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Identification of Features Associated with Plant Cell Wall Recalcitrance to Pretreatment by Alkaline Hydrogen Peroxide in Diverse Bioenergy Feedstocks Using Glycome Profiling

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ABSTRACT

A woody dicot (hybrid poplar), an herbaceous dicot (goldenrod), and a graminaceous monocot (corn stover) were subjected to alkaline hydrogen peroxide (AHP) pretreatment and subsequent enzymatic hydrolysis in order to assess how taxonomically and structurally diverse biomass feedstocks respond to a mild alkaline oxidative pretreatment and how differing features of the cell wall matrix contribute to its recalcitrance. Using glycome profiling, we determined changes in the extractability of non-cellulosic glycans following pretreatment and screened a panel of 155 cell wall glycan-specific monoclonal antibodies against these extracts to determine differences in the abundance and distribution of non-cellulosic glycan epitopes in these extracts and assess pretreatment-induced changes in the structural integrity of the cell wall. Two taxonomically-dependent outcomes of pretreatment were identified that both improved the subsequent enzymatic hydrolysis yields but differed in their impacts on cell wall structural

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32 integrity. Specifically, it was revealed that the goldenrod exhibited decreases in all classes of
33 alkali-extractable glycans indicating their solubilization during pretreatment, which was
34 accompanied by an improvement in the subsequent extractability of the remaining cell wall
35 glycans. The corn stover did not show the same decreases in glycan abundance in extracts
36 following pretreatment, but rather mild increases in all classes of cell wall glycans, indicating
37 overall weaker associations between cell wall polymers and improved extractability. The hybrid
38 poplar was relatively unaffected by pretreatment in terms of composition, enzymatic hydrolysis,
39 and the extractability of cell wall glycans due presumably to its higher lignin content and denser
40 vascular structure.
41 Keywords: glycome profiling, cell wall recalcitrance, bioenergy, AHP pretreatment, plant cell
42 walls
43

44 **BROAD IMPACTS AND HIGHLIGHTS**

45 The cell wall matrix of plants provides an effective barrier to the chemical and/or
46 biological deconstruction to its monosaccharide components for use in biofuels applications.
47 The properties of the cell wall matrix impacting its recalcitrance set by many features of cell wall
48 macromolecules across a wide range of length scales. Changes in the physical and chemical
49 properties of cell wall polymers and the overall cell wall environment are important outcomes of
50 pretreatments. Quantitatively identifying how properties of structurally and taxonomically
51 diverse cell walls are impacted by pretreatments and how this correlates to reduced recalcitrance
52 is an important goal with implications for both designing plants with properties suited for
53 deconstruction[1, 2] and designing effective deconstruction strategies. This work applied glycome
54 profiling to identify how a mild alkaline oxidative pretreatment impacts the composition and
55 structural organization of the cell wall and identified two distinct mechanisms by which this
56 pretreatment overcomes cell wall recalcitrance in either herbaceous dicots or graminaceous
57 monocots. These results highlight both the taxonomic differences in cell wall organization and
58 the differences in their response to pretreatment.
59

60 **INTRODUCTION**

61 The lignified cell walls of vascular plants are the result of nearly half a billion years of
62 evolution to resist biological degradation while serving the structural and physiological needs of

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63 the plant.[3, 4] It is believed that the majority of terrestrial carbon in the biosphere is sequestered
64 within plant cell walls,[5] yet this vast resource of reduced carbon is used primarily by humans for
65 its existing structural value (as fiber/paper and as a structural/building material), as a fuel for
66 combustion, or as ruminant forage rather than for the value contained in its existing chemical
67 constituents. This is due to the recalcitrance of the cell wall to deconstruction by chemical and
68 biological treatments and is set by features that are both structural and chemical that cut across
69 the length scales at the molecular, macromolecular, and cellular levels.[6, 7]
70 The plant cell wall matrix is a complex network of cellulose and other matrix
71 polysaccharides including hemicelluloses and pectin, lignin, and structural proteins and presents
72 an obvious barrier to water and cellulolytic enzyme penetration, in particular due to lignin's
73 capacity to set cell wall hydrophobicity and porosity. However, underpinning this lignin barrier
74 is a network of non-cellulose, cell wall matrix polysaccharides providing structure and
75 organization. The major classes of matrix polysaccharides include the xylans (GAXs and GXs),
76 glucomannans (GMs), xyloglucans (XyGs), mixed-linkage glucans (MLGs), pectins, and cell
77 wall protein glycosylations.[8-10] The abundance, composition, and substitution patterns of these
78 glycans vary temporally during plant growth and cell wall expansion, spatially within cell walls
79 and between plant tissues, and taxonomically across diversity plant. In dicots, XyGs and GAXs
80 are the predominant non-cellulosic glycans in the primary cell walls with GXs the predominant
81 hemicellulose in dicot the secondary cell walls.[11] In grasses, GAXs have been found to occupy a
82 significant fraction of the interstitial space between cellulose microfibrils in the primary cell
83 walls in addition to mixed-linkage MLGs and grass-specific XyGs and GMs.[2, 12] Pectic
84 polysaccharides are complex and can comprise up to 30% of the primary cell walls of dicots and
85 significantly less in grasses[13] where some of this function is thought to be performed by other
86 glycans including MLGs and GAXs.[2] Structural proteins may comprise up to 10% of the cell
87 wall in some plant tissues and can be significantly glycosylated, for example with
88 arabinogalactans (AGs).[8]
89 Matrix polysaccharide content, diversity, interactions, and distribution play a role in
90 recalcitrance by setting the accessibility of cellulose to celluloytic enzymes and defining the
91 porosity of the cell wall.[13-15] This network of macromolecules is built up from a combination of
92 physical entanglement of structural polymers as well as non-covalent and covalent cross-links
93 between macromolecules.[16] These non-covalent interactions are important and form the

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94 principle mechanism of association between cell wall glycans.[15] Taken together, this complex
95 composite structure presents a challenge for characterization and a number of approaches have
96 been developed recently with a focus on relating structural features of the cell wall to its
97 recalcitrance as reviewed recently by Foston et al.[17]

98 Immunological methods using glycan directed mAbs are widely used tools to investigate
99 plant cell wall structure.[18, 19] Besides the cell wall composition and structure, mAbs can be used
100 for qualitative and quantitative detection of carbohydrate epitopes in plant sequential extracts.
101 This has been performed in order to characterize pretreatments using one approach that plotted
102 polysaccharides from three increasingly severe cell wall extracts (CDTA, NaOH, cadoxen) onto
103 a microarray, which was then probed with mAbs and CBMs[20] in order to identify changes in
104 content and extractability of xylans, XyGs, and MLGs epitopes in hydrothermal pretreated wheat
105 straw.[21] Recently, Pattathil *et al*. assembled a collection of glycan-specific antibodies[22] shown in
106 Supplemental Table S1 and an ELISA-based screen was used to categorize these mAbs with
107 respect to binding affinity for structurally diverse plant cell wall glycans.[22, 23] This technique has
108 been applied to evaluate the structural, accessibility, and extractability changes of cell wall
109 glycans in hybrid poplar during dilute acid pretreatments[24] and to compare differences in the
110 structural features underpinning cellulose digestibility in switchgrass and hybrid poplar.[25]

111 Chemical pretreatments are one route to overcoming cell wall recalcitrance[26] and can be
112 coupled to an enzymatic depolymerization of cell wall polysaccharides whereby the sugars
113 monomers generated can be subsequently biologically converted to fuels and chemicals,
114 providing a path forward for developing a bio-based fuels and chemicals industry that is
115 renewable and petroleum-displacing. Ultimately this recalcitrance to cell wall deconstruction
116 lies in the challenge for cellulolytic enzyme infiltration into the cell wall which can be seen as a
117 combination of cell wall porosity, hydrophobicity (or water penetration/swelling), and glycan
118 accessibility. Alkaline hydrogen peroxide (AHP) pretreatment is comparable to alkaline
119 hydrogen peroxide pulp bleaching stages in the paper industry with the difference that higher pH
120 and higher hydrogen peroxide loadings are employed to affect a mild delignification rather than
121 brightening as the outcome.[27, 28] AHP pretreatment has several potential advantages compared to
122 other pretreatment processes including minimal loss of polysaccharides,[29] operation at low
123 temperature and pressure, and minimal formation of inhibitors for fermentation,[30] and potentially
124 high enzymatic digestibilities in grasses. In particular, we have recently been able to achieve

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125 ethanol titers greater than 50 g/L from undetoxified hydrolysates of 12.5% (w/w) H_2O_2 loading
126 AHP-pretreated corn stover and switchgrass including complete glucose and xylose utilization
127 using *Saccharomyces cerevisiae* strains metabolically engineered and evolved for xylose
128 fermentation.[30]

129 It has been relatively well-established that hydrothermal pretreatments such as dilute
130 acid[31] or liquid hot water[32] overcome recalcitrance through thermal effects by melting and
131 redistributing lignin and catalyzing xylan hydrolysis and solubilization. This effect on xylan
132 removal has been validated using similar characterization approaches for quantifying glycan
133 extractability and epitope abundance.[21, 24] As AHP pretreatment is mechanistically different from
134 acidic hydrothermal pretreatments and targets lignin solubilization at low temperature while
135 preserving carbohydrates considerably, glycome profiling should be able to provide new insight
136 into matrix polysaccharide-specific contributions to cell wall recalcitrance.

137 Specifically, in this study we investigate the response of cell wall glycans of diverse
138 plants including hybrid poplar (woody dicot), goldenrod (herbaceous dicot), and corn stover
139 (monocot grass) to AHP pretreatment with increasing H_2O_2 loadings. The cell wall response to
140 pretreatment was characterized by compositional changes and overall mass loss of the cell wall,
141 glucan and xylan enzymatic yields using a commercial enzyme cocktail, and glycome profiling
142 of the sequential glycan extracts of the untreated and AHP-pretreated cell walls at 12.5% (w/w)
143 H_2O_2 loading on biomass. Using this information, we draw conclusions about the structural
144 changes associated with AHP pretreatment and additionally are able to gain insights into the role
145 that differences in plant cell wall architecture have on cell wall recalcitrance.
146

147 **EXPERIMENTAL**

148 **1. Pretreatment**

149 Biomass consisted of a commercial hybrid corn stover (Pioneer Hi-Bred 36H56)
150 provided through the Great Lakes Bioenergy Research Center (GLBRC), debarked hybrid poplar
151 (*Populus nigra* var. *charkoviensis* x *caudina* cv. NE-19) grown at the University of Wisconsin
152 Arlington Agricultural Research Station and provided through the GLBRC, and goldenrod
153 (*Solidago canadensis*) collected locally in East Lansing, MI and obtained from Dr. Jonathan
154 Walton (MSU, Plant Biology). Biomass was initially milled with a Wiley MiniMill (Thomas
155 Scientific) to pass a 2 mm screen and air-dried to ~5% moisture. The milled biomass was

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156 subjected to alkaline hydrogen peroxide (AHP) pretreatment using H_2O_2 loadings of either
157 12.5%, 25%, 50% (g H_2O_2 per g biomass). These were performed in shake flasks with a 100 g
158 total mass and 2% (w/v) solids concentration for 24 h at 30°C with orbital shaking at 170 rpm
159 and periodic pH adjustment to 11.5. After pretreatments, the liquid in the samples were removed
160 by vacuum filtration (#113 Whatman filter paper) and the slurry was washed several times with
161 deionized water to remove solubles and air-dried at room temperature. The mass yield during
162 pretreatment was quantified as the ratio between the mass (dry basis) after pretreatment and the
163 initial mass before pretreatment.
164

165 **2. Composition analysis, enzymatic hydrolysis and digestibility determination**

166 The pretreated biomass was subjected to a two-stage acid hydrolysis according to NREL
167 composition analysis[33] to determine neutral polysaccharide content, Klason lignin, and ash with
168 the difference that Aminex HPX-87H (Bio-Rad, Hercules, CA) column was used to quantify the
169 glucan, xylan+galactan+mannan, arabinan, as well as acetate content. The total uronic acids were
170 assayed enzymatically (K-Uronic, Megazyme, Wicklow, Ireland). The extractives content was
171 determined by a sequential 3-step extraction including 70% ethanol, followed by 1:1 (v/v)
172 methanol and chloroform mixture, then acetone. Three extraction cycles for each solvent were
173 performed and followed by centrifugation at 10500 x g for 10 minutes for each cycle. Before the
174 enzymatic hydrolysis, the pretreated biomass was ball-milled for 3 cycles using a QIAGEN
175 TissueLyser II equipped with 25 mL Teflon jars and 20 mm diameter Teflon balls at 30 Hz for 2
176 minutes with liquid nitrogen cooling. The ball-milled samples were incubated with Cellic CTec2
177 (Novozymes, Bagsværd, Denmark) at a loading of 30 mg protein/g glucan at 50°C, 10% solid
178 loading and 5 mL total volume in 0.05 M Na-citrate buffer pH 4.8, for 24 hours or 72 hours. The
179 glucan and xylan yields (based on only glucan and xylan remaining after pretreatment) were
180 determined by the HPLC analyzable glucose and xylose concentrations after hydrolysis divided
181 by the original glucan and xylan contents in the pretreated samples.
182

183 **3. Sequential extraction, glycome profiling**

184 Sequential cell wall extractions and glycome profiling were carried out as described
185 previously.[22, 24, 34] The six extractions included (in order of extraction) oxalate to remove "loose"
186 pectins, carbonate to remove "more tightly" bound pectins, 1M KOH to remove "loose"

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187 hemicelluloses along with tightly bound pectins, 4M KOH to remove "tightly" bound
188 hemicelluloses along with tightly bound pectins, acid chlorite to oxidize and solubilize lignin and
189 release lignin-embedded hemicelluloses, and a 4M KOH post-chlorite treatment to remove
190 additional lignin-bound polysaccharides. Plant glycan-directed monoclonal antibodies were from
191 laboratory stocks (CCRC, JIM and MAC series) at the Complex Carbohydrate Research Center
192 (available through CarboSource Services; http://www.carbosource.net) or were obtained from
193 BioSupplies (Australia) (BG1, LAMP). A description of the mAbs used in this study can be
194 found in the Supporting Information, Table S1, which includes links to a web database,
195 WallMAbDB (http://www.wallmabdb.net) that provides detailed information about each
196 antibody.
197

198 **RESULTS AND DISCUSSION**

199 **1. Changes in composition and mass loss**

200 Three types of biomass representing three classes of plants that may offer promise as
201 feedstocks for cellulosic biofuels were tested in this study. Corn stover represents the
202 agricultural residue with the highest production and availability for bioenergy applications in the
203 U.S.,[35] while short-rotation hybrid poplar has agronomic, logistical, and environmental
204 advantages as a feedstock.[36] "Low-input high-diversity" bioenergy landscapes have many
205 attractive sustainability attributes[37] and comprise mixed communities of plants on marginal or
206 degraded lands, and in this study we use goldenrod (*Solidago canadensis*) as a representative
207 herbaceous dicot that may be present in these landscapes. The composition of the untreated
208 biomass is presented in Table 1. Notable differences include the low content of pectic
209 polysaccharides (as uronic acids) in the corn stover, which is 5-fold lower than the goldenrod and
210 2-fold lower than the hybrid poplar. The goldenrod has a substantially higher extractives content
211 (23.5%) relative to the other two biomass types. Additionally, lignin content of the corn stover is
212 nearly half that of the goldenrod and poplar.

213 AHP pretreatment was performed at increasing H_2O_2 loadings (12.5, 25, and 50% w/w on
214 biomass) which would be significantly higher than would be economically practicable
215 industrially. The reason for these high loadings was to compare and analyze how the cell walls
216 from phylo-genetically diverse differ in their susceptibility to low temperature mild oxidative
217 delignification and hemicellulose extraction and as a screen for overall differences in enzymatic

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218 hydrolyzability. The total cell wall mass loss (excluding extractives), xylan loss, and lignin loss
219 for the three biomass types following pretreatment with increasing H_2O_2 loadings show distinct
220 responses between the biomass types (Figure 1). For poplar, minimal material was solubilized
221 with pretreatment (<1% by mass), while up to 20% and 25% of the mass the cell walls of the
222 goldenrod and corn stover, respectively, was solubilized by pretreatment at the higher H_2O_2
223 loadings. The mass of corn stover decreased continuously with increasing H_2O_2 loading, while
224 the sample mass of poplar and goldenrod decreased abruptly with the mildest treatment (12.5%
225 H_2O_2). For individual cell wall components, AHP pretreatments resulted in minimal changes in
226 glucan for all biomass types representing preservation of cellulose (data not shown) which is
227 consistent with our previous findings.[27, 29] while the xylan content decreased only for the corn
228 stover (Figure 1B). For goldenrod, the Klason lignin content was only slightly reduced by AHP
229 pretreatment and did not change significantly by increasing H_2O_2 loading. The simultaneous
230 removal of xylan and Klason lignin in corn stover was increased by increasing H_2O_2 loading.
231 This is a well-known property of grass cell walls and is due to the higher solubility of grass
232 lignins[38] and alkali-only extraction of lignin and xylans in grasses is known to be significantly
233 higher in grasses than in woody dicots.[25, 39] Besides alkali solubility, cleavage of ester and ether
234 cross-links between xylan and lignin or lignin and lignin mediated by ferulate[12, 40] in grasses are
235 thought to be an important target of AHP pretreatment[29] and likely contribute to these outcomes.
236
237 **2. Enzymatic hydrolysis yields of poplar, goldenrod, and corn stover**
238 Figure 2 shows the enzymatic hydrolysis yields of glucose for poplar, goldenrod, and
239 corn stover subjected to increasing H_2O_2 loadings for 24 and 72 hr hydrolysis times using a
240 commercial cellulase (Cellic CTec2) with no xylanase supplementation. These results represent
241 screening only and were not focused on optimizing enzyme cocktail or loading. As such,
242 improved glucose (and xylose) yields at lower enzyme loadings would be observed if xylanase
243 and pectinase were used. These results show that the glucose yields were increased with
244 increasing H_2O_2 loading for the three types of biomass. Similar to the results for compositional
245 changes (Figure 1), the yield changes with pretreatment are significantly different between the
246 three classes of plants tested. For poplar, the glucose yields were significantly lower than
247 goldenrod and corn stover with the highest glucose yields approaching 40%. For goldenrod, the
248 glucose yields "saturate" at approximately 70% at the mildest pretreatment condition. For corn

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249 stover, the glucose yield approaches 100% with increased H_2O_2 loading and it is known that corn
250 stover as well as many other cereal stovers are often considerably more digestible than many
251 undomesticated grasses.[29, 40] Gould[41] determined that diverse graminaceous monocots responded
252 considerably better to AHP pretreatment at 100% (w/w) H_2O_2 loadings than herbaceous
253 dicots/forbs (including goldenrod) and that the 7 grasses tested had on average more than double
254 the improvement in digestibility of the 11 forbs tested following AHP pretreatment, although the
255 goldenrod showed the largest improvement in digestibility of the dicots tested and was among
256 the highest in terms of absolute glucose release per gram of biomass.
257

258 **3. Glycome profiling of poplar, goldenrod, and corn stover**

259 Glycome profiling (GP) provides quantitative information on both how pretreatment
260 impacts the strength of association between cell wall glycans and other cell wall matrix polymers
261 and how pretreatment impacts cell wall glycan composition. For GP, the samples were subjected
262 to increasingly severe extractions to sequentially remove the cell wall polymers, followed by
263 quantification of recovered materials in each extraction step, and probing of the binding strength
264 for the diverse array of antibodies covering a range of non-cellulose cell wall polysaccharide
265 epitopes.[22, 24, 34] The categories of glycan-specific mAbs were determined previously based on
266 hierarchical clustering of the antibodies against a panel of 54 known plant polysaccharides,[22]
267 with complete data on the binding specificity and cross-reactivity for each mAb on the web
268 database, WallMAbDB (http://www.wallmabdb.net). It should be noted that the GP approach
269 does not yield information on small glycan molecules (*e.g.* oligomeric glycans and
270 monosaccharides) as only larger cell wall glycans are able to effectively adsorb to the ELISA
271 plates.[22] For the sequential extractions, the strength of association between individual glycans
272 and other cell wall matrix polymers may be hypothesized to be mechanistically due to
273 differences in: (1) the strength of non-covalent association or physical entanglement between
274 polymers or "encrustation" within lignin, (2) location within the cell wall (surface vs. interior),
275 and (3) tissue type (*e.g.* lignified parenchyma and sclerenchyma versus low lignin pith tissues).
276 The role of lignin in setting the alkali-extractability of cell wall glycans was recently
277 demonstrated by Pattathil et al.,[42] whereby it was identified that alfalfa lines with disrupted
278 monolignol synthesis resulting in a low-lignin phenotype contained considerably more alkali-
279 extractable glycans than the control line where much more of the cell wall glycans were

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280 extractable only after chlorite delignification.

281 The GP results from this work are presented in Figure 3 for the three biomass categories
282 for either no pretreatment or AHP pretreatment at 12.5% (w/w) H_2O_2 loading on the biomass.
283 Substantial differences can be observed between biomass types and for biomass subjected to
284 pretreatment as quantified for both mass partitioning of extracted glycans (top panel of Figure 3)
285 and differences in the abundance of glycan epitopes in these extracts (heat map in the lower part
286 of Figure 3). For the extract mass partitioning of glycans, it is clear that the two dicots have
287 similar profiles for the four most severe extracts, *i.e.* the 1 M KOH, 4 M KOH, chlorite, and 4 M
288 KOH PC. The goldenrod shows a very high content of the oxalate- and carbonate-extractable
289 polysaccharides relative to the other two types of biomass. This may be a consequence of the
290 goldenrod having a higher proportion of pectic polysaccharide-rich leaves compared to the
291 poplar which consists of only stem heartwood, and the corn stover, which as a graminaceous
292 monocot is known to have low pectic polysaccharide content.[43]

293 A number of noteworthy differences are apparent in comparing the glycan epitope
294 abundances within the six extracts for the three types of untreated biomass. One difference is
295 that the XyG epitopes show significantly different partitioning between the three biomass types
296 and the goldenrod is the only biomass exhibiting abundant XyG epitopes in the 1 M KOH
297 extract. The xylan epitopes are more abundant in the corn stover extracts and more abundantly
298 distributed into the two most severe extracts (chlorite and 4 M KOH PC) that might correspond
299 to "lignin-bound" xylan. Pectic polysaccharides and AG domains show different partitioning
300 behavior between the biomass types as well, with the cell wall extracts from goldenrod
301 exhibiting the most abundant content of these classes of epitopes. Two intense MLG epitopes
302 are present in all the corn stover cell wall extracts corresponding to antibodies LAMP2H12H7
303 and BG1 and the abundance of these epitopes increase in extracts (particularly in the 2 mildest
304 and the chlorite delignification) following AHP pretreatment. Weak epitope binding for both of
305 these antibodies is present in some of the poplar extracts and goldenrod extracts. These
306 observations are consistent with the primary cell wall models proposed by Carpita,[12] where for
307 grasses (Type II cell wall), the MLGs and GXs have a more important structural role and may act
308 in the capacity that XyGs and pectic polysaccharides in dicots (Type I cell wall).

309 Pretreatment can conceivably alter the binding of mAbs to their glycan epitopes from the
310 same extraction condition in three ways by: (1) altering the cell wall structural integrity to shift

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311 the glycan epitope into a more easily (or more difficult) extractable category, (2) solubilizing the
312 glycan epitope during pretreatment, and (3) structurally altering the glycan epitope, for example,
313 through alkali-induced deacetylation or demethylesterification. These three modes of action are
314 used to interpret the changes associated with pretreatment. To better visualize the effects of
315 pretreatment on glycan extractability, the GP data are replotted in Figures 4 and 5 after
316 normalizing to epitope abundance per mass of original cell wall. In this representation, glycan
317 epitopes that are increased in their abundance in individual extracts after pretreatment will
318 appear to the left of the x-y line, while epitopes that are decreased will appear to the right. It can
319 be observed that for the poplar, pretreatment has very little effect on the total extractable glycans
320 in most of the six fractions. The apparent increase in the xylan epitopes in oxalate and carbonate
321 extracts suggest that the extractability of xylan by mild solvents may be enhanced by
322 pretreatment (Figure 4, subplot B). However, considering that the total content of carbohydrates
323 in these extracts are unchanged and that the xylan-specific antibodies were developed for
324 deacetylated, alkali-extracted xylans, this result likely indicates that easily extractable xylans
325 were deacetylated by pretreatment and that the abundance of deacetylated xylan epitopes
326 increase as a consequence. Another possibility is that a small fraction of the total xylan becomes
327 more easily extractable following pretreatment. The slight differences in other epitopes in the 4
328 harshest extracts (Figure 5, subplots A-C) suggests that AHP pretreatment results in only minor
329 alterations in the extractability of other major cell wall glycans indicating minimal impact on the
330 structural and compositional organization of the cell wall in agreement with the results in Figures
331 1 and 2. An exception is the xyloglucan epitopes in the 1M KOH extract (Figure 5A), which are
332 slightly improved in their extractability by pretreatment.

333 For goldenrod, the GP results show that xylan epitopes and XyG epitopes in the oxalate
334 extract increased considerably (Figure 4, subplots D-F); potentially as a consequence of
335 pretreatment-induced deacetylation or by pretreatment increasing the extractability in agreement
336 with the increase in total glycan mass in the oxalate extract with pretreatment (Figure 3). Unlike
337 the poplar, the epitopes for HG backbones, RG-I/AG, and AG are decreased in both the oxalate
338 and carbonate extracts, indicating that these pectic polysaccharides in goldenrod are likely
339 solubilized during AHP pretreatment. In the four most severe extracts, a number of important
340 trends are apparent (Figure 5, subplots D-F). The first is that virtually all epitopes are decreased
341 as a consequence of pretreatment in the 1M KOH extract (corresponding to alkali-soluble

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342 glycans not closely associated with lignin), while slight increases in the abundance of epitopes
343 are observed after pretreatment in the 4M KOH, chlorite and 4M KOH post-chlorite extracts
344 (corresponding to lignin-embedded glycans). These results indicate that the likely target of AHP
345 pretreatment for improving enzymatic hydrolysis in goldenrod is glycan (XyG and xylan)
346 solubilization to improve cell wall accessibility to glycolytic enzymes and minor delignification
347 which slightly improves the extractability of lignin-embedded polysaccharides.
348 The corn stover results show considerable differences in both their glycan extraction plots
349 and antibody binding profiles relative to the hybrid poplar and goldenrod (Figure 3). From the
350 glycan mass extraction profile at the top of the panel, it can be observed that, unlike the
351 goldenrod, the amounts of extractable glycans in the three most severe extracts were significantly
352 altered by pretreatment. One noticeable alteration is that the glycan epitopes in the 4M KOH
353 post-chlorite extract were shifted into the chlorite extracts after AHP pretreatment. This
354 phenomenon of the pretreatment changing the glycan extractability profile was not shown for
355 dilute acid pretreated hardwood.[24] This result supports the identified changes in composition
356 shown in Figure 1, and together with Figure 2, make clear that alteration of the non-cellulosic
357 glycan extractability directly impacts the glucose hydrolysis yield in the subsequent enzymatic
358 treatment. In the two mildest extracts from corn stover, the XyG and xylan epitopes were
359 increased, which could be a consequence of improving extractability or likely due to
360 deacetylation of these glycans by pretreatment (Figure 4, subplots G and H). Unlike goldenrod
361 and poplar, the epitopes for pectic polysaccharides were increased in the two mildest extracts,
362 possibly indicating differences in their structural role between monocots and dicots in pectic
363 polysaccharides.[12] Additionally, the results for changes in epitope abundance as a result of
364 pretreatment for the four harshest extracts were considerably different for the corn stover than for
365 the goldenrod. Compared to goldenrod, where all glycan epitopes were decreased by
366 pretreatment in the 1M KOH extract but slightly increased in the lignin-associated extracts
367 (Figure 5, subplots D-F), the corn stover glycan epitopes showed minimal change or slight
368 increases across all four extracts except in the 4M KOH post-chlorite extract, in which the lignin-
369 embedded glycan epitopes are decreased by pretreatment (Figure 5, subplots G-I). These
370 reductions of corn stover glycans in the harshest extracts are likely a consequence of these
371 epitopes being already removed by less harsh extractions. Increases in the 2 MLG epitopes were
372 observed with pretreatment for corn stover (Figure 3) for all extracts except the 4M KOH post

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373 chlorite treatment.

374 These differing responses to AHP pretreatment between monocots and dicots have
375 important implications for structural features of the cell wall contributing to recalcitrance as well
376 as the mechanism or target of pretreatment. The composition and structure of the cell wall are
377 obviously important and many properties of the cell wall impacting recalcitrance have been
378 described in the literature including cell wall hydrophobicity,[44] porosity,[45, 46] xylan content,[47]
379 lignin content, cross-linking, and higher order structure.[48] Jung et al.[1] noted that lignified
380 secondary cell walls were the primary obstacle hindering ruminant digestibility in dicots with
381 stem secondary xylem (*i.e.* woody biomass) the most recalcitrant, while increasing lignification
382 in grasses hinders, but does not completely inhibit digestion. The current work identified that
383 AHP pretreatment has a relatively minor impact on the hybrid poplar composition, hydrolysis
384 yields, and glycan extractability profiles.

385 Substantial work has been devoted to understanding the cell wall properties contributing
386 to ruminant digestibility of grasses and properties including ferulate content, total lignin content,
387 syringyl:guaicyl ratio of lignin monomers, and degree of arabinosylation of xylans have all been
388 linked to differences in hydrolysis yields.[1, 48-50] Pretreatments may impact any of these afore-
389 mentioned cell wall properties to improve cell wall digestibility. DeMartini et al.[25] found that
390 treatment of switchgrass with alkali alone to solubilize xylan (and lignin) from the cell wall was
391 sufficient to result in glucan enzymatic hydrolysis yields approaching the theoretical maximum,
392 while for hybrid poplar, chlorite delignification was necessary to improve enzymatic hydrolysis
393 significantly past alkali-only treatment. This is consistent with models for grass cell walls that
394 include alkali-labile ferulate ester cross-links between cell wall polymers as an important
395 structural feature controlling lignin integration into cell walls.[40] Our previous work identified
396 that lignin and ferulate removal by AHP pretreatment are important predictors of digestibility in
397 diverse grasses.[29] We have previously shown that AHP pretreatment results in the destruction of
398 β-O-4 bonds in grasses[29] and, for example, the content of free phenolics in grass lignins may
399 enable improved alkali solubilization or potentially participate in the initiation β-O-4 scission
400 reactions.

401 Re-engineering plant cell walls for improved bioconversion outcomes is currently the
402 subject of substantial research interest,[51] and the findings of this work and the literature suggest
403 strategies for tailoring bioenergy feedstock phenotypes to an alkaline-oxidative pretreatment

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404 process. Specifically, low initial lignin content and/or the capacity of the pretreatment to
405 effectively remove lignin are recognized as important contributors to high enzymatic hydrolysis
406 yields. As such, engineered plant phenotypes that would optimally couple to an alkaline-
407 oxidative pretreatment might include decreased lignin content (without impacting plant fitness)
408 and increasing alkali-labile bonds in lignin for example through the introduction of ester cross-
409 links[52] or increasing the β-O-4 content through increasing the S/G ratio.[53]
410

411 **CONCLUSIONS**

412 Untreated and AHP-pretreated biomass from phylogenetically diverse plants were
413 compared to understand fundamental features impacting cell wall recalcitrance. We found that
414 enzymatic hydrolysis yields, cell wall biopolymer and total mass solubilization, cell wall glycan
415 extractabilities, and glycan epitope abundances in these extracts differed significantly in their
416 response to AHP pretreatment for a woody dicot (hybrid poplar), an herbaceous dicot
417 (goldenrod), and an graminaceous monocot (corn stover). Using glycome profiling, we
418 identified different mechanisms for how AHP pretreatment overcomes cell wall recalcitrance in
419 the goldenrod versus the corn stover, while it was relatively ineffective on the poplar. For the
420 corn stover, mild alkaline-oxidative pretreatment resulted in slight delignification and
421 presumably disruption of cell wall polymer cross-linking. This had the consequence of
422 disrupting the structural integrity of the cell wall which was manifested through improved
423 extractability of important structural glycans including xylans, MLGs, and XyGs and presumably
424 allowed for improved accessibility for glycolytic enzymes into the cell wall during hydrolysis.
425 Goldenrod was found to respond differently in the extractability profiles where all classes of
426 glycan epitopes exhibited considerable decreases in the 1M KOH extracts following pretreatment
427 rather than an increase for the case of corn stover. Besides these differences, it was revealed that
428 the pectic polysaccharides (HG, RG-I, and AG) were not only significantly more abundant in the
429 goldenrod than in the corn stover, but were solubilized by pretreatment as indicated by their
430 decrease following pretreatment in the three mildest extracts for goldenrod. For corn stover, the
431 pectic polysaccharides as well as the significantly more abundant MLGs showed mild increases
432 in extractability following pretreatment indicating "loosening" from the cell wall rather than
433 solubilization. These results call attention to the important role that differences in cell wall
434 structure (*e.g.* MLGs in graminaceous monocots and pectic polysaccharides in herbaceous

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435 dicots) and organization (*e.g*. ester cross-linking) play in setting the cell wall recalcitrance to
436 deconstruction by pretreatment and enzymatic hydrolysis. Thus, the pretreatment conditions that
437 are feedstock-specific are likely to be more effective than general approaches, and the future
438 work in breeding and engineering plants with cell walls designed for specific deconstruction
439 approaches should make use of positive synergistic interactions between specific pretreatments
440 and particular cell wall features.
441

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447 antibodies used in this work was supported by the NSF Plant Genome Program (DBI-0421683
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449 1336622).
450

451 **ABBREVIATIONS**
452 AG: arabinogalactan; AHP: alkaline hydrogen peroxide; AIR: alcohol insoluble residue; AX:
453 arabinoxylan; CBM: cellulose binding module; CCRC: Complex Carbohydrate Research Center;
454 CDTA: (1,2-cyclohexylenedinitrilo)tetraacetic acid; CS: corn stover; ELISA: enzyme-linked
455 immunosorbent assay; GAX: glucuronoarabinoxylan; GM: glucomannan; GP: glycome
456 profiling; GX: glucuronoxylan; HGA: homogalacturonic acid; HPLC: high pressure liquid
457 chromatography; mAb: monoclonal antibody; MLG: mixed-linkage glucan; RG-I:
458 rhamnoglucuronan-I; SG: switchgrass; XyG: xyloglucan.
459

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557 **Table 1:** Composition of untreated biomass. Neutral polysaccharides, lignin, and ash were
558 determined according to NREL Laboratory Analytical Protocol NREL/TP-510-42619. Acetyl
559 content was determined by HPLC following two-stage acid hydrolysis. Uronic acids were
560 assayed enzymatically (K-Uronic, Megazyme). Extractives were determined gravimetrically
561 following sequential extraction with ethanol, methanol-chloroform, and acetone. Compositions
562 are reported on a whole sample basis rather than an extractives-free basis. Errors represent data
563 range of duplicate measurements.
564
565
566 **Figure 1.** Compositional changes (extractives-free basis) associated with AHP pretreatment with
567 increasing H_2O_2 loading showing solubilization of total cell wall mass (A), xylan (B), and lignin
568 (C). The results indicate substantially different responses to AHP pretreatment by the three
569 biomass types in all three categories. The basis for these differences arising from the structural
570 organization of cell wall glycans are further explored by glycome profiling.
571
572 **Figure 2.** Enzymatic hydrolysis yields of cell wall glucan to glucose in untreated and AHP
573 pretreated biomass with increasing H_2O_2 loading for poplar, goldenrod, and corn stover with
574 hydrolysis (Cellic CTec2) for 24 h and 72 h.
575
576 **Figure 3.** Glycome profiling of hybrid poplar, goldenrod and corn stover biomass samples before
577 and after AHP pretreatment (12.5% H_2O_2 loading). Sequential cell wall extracts were made from
578 untreated and pretreated biomass samples using increasingly harsh reagents as explained in
579 materials and methods. Various extracts obtained were ELISA screened using 155 mAbs
580 directed against most major plant cell wall glycans (See Supplemental Table S1). The resulting
581 binding response data are represented as heatmaps with yellow-red-black scale indicating the
582 strength of the ELISA signal (yellow, red and dark-blue colors depict strong, medium, and no
583 binding, respectively). The mAbs are grouped based on the cell wall glycans they recognize as
584 depicted in the panel at right hand side of the figure. The actual amounts of materials extracted
585 out at each extraction condition are depicted as bar graph at the top of heatmaps with color codes
586 for reagents used for extraction.
587

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588 **Figure 4:** Comparison of the changes in the relative abundance of three glycan epitope
589 categories due to AHP pretreatment from the oxalate (blue dots) and carbonate (red dots) extracts
590 in glycome profiling, for poplar (A, B and C, for xyloglucans, xylans and pectic
591 polysaccharides), goldenrod (D, E and F for xyloglucans, xylans and pectic polysaccharides) and
592 corn stover (G, H and I for xyloglucans, xylans and pectic polysaccharides). Data are replotted
593 from Figure 3, but are normalized to represent mAb binding strength per mass of original AIR to
594 make absolute values comparable between conditions and samples. The red dash line represents
595 x = y and denotes the case where the abundance of these glycan epitopes was unchanged after
596 AHP pretreatment in these extracts. Data points above and below the dash lines represent
597 increased or decreased abundance of the glycan epitopes appearing in each extract, respectively.
598
599 **Figure 5:** Comparison of the changes in the relative abundance of three glycan epitope
600 categories due to AHP pretreatment from each of the four harshest extracts including 1M KOH
601 (green triangles), 4M KOH (blue diamonds), chlorite (red squares), and 4M KOH PC (purple
602 crosses) in glycome profiling. Data are normalized to represent relative abundance per mass
603 original AIR.
604
605

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606 Table 1.
607

	Hybrid Poplar (wt/wt %)	**Goldenrod (wt/wt %)**	**Corn Stover (wt/wt %)**
Glc	48.3±2.0	27.1±0.8	38.4±0.3
Xyl+Man+Gal	16.9±0.6	13.6±0.8	25.2±0.2
Ara	1.33±0.07	2.96±0.03	3.92±0.02
Acetyl	4.14±0.09	2.59±0.06	3.20±0.10
Total Uronic Acids	1.88±0.10	4.75±0.03	0.88±0.02
Lignin (Klason)	20.85±1.1	19.92±0.7	12.57±1.2
Extractives	5.79±0.22	23.5±0.23	12.0±0.74
Ash	1.95±0.48	6.08±1.02	3.03±0.29

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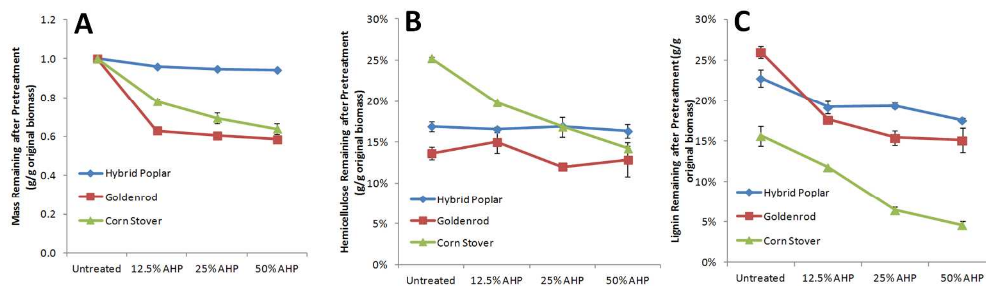

Figure 1: Compositional changes (extractives-free basis) associated with AHP pretreatment with increasing H_2O_2 loading showing solubilization of total cell wall mass (A), xylan (B), and lignin (C). The results indicate substantially different responses to AHP pretreatment by the three biomass types in all three categories. The basis for these differences arising from the structural organization of cell wall glycans are further explored by glycome profiling.
789x230mm (96 x 96 DPI)

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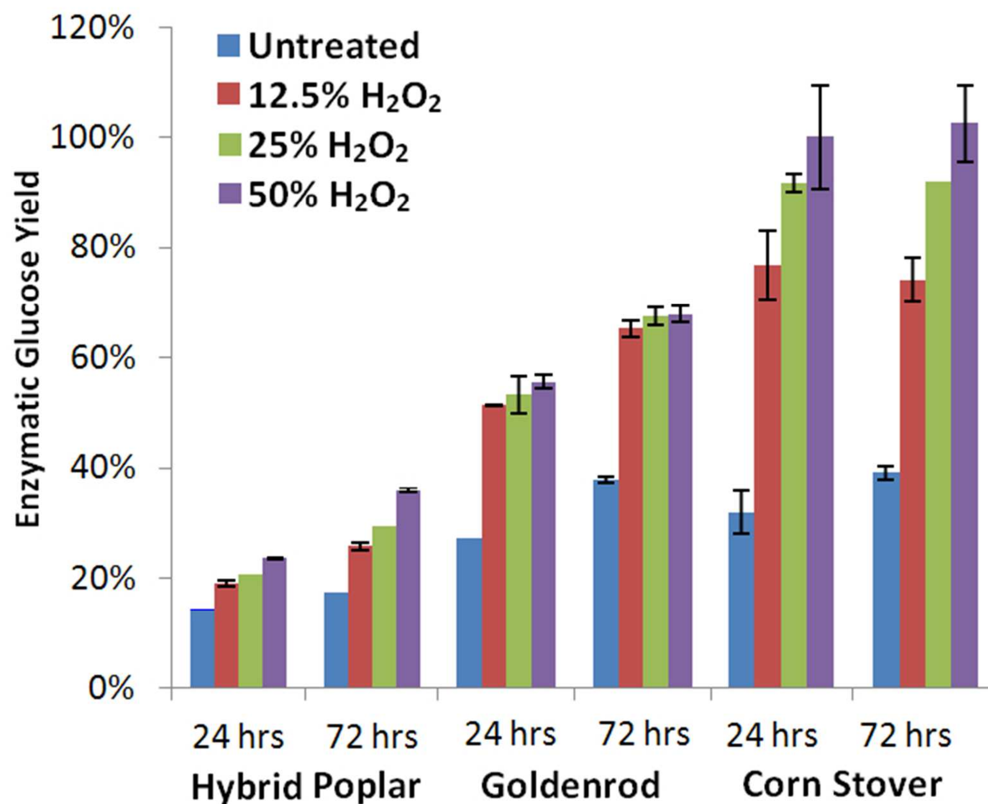

Figure 2: Enzymatic hydrolysis yields of cell wall glucan to glucose in untreated and AHP pretreated biomass with increasing H_2O_2 loading for poplar, goldenrod, and corn stover with hydrolysis (Cellic CTec2) for 24 h and 72 h.
176x144mm (96 x 96 DPI)

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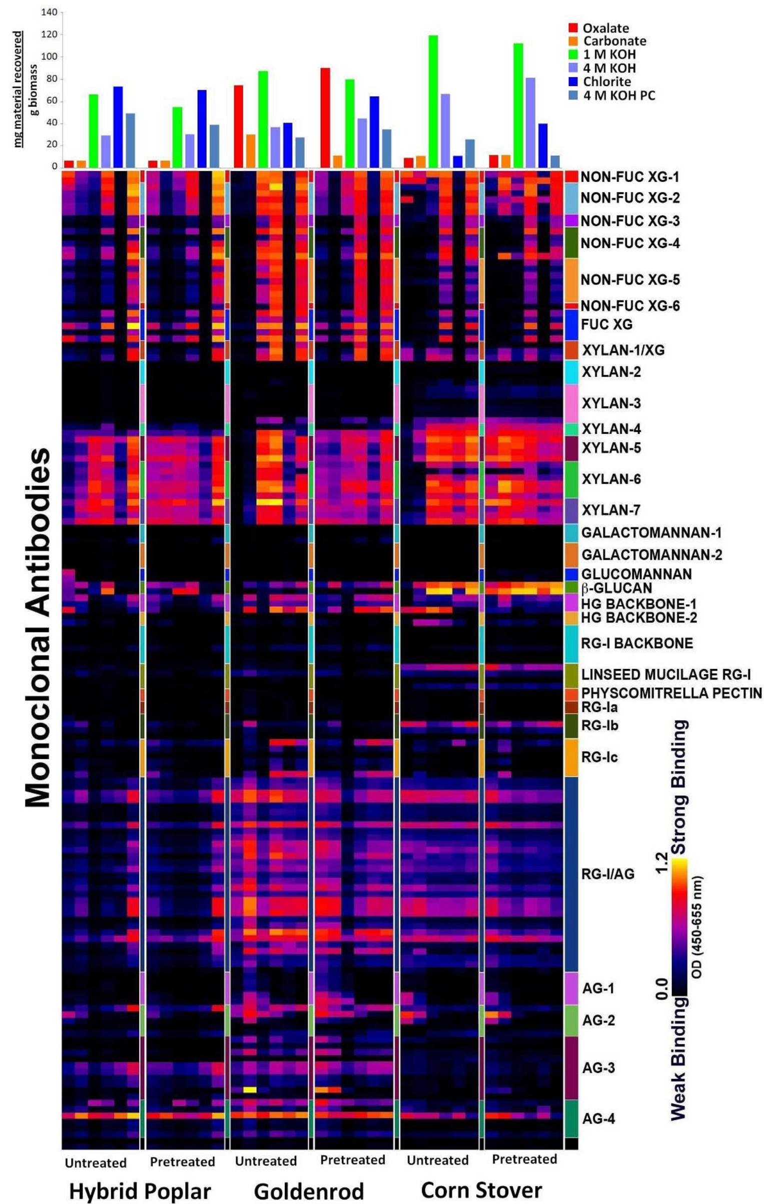

Figure 3: Glycome profiling of hybrid poplar, goldenrod and corn stover biomass samples before and after AHP pretreatment (12.5% H_2O_2 loading). Sequential cell wall extracts were made from untreated and pretreated biomass samples using increasingly harsh reagents as explained in materials and methods. Various extracts obtained were ELISA screened using 155 mAbs directed against most major plant cell wall glycans (See supplemental Table S2). The resulting binding response data are represented as heatmaps with yellow-red-black scale indicating the strength of the ELISA signal (yellow, red and dark-blue colors depict strong, medium, and no binding, respectively). The mAbs are grouped based on the cell wall glycans they recognize as depicted in the panel at right hand side of the figure. The actual amounts of materials extracted out at each extraction condition are depicted as bar graph at the top of heatmaps with color codes for reagents used for extraction.
544x806mm (96 x 96 DPI)

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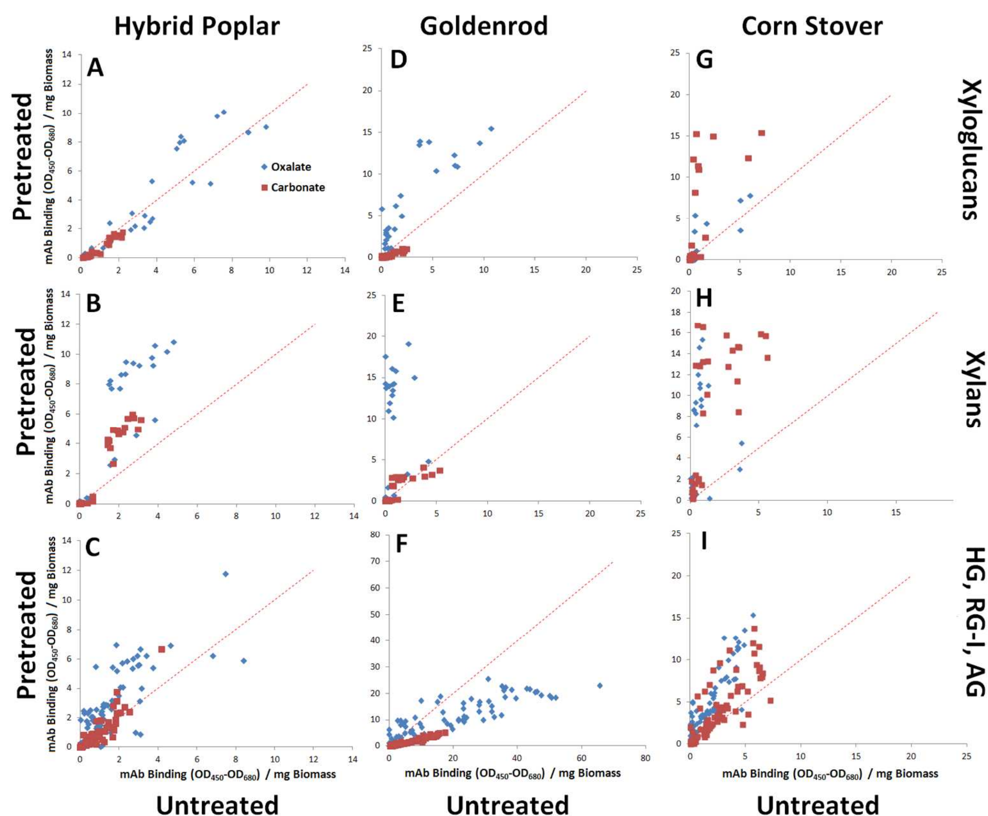

Comparison of the changes in the relative abundance of three glycan epitope categories due to AHP pretreatment from the oxalate (blue dots) and carbonate (red dots) extracts in glycome profiling, for poplar (A, B and C, for xyloglucans, xylans and pectic polysaccharides), goldenrod (D, E and F for xyloglucans, xylans and pectic polysaccharides) and corn stover (G, H and I for xyloglucans, xylans and pectic polysaccharides). Data are replotted from Figure 3, but are normalized to represent mAb binding strength per mass of original AIR to make absolute values comparable between conditions and samples. The red dash line represents x = y and denotes the case where the abundance of these glycan epitopes was unchanged after AHP pretreatment in these extracts. Data points above and below the dash lines represent increased or decreased abundance of the glycan epitopes appearing in each extract, respectively.
438x365mm (96 x 96 DPI)

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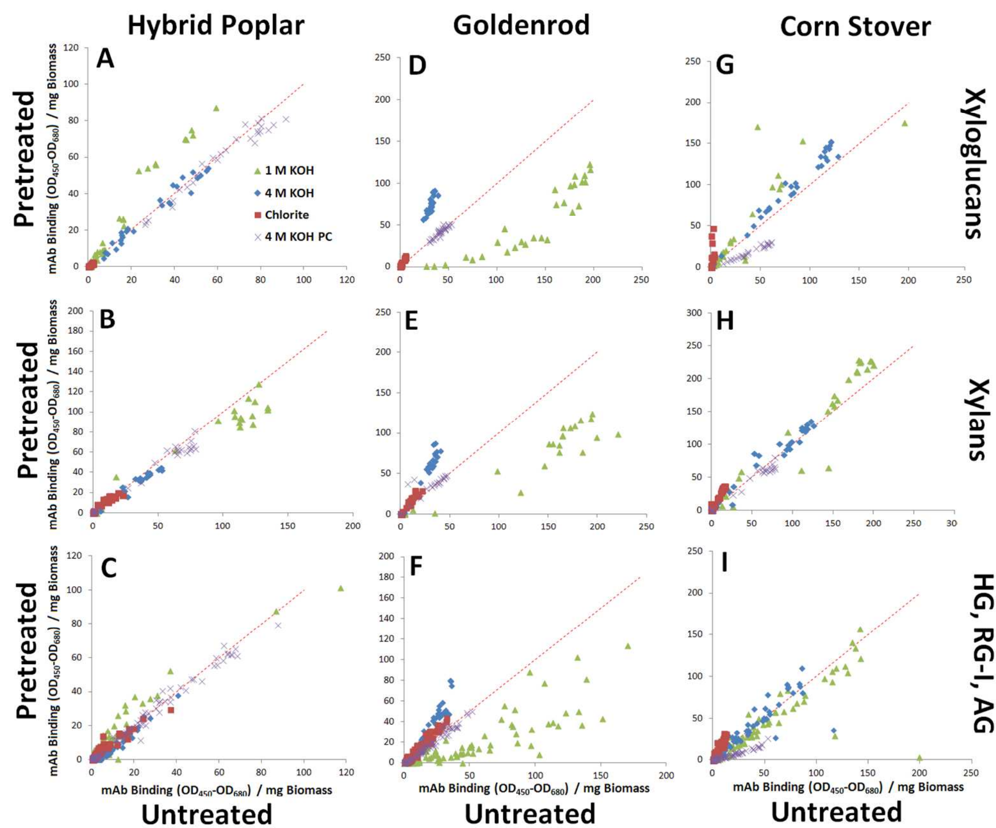

Comparison of the changes in the relative abundance of three glycan epitope categories due to AHP pretreatment from each of the four harshest extracts including 1M KOH (green triangles), 4M KOH (blue diamonds), chlorite (red squares), and 4M KOH PC (purple crosses) in glycome profiling. Data are normalized to represent relative abundance per mass original AIR.
410x337mm (96 x 96 DPI)

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