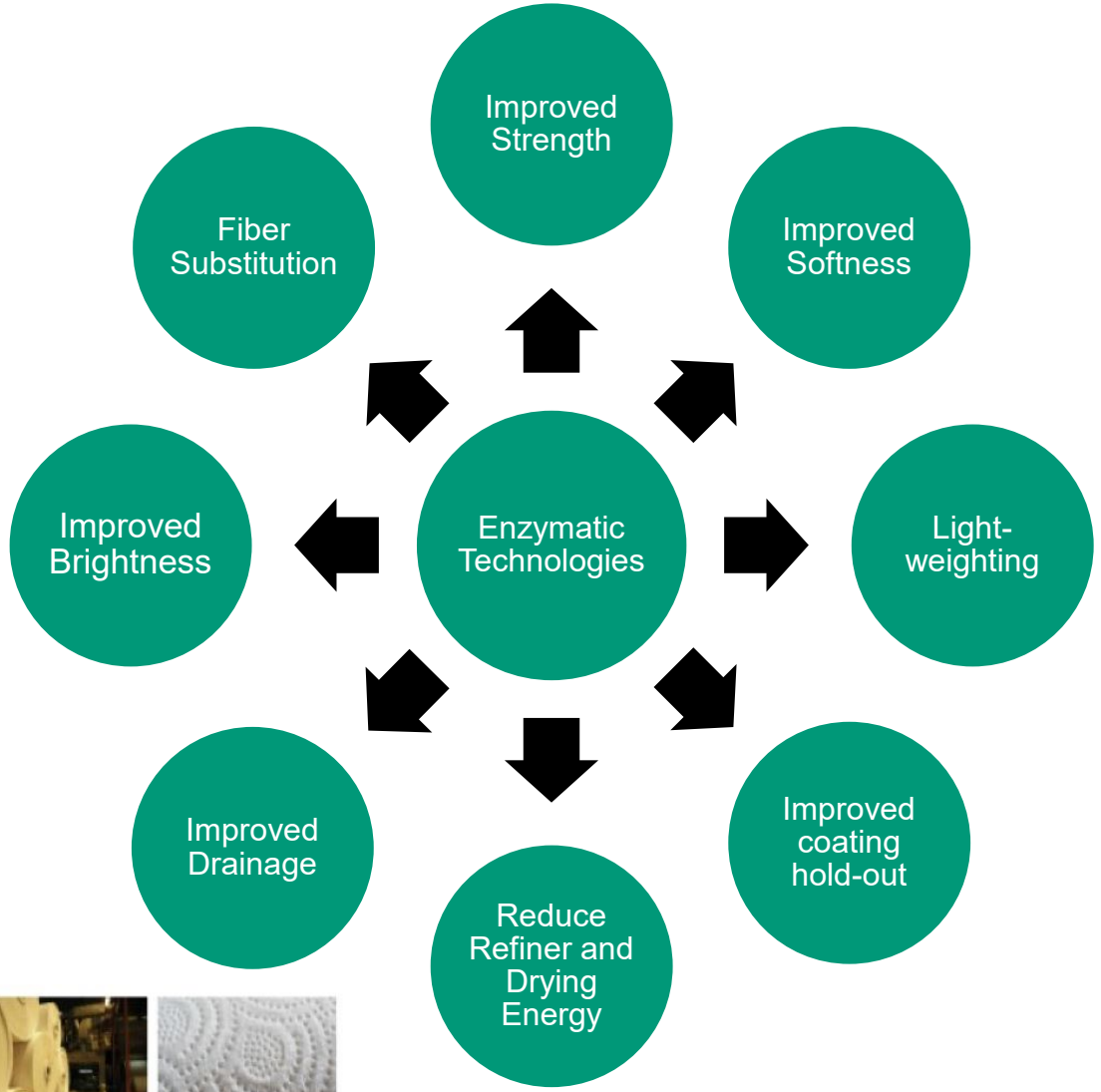
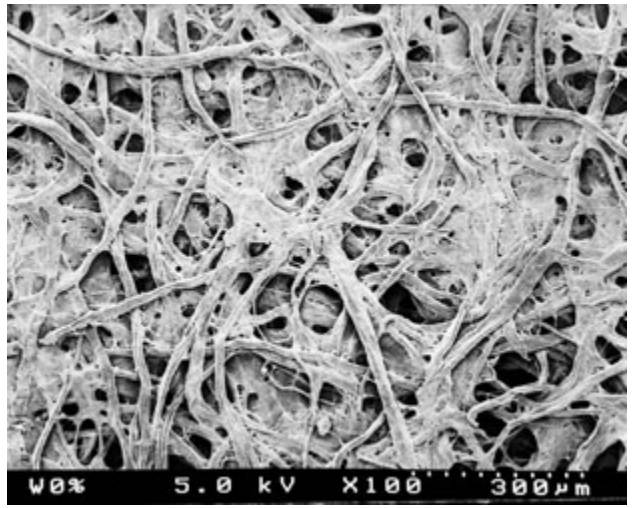
- Buckman
  - Brandico Barr
  - Emily Weatherman
  - Bernard Janse
  - Kevin MacDonald
  - Daniel Glover

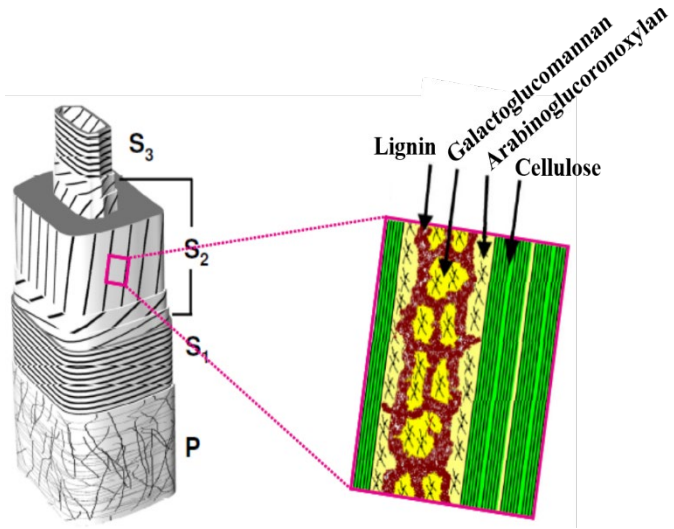
Benefits of Enzymatic Technologies



Understanding the Substrate

- The end properties of a sheet are related to several factors
  - Furnish
  - Mechanical treatment
  - Chemical/enzymatic treatment
- We are effectively sculpting from a finite structure to maximize physical characteristics
- Understanding the impact of factors on the “sculpted” surface is critically important





Diagnostic Methods

- Compositional Analysis
  - X-ray photoelectron spectroscopy (XPS)
  - Fourier-transform infrared spectroscopy (FTIR)
- Surface Imaging
  - Atomic force microscopy (AFM)
  - Scanning electron microscopy (SEM)
  - Transmission electron microscopy (TEM)
- Index of crystallinity
  - X-ray diffraction (XRD)
  - Nuclear magnetic resonance spectroscopy (NMR)

Limitations of Current Methods

- Low-throughput
- Time-consuming
- Resolution of different biopolymers
  - Surface versus total
  - Amorphous versus crystalline
- Require access to specialized equipment/expertise
- Low portability

The Challenge

Utilize a method that can rapidly and reproducibly characterize the surface of the fiber to address key questions:

1. Can we see differences in furnish?
2. How do chemistries impact the fiber surface?
3. Can the product be used to optimize enzymatic selection?

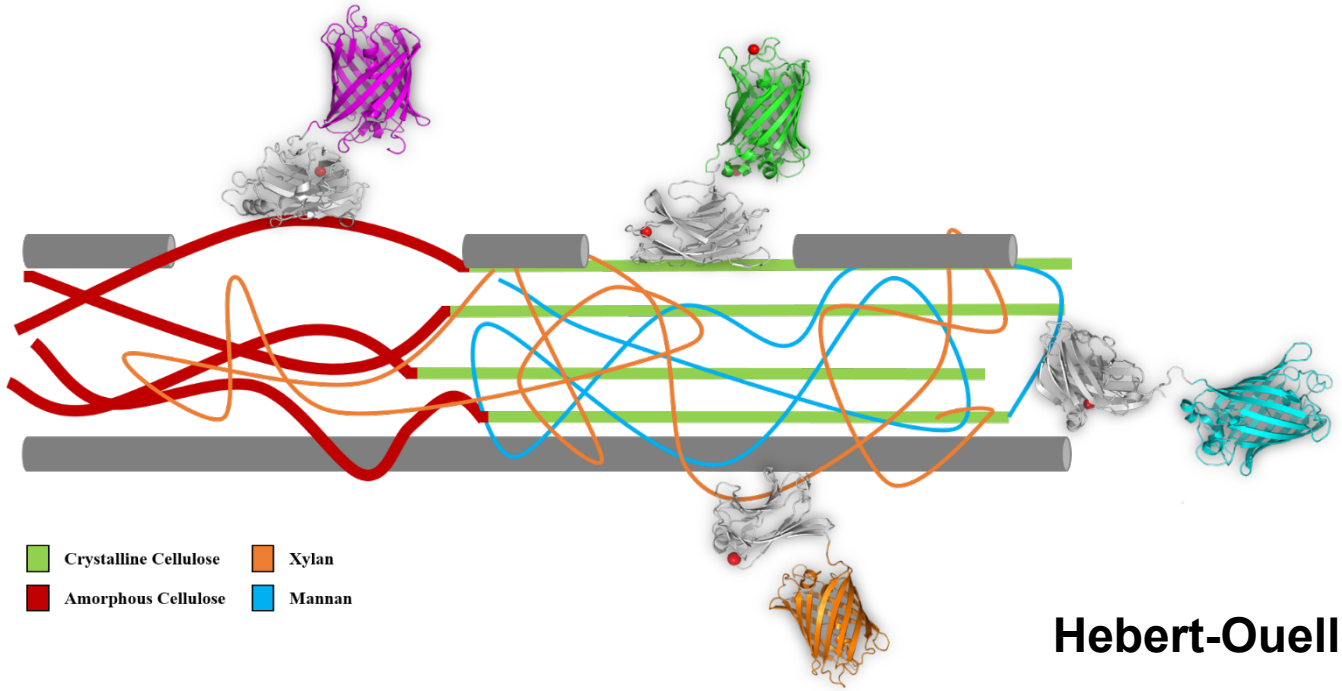


The Solution

- Carbohydrate-Binding Motif (CBM)
- Fluorescence-conjugation
- High-throughput
- Patented technology

Khatri et al. (2016) Specific tracking of xylan using fluorescent-tagged carbohydrate-binding module as molecular probe

Multiplex Test Potential

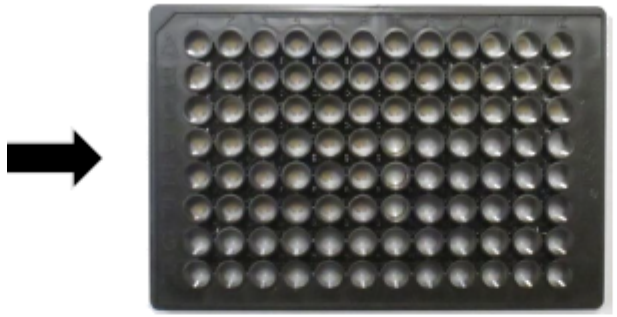


Hebert-Ouellet *et al. 2017*

- Different fluorophores with distinct emission spectra
- Parallel assessment of binding
- Resolve differences in the amount of each substrate

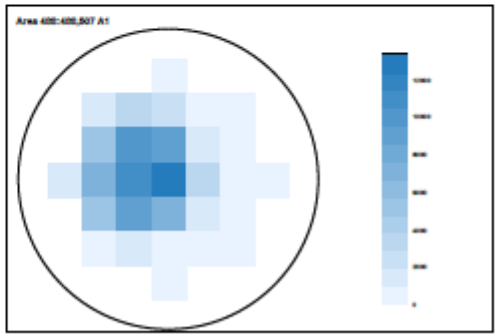
Probe Assay Format

- Crystalline cellulose probe
- Amorphous cellulose probe
- Xylan probe
- Mannan probe



Washing (Tween 20) remove the non specific binding

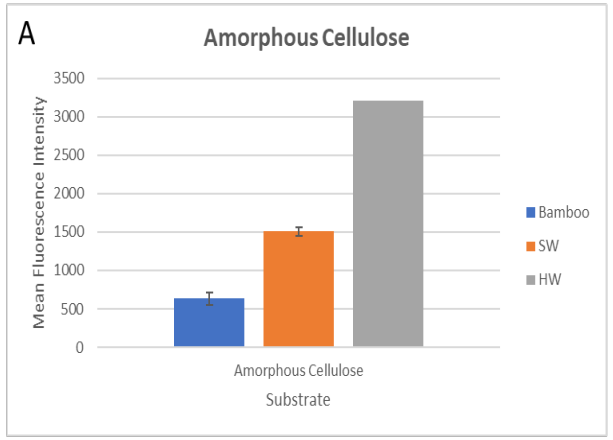
Area scanning

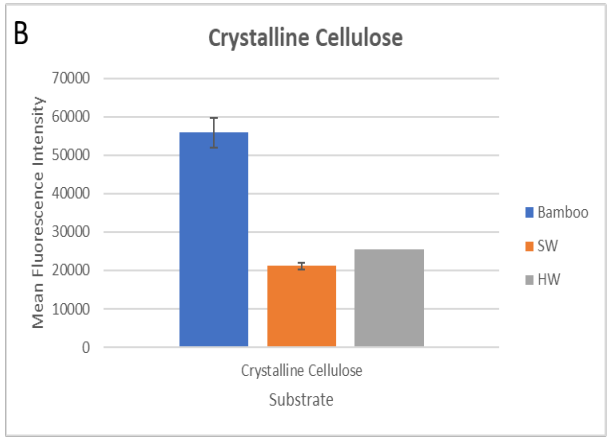


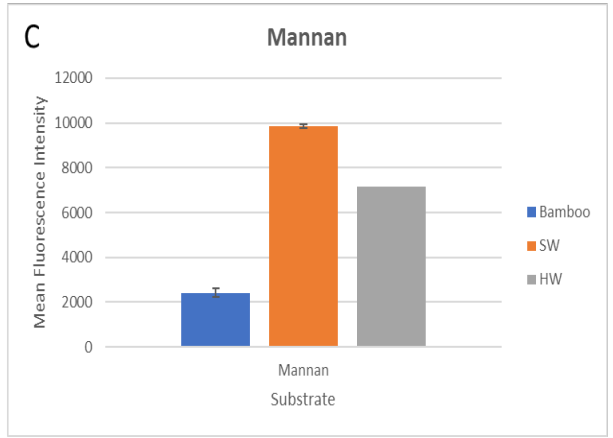
Total Duration: ~4 hours including sample blocking, washing & analysis

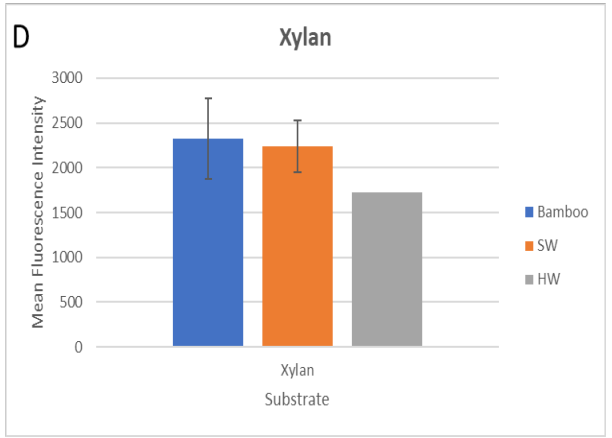
1. Differences in Furnish

- Comparison of bleached SW, HW and Bamboo fiber
- Differences can be resolved
- Bamboo has high crystalline content and low amorphous relative to more traditional fiber types



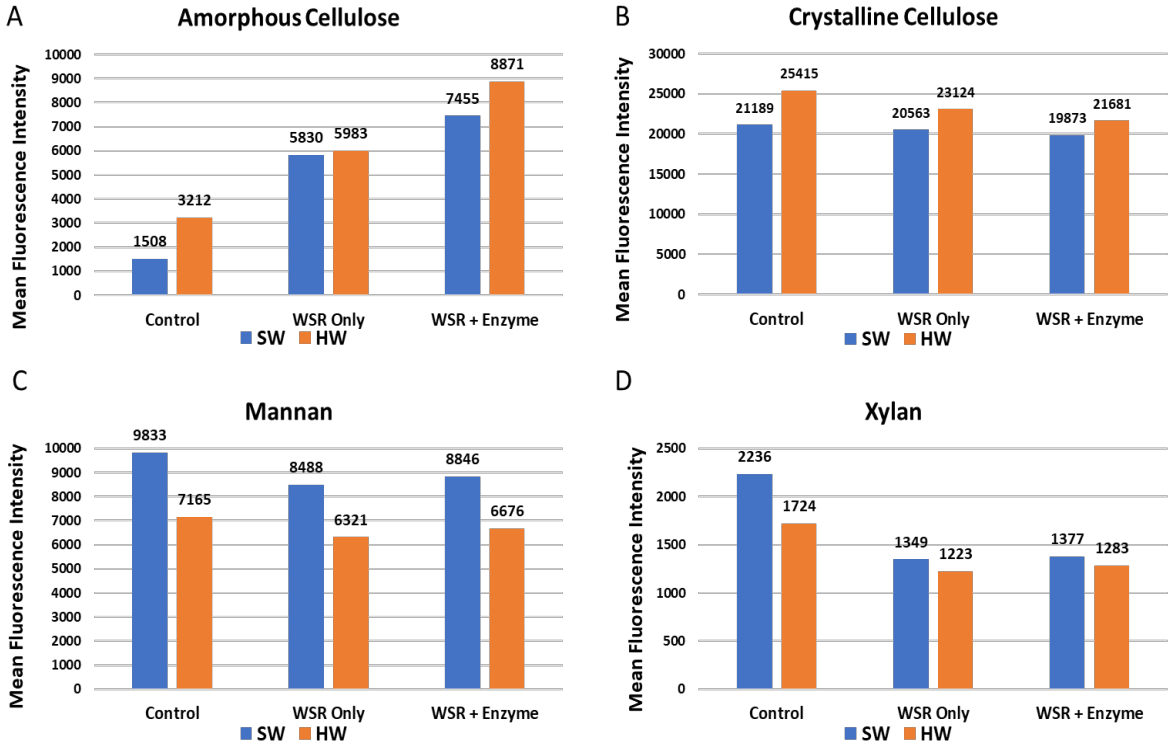






Comparison of four surface substrates using fiber surface characterization. A) Amorphous cellulose, B) Crystalline cellulose, C) Mannan, and D) Xylan. Values provided represent the mean of at least three samples.

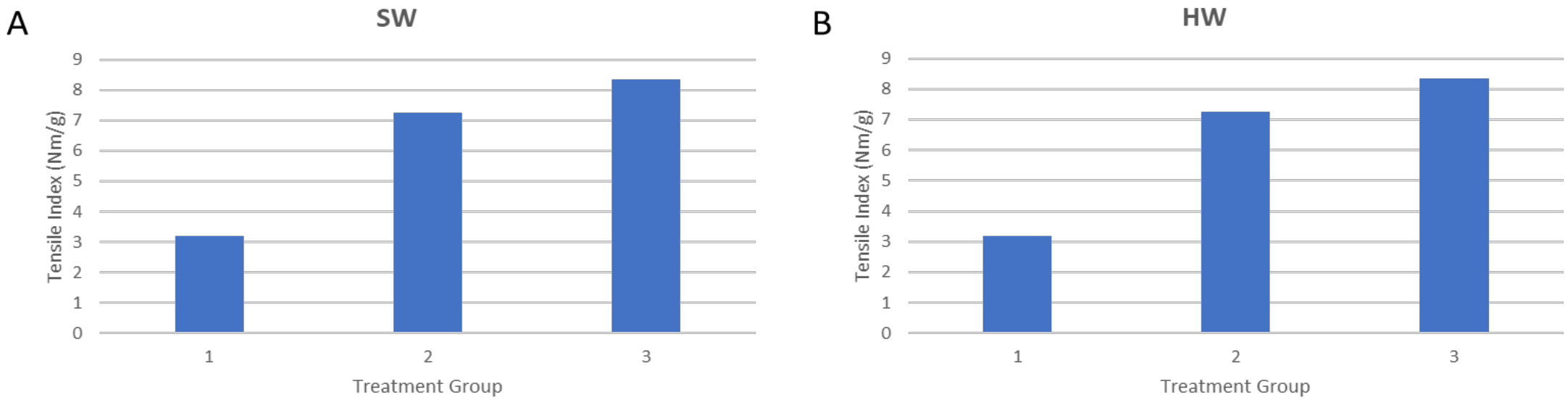
2. Impact of Chemistry



Surface characterization results for the four substrates across three treatment groups on hardwood (HW) and softwood (SW) furnish. A) Amorphous cellulose, B) Crystalline cellulose, C) Mannan, and D) Xylan. Values provided represent the mean of at least three samples.

- Substrate prevalence was impacted differently
- Non-enzymatic treatment caused shifts e.g. amorphous
- Cellulase action was specific to cellulose measures
  - Little impact on hemicellulose

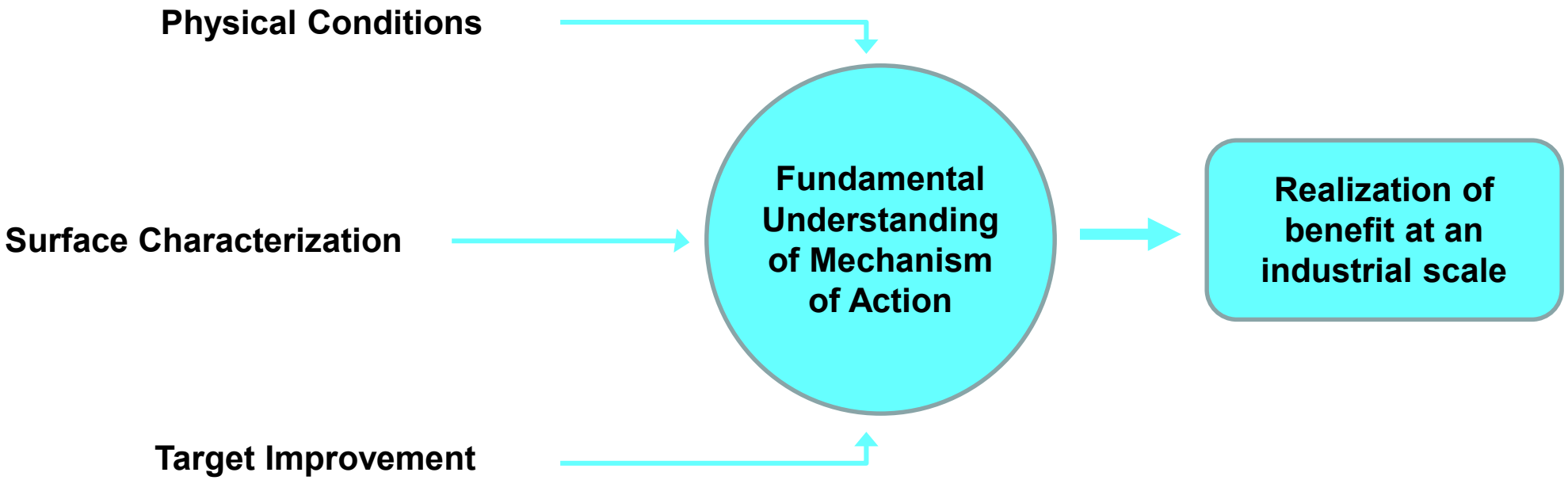
2. Impact of Chemistry



Summary of wet tensile data for furnish. Treatment Groups: 1) Untreated Control, 2) 4kg/t wet strength resin, 3) 1kg/t Enzyme + 4kg/t wet strength resin. A) Softwood (SW), B) Hardwood (HW). Values provided are the mean of at least seven measurements.

- Surface characterization changes correlate to physical properties
- Impact of products can be predicted
  - Amorphous: Crystalline relationship appears important (Hebert-Ouellet *et al. 2017)*

3. Enzymatic Technology Selection



Case Study: Enzymatic Technology Selection

		Treatment		
		Control	Enzyme 1	Enzyme 2
Refining Energy	% Improvement	-	28.1	22.3
Drainage	CSF (mL)	571	580	590
	vs. control		+9	+19
Fiber Length	mm, weighted	2.18	2.13	2.19

- Goal: Reduce energy while maintaining strength, drainage and fiber length
- Completed surface characterization and rapid prototyped six formulations
- Based upon lab testing selected 2 shortlist formulations
- Demonstrated >20% energy savings

Conclusion

- Fiber surface characterization represents a rapid means to baseline substrate properties
- Impact of physical and chemical process changes is observable and predictive
- Opportunities exist to optimize the application of enzymatic technologies based upon the desired goal

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Thank you

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