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Title: Impact of ball milling on maize (*Zea mays* L.) stem structural components and on enzymatic hydrolysis of carbohydrates

Year: 2014

Version: Pre-print

Please cite the original version:

Sipponen, Mika Henrikki & Laakso, Simo & Baumberger, Stéphanie. 2014. Impact of ball milling on maize (*Zea mays* L.) stem structural components and on enzymatic hydrolysis of carbohydrates. *Industrial Crops and Products*. Volume 61. P. 130-136. ISSN 0926-6690 (printed). DOI: 10.1016/j.indcrop.2014.06.052.

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1 **Impact of ball milling on maize (*Zea mays* L.) stem structural components and on**
2 **enzymatic hydrolysis of carbohydrates**

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10 **Abstract**

11 The impact of ball milling on structural components of maize (*Zea mays* L.) stem was
12 investigated in relation to enzymatic hydrolysis of carbohydrates. Ball milling of extractive-
13 free maize stem material was carried out with different milling times up to 12 h.
14 Carbohydrate conversion from ball-milled maize stem material with cellulolytic preparation
15 Onozuka R-10 increased to 79% with increasing ball milling time up to 4 h, but did not
16 thereafter increase further. Ball milling caused drastic depolymerization of hemicelluloses as
17 revealed by increasing amounts of monosaccharides released in absence of enzymes. Infrared
18 spectroscopy showed that cellulose crystallinity decreased within the first 6 h milling
19 thereafter reaching a plateau. Thioacidolysis of solid residue fractions obtained after
20 treatment of ball-milled samples with Onozuka R-10 suggested that no extensive degradation
21 of lignin occurred during the milling. The effect of lignin structure on enzymatic hydrolysis
22 of associated carbohydrates was further studied independently of cellulose crystallinity. Two
23 lignin-carbohydrate fractions comprising hemicellulose and lignin with either high or low
24 amount of aryl ether linkages were used as model materials. Lignins abundant in aryl ether
25 linkages appeared more detrimental than condensed lignins to enzymatic hydrolysis of
26 associated carbohydrates.

27 **Keywords:** Ball-milling, enzymatic, hydrolysis, thioacidolysis, maize stem

28

29 **1 Introduction**

30

31 Residues of cereal crops could supply sustainable raw material for biofuel production.

32 Conversion of lignocellulose into valuable products via so-called sugar platform involves

33 four main steps: pretreatment, hydrolysis, fermentation, and product recovery. Initial

34 mechanical treatment, for instance by shredding, precedes pretreatment, and is used to

35 decrease particle size of the lignocellulosic feedstock suitable for mass transfer operations.

36 Enzymatic hydrolysis of lignocellulose is inefficient without further pretreatment that

37 disintegrates the cell wall components and renders carbohydrates susceptible for enzymes.

38

39 Mechanical treatments can facilitate enzymatic hydrolysis by disrupting the lignin-

40 carbohydrate network and by increasing accessible surface area of carbohydrates. In addition

41 to the particle size reduction occurring in all mill types, ball milling has been shown to

42 decrease cellulose crystallinity of rice straw, sugarcane bagasse and straw, leading to

43 improved enzymatic digestibility (Hideno et al., 2009; da Silva et al., 2010). While ball

44 milling is typified by high energy consumption, making it too expensive for large scale

45 pretreatment processes, it is an efficient laboratory scale technique used for example in

46 sample preparation prior to extraction (Balakshin et al., 2011; Zoia et al., 2011), size-

47 exclusion chromatography (Salanti et al., 2012) or liquid-state NMR spectroscopy (Lu and

48 Ralph, 2003). In view of the positive effect of ball milling on enzymatic hydrolysis, better

49 understanding of the structural changes induced by ball milling could turn out useful for

50 development of improved hydrothermal or thermochemical pretreatments.

51

52 It is well known that ball milling can have drastic effects on plant cell walls. Operation mode
53 of the ball mill, as well as the materials of the milling jar and balls, and the amount and type
54 of feedstock all affect outcome of the milling. Schwanninger et al. (2004) showed that the
55 effect of even short (5 s to 2 min) duration vibratory ball milling can be visualized from the
56 Fourier transform infrared (FTIR) spectra, and that in the case of wood, the main changes
57 were attributable to the decrease in the degree of crystallinity and/or degree of polymerisation
58 of cellulose. Also planetary ball milling induces changes in lignocellulose, at least when
59 using zirconium milling jar and balls, as demonstrated by production of arabinoxylan
60 oligosaccharides from wheat and rye bran (Van Craeyveld et al., 2009). In addition to
61 cleavage of glycosidic linkages of carbohydrates, covalent bonds between lignins and
62 carbohydrates may be cleaved. Balakshin et al. (2011) mentioned that the benzyl ether bond
63 in the lignin carbohydrate complex (LCC) in wood is very likely degraded in planetary ball
64 milling. Furthermore, degradation of interunit linkages of wood lignins has been suggested as
65 a result of vibratory, but not rotary, ball milling (Ikeda et al., 2002; Schwanninger et al.,
66 2004; Fujimoto et al., 2005; Guerra et al., 2006). As contrasted to these studies on wood
67 materials, effects of ball milling on grasses and grass lignins have not been as
68 comprehensively elucidated. In particular, though maize (*Zea mays* L.) is a widespread crop
69 of genetic, agronomical and industrial interest (Barrière et al., 2009), little is known about the
70 impact of mechanical treatments on maize stem structural components and enzymatic
71 hydrolysis.

72

73 The objective of the current paper was to investigate the impact of mechanical disruption of
74 the cell wall components on enzymatic hydrolysis of maize stem carbohydrates. To our
75 understanding, this is the first study that utilizes maize stem material in ball milling from the
76 viewpoint of enzymatic hydrolysis. In the first step, the overall effect of ball milling on
77 enzymatic hydrolysis was studied using a commercial cellulase preparation. Stability of

78 lignin aryl ether linkages towards mechanical degradation was studied by analyzing the
79 milled solid fractions before and after enzymatic hydrolysis by thioacidolysis-GC/MS. In the
80 second step the aim was to study in detail the role of lignin in enzymatic hydrolysis of
81 carbohydrates. To achieve this, two alkali-soluble lignin-carbohydrate (LC) fractions were
82 used as models for enzymatic hydrolysis. Separate treatments of the LC fractions with the
83 cellulase preparation and with a purified endoxylanase were performed to elucidate the effect
84 of lignin structure on carbohydrate conversion. Evidence was obtained that lignin structure
85 has a significant role in enzymatic hydrolysis of polysaccharides.

86

87 **2 Materials and methods**

88

89 **2.1 Materials**

90 Extractive-free residue from F2 line (INRA) maize stem material contained 17.3% Klason
91 lignin and anhydrous sugars in the following percentages: 40.0% glucose, 20.2% xylose, 2.4
92 % arabinose, 0.6% galactose (Sipponen et al., 2013). Two lignin-carbohydrate (LC) fractions,
93 LC1 and LC2, isolated from the above maize stem material by room temperature alkaline
94 extractions were used as model materials for enzymatic hydrolysis (Sipponen et al., 2013).
95 LC1 and LC2 contained 38.3% and 18.7% Klason lignin and 50.3% and 70.7% carbohydrate
96 of which arabinoxylan comprised 93% and 89%, respectively. In addition, LC1 contained 12-
97 times more hydroxycinnamic acids and lignin interunits only involved in aryl ether linkages
98 compared to LC2 as revealed by thioacidolysis (Sipponen et al., 2013). A cellulolytic
99 preparation “Onozuka R-10” (Serva Electrophoresis, Germany) from *Trichoderma viride* was
100 used in the saccharification assays. Purified endoxylanase PaXyn11A belonging to the GH11
101 family, and fused to a family 1 CBM (carbohydrate binding module), was a kind gift from

102 Mrs. Mireille Haon (INRA, Marseille). Details of the expression and secretion of PaXyn11A
103 in the yeast *Pichia pastoris* has been published (Couturier et al., 2011).

104

105 **2.2 Ball milling procedure**

106 Extractive-free maize stem material (3.0 g) was weighed into a 50 mL stainless steel vessel
107 with internal agate surface, and loaded with 28.6 g of agate balls (20 balls, 10 mm diameter).
108 The vessel was fixed into a Retsch PM 100 planetary ball mill, operated at 600 rpm
109 frequency at room temperature for a predetermined time with 20 min-20 min work-pause
110 sequence. The actual working time of the mill excluding the paused time is presented in this
111 work.

112

113 **2.3 Extraction of ball-milled material with aqueous dioxane**

114 To study disintegration of lignin-carbohydrate network during ball milling, 15 mg of milled
115 material was extracted with 1 mL of dioxane/water (9:1, v/v), for 24 h at room temperature.
116 Mass extinction coefficient of the soluble matter at 280 nm was calculated based on the total
117 amount of dry matter of milled material weighed to extraction. The extractions were carried
118 out in duplicate.

119

120 **2.4 Determination of enzyme activities**

121 Endoglucanase activity was determined according to Ghose (1987) using 1.5% (w/v) CMC-
122 Na salt, low viscosity (Sigma) as substrate. The assay for endoxylanase activity was adapted
123 from Nakamura et al. (1993) using 1.5% (w/v) beechwood xylan (Sigma-Aldrich) or
124 larchwood xylan (EGA-Chemie) as substrate in 50 mM Na-citrate buffer (pH 4.8) at 50 °C

125 for 10 min. The amount of reducing sugars formed in the endoxylanase and endoglucanase
126 assays were determined relative to xylose and glucose standards, respectively (Miller, 1959).
127 One international unit of activity refers to the amount of enzyme that releases one μmol of
128 xylose or glucose in one minute under the assay conditions. The determinations were carried
129 out in duplicate and the results are given in the supplementary material in Table S1.

130

131 **2.5 Determination of protein content**

132 Protein contents were assayed according to the Bradford (1976) method using Coomassie
133 Plus reagent (Thermo) and BSA (Sigma) standards. Absorbance at 595 nm was measured
134 using a Spectra max 340PC microplate reader (Molecular devices, USA). The determinations
135 were carried out in duplicate.

136

137 **2.6 Enzymatic treatments**

138 Lignin-carbohydrate fractions (LC1, LC2) or ball-milled extractive-free maize stem samples
139 were mixed to a low consistency (1-2% w/v) suspension in 0.05 M Na-acetate buffer (pH 5)
140 at constant amount of Onozuka R-10 per sample dry matter (4.5 mg protein/g). Hydrolysis
141 reactions were carried out in screw-capped test tubes at 45 °C in continuous free-fall
142 agitation. Sodium azide (0.025%, w/v) was used to prevent microbial growth. Reaction time
143 was 40 h for the LCs and 72 h for the ball-milled samples. Under identical reaction
144 conditions, LC1 and LC2 were treated with PaXyn11A (33 mg protein/g). To obtain enough
145 residual material for lignin analysis, hydrolysis of ball-milled materials was carried out in
146 identical conditions as above, but in stirred suspensions containing 1 g of solids. After
147 reaction, the suspension was centrifuged and 1 mL aliquot was withdrawn from the
148 supernatant before it was decanted off. The solid fraction was washed three times with

149 deionized water, or in the case of LC1 and LC2, washed twice with 10 mL of 0.05 M Na-
150 acetate buffer (pH 5), 10 mL of 0.2% (w/v) Tween 20 (Sigma-Aldrich) in the same buffer
151 solution with 15 min incubation in an ultrasonic water bath at 50 °C, and finally three times
152 with 10 mL of deionized water (pH 5). Control experiments containing LC1, LC2 or ball-
153 milled samples in the buffer without enzymes were carried out in parallel with the enzymatic
154 treatments that were carried out in duplicate.

155

156 **2.7 Lignin and carbohydrate analysis**

157 Lignin content was determined gravimetrically from the ball-milled samples and from the
158 corresponding hydrolysis residues after the Onozuka R-10 treatment according to the
159 protocol adapted from Dence (1992). The acid-insoluble residue from the two-stage sulfuric
160 acid hydrolysis was corrected for its ash content and termed Klason lignin. To determine total
161 carbohydrate content of the liquid sample after enzymatic reaction, monosaccharides were
162 analyzed either directly or after post-hydrolysis (Sluiter et al., 2008). The post-hydrolysis was
163 carried out by mixing 2.5 mL of the supernatant dilution with 2.5 mL 8% w/v H₂SO₄ in a
164 glass screw-cork tube and carrying hydrolysis in an oil bath at 121 °C for 1 h.

165 Monosaccharides were analyzed with high-performance anion-exchange chromatography
166 (HPAEC) as described in the literature (Sipponen et al., 2013). The difference between the
167 amount of sugar obtained from the liquid sample after and before the post-hydrolysis was
168 interpreted as the content of polymeric carbohydrate in the samples. The hydrolysis
169 treatments and sugar analyses were carried out in duplicate. Calculation of anhydrosugars
170 was made by multiplying the masses of pentose and hexose monosacchrides by 0.88 and
171 0.90, respectively.

172

173 **2.8 ATR-FTIR spectroscopy**

174 Attenuated total reflection Fourier transform infrared (ATR-FTIR) spectra were recorded
175 using a Nexus 470 FT-IR (American Nicolet Company). Averaged spectra were calculated
176 from 5-10 individual acquisitions from each of the materials with 10 scans at resolution of 4
177 cm^{-1} .

178

179 **2.9 Thioacidolysis**

180 Thioacidolysis of untreated or ball-milled maize stem materials (15 mg) was carried out
181 according to the literature (Lapierre et al., 1995), using heneicosane $\text{C}_{21}\text{H}_{44}$ (Fluka) as
182 internal standard (IS). Lignin-derived trithioethylated *p*-hydroxyphenyl (H), guaiacyl (G) and
183 syringyl (S) monomers were analyzed as their trimethylsilyl derivatives by gas
184 chromatography-mass spectrometry (GC-MS). The quantitative determination of the H, G,
185 and S monomers was performed from ion chromatograms reconstructed at m/z 239, 269 and
186 299, respectively, as compared to the IS signal measured from the ion chromatogram
187 reconstructed at m/z (57+71+85). The molar yield of the detected monomers was calculated
188 on the basis of the Klason lignin content of the sample.

189

190 **2.10 Solubility in pyridine**

191 Solubility in pyridine of LC1 and LC2 as well as their corresponding enzymatic residues was
192 studied using spectrophotometry. Accurately weighed sample (5-10 mg) was mixed with 20
193 mL of spectroscopy grade pyridine (Merck), and the suspension was incubated 48 h at room
194 temperature with occasional shaking. The solution was passed through 0.45 μm GHP syringe
195 filter (Pall), and absorbance against pyridine was measured at 317 nm and 380 nm in a
196 capped quartz cuvette. Mass extinction coefficients at 317 nm and 380 nm were calculated

197 based on the total amount of sample mixed with pyridine. Duplicate absorbance
198 measurements from each single solution were made.

199

200 **3 Results and discussion**

201

202 **3.1 Effect of ball milling on enzymatic hydrolysis of maize stem carbohydrates**

203 Recalcitrance of lignocellulosic materials towards enzymatic hydrolysis limits
204 saccharification of these renewable resources. One of the targets of the current paper was,
205 therefore, to study the effect of ball milling of maize stem on enzymatic hydrolysis of its
206 carbohydrates. Enzymatic treatment of the milled material resulted in increased conversion of
207 carbohydrates to monosaccharides when the milling time was increased up to 5 h (Fig. 1).
208 Thereafter, the carbohydrate conversion maintained at 77%...79% regardless of the milling
209 time (6 h...12 h). In accordance with the observed carbohydrate conversions, Klason lignin
210 content in the solid residues from the enzymatic hydrolysis increased from 24.3% to 63.8%
211 during the first six hours of milling, and increased further as the milling time reached 12 h
212 (67.2%). Ball milling alone rendered increasing amounts of maize stem material water-
213 soluble with increasing milling time (Fig. 1). After 12 h milling 82% of the solid material was
214 recovered as insoluble residue from aqueous suspension as opposed to 95% without milling.

215

216 The observed maximum carbohydrate conversion is in accordance with the literature results
217 with other grass materials. Total carbohydrate conversions from ball-milled rice straw and
218 sugarcane bagasse of 78% and 82% have been reported (Hideno et al., 2009; da Silva et al.,
219 2010). Hideno et al. (2009) further showed that carbohydrate conversion from ball-milled rice
220 straw could not be increased with increasing cellulase activities beyond 2 FPU/g-rice straw. It

221 thus appears that the action of hydrolytic enzymes is limited by structural factors of the solid
222 material. In the present study the conversion of cellulose was higher than that of
223 hemicellulose (Table 1). In fact, after 3 h milling conversion of cellulose reached the level of
224 90% while conversion of arabinoxylan remained low (arabinose < 29% and xylose < 63%),
225 suggesting that presence of lignin associated to hemicelluloses, within lignin-carbohydrate
226 complexes (LCCs), might be involved in formation of such resistant entities. In order to
227 identify these structural elements, the effect of ball milling on main structural components of
228 maize stems was studied, and the effects are discussed based on the observed carbohydrate
229 conversions through enzymatic hydrolysis.

230

231 **3.2 Effect of ball milling on maize stem cell wall components**

232 **3.2.1 Extractable phenolic and carbohydrate fractions**

233 During ball milling the samples were pulverized in less than thirty minutes, and Klason lignin
234 contents of the milled samples remained unchanged at 17.1% with up to 12 h milling. To
235 study disintegration of the lignin-carbohydrate network, ball-milled samples were extracted
236 with dioxane:water (9:1, v/v) at room temperature, and the extracts were analyzed by UV-
237 spectroscopy. Calculated from the Beer-Lambert law and based on the measured absorbance
238 at 280 nm, mass extinction coefficient of the aqueous dioxane extracts showed linearly
239 increasing trend with the increasing milling time (Fig. 2). This increase could be a priori
240 explained by an increased proportion of extractable phenolics or by a change in the
241 composition of the phenolic extract. A closer look into the absorbance spectra normalized at
242 280 nm showed increasing relative absorbance in wavelengths exceeding 280 nm (Fig. 2).
243 The maximum increase in absorbance at 315 nm from 1 h to 12 h milled samples could be
244 attributed to dissolution of material containing *p*-coumaric acid and ferulic acid (Higuchi et
245 al., 1967), such as lignin, LCCs, and arabinoxylan fragments stemming from hemicellulose

246 depolymerization. Evidence of this depolymerization was brought by analysis of the free
247 monomeric sugars released into the aqueous extracts in absence of enzymes. In addition to
248 generation of arabinose, glucose, and xylose, appearance of galactose was noticed (Fig. 2).
249 While glucose could originate either from cellulose or xyloglucan, arabinose, xylose, and
250 galactose arised from hemicellulose depolymerization. This is consistent with an earlier work
251 showing production of oligomeric arabinoxylans as a result of ball milling (Van Craeyveld et
252 al., 2009). Enzymatic hydrolysis of cellulose from the milled samples was probably
253 facilitated by the depolymerization of hemicelluloses, but nonetheless enzymatic conversion
254 of arabinoxylan remained low (Table 1).

255

256 **3.2.2 Infrared spectra and cellulose crystallinity**

257 To analyze direct changes in cellulose, Attenuated Total Reflectance Fourier transform
258 infrared spectroscopy (ATR-FTIR) was used to study the effect of ball milling on maize stem
259 cell walls as analyzed before and after enzymatic hydrolysis. Compared to traditional
260 transmission FTIR measured using KBr-pelleting technique, ATR-FTIR is essentially a
261 surface analysis technique and thus advantageous for studying enzymatic reactions on solid
262 cellulose surfaces. Samples were selected for the ATR-FTIR analysis based on the observed
263 enzymatic carbohydrate conversions (Fig. 1). Analysis of the whole milled material before
264 enzymatic treatment indicated clear changes in the spectra as a function of milling time (Fig
265 3). Intensity of the broad band at 1200-1300 cm^{-1} increased when ball milling time increased
266 from 0 h to 2 h with little change thereafter. Though this band at 1200-1300 cm^{-1} may
267 originate from structures assignable to both lignin and carbohydrates or LCCs (G-ring, CH-,
268 C=O, OH, COOH, C-C, C-O, C=O) (Schwanninger et al., 2004), decreasing intensity of the
269 band was interpreted to be due to increasing amount of lignin on the particle surfaces as a
270 result of reduced particle size by milling. Other apparent change was the increased intensity

271 of the band at 894 cm^{-1} as a function of increasing milling time. The ratio of band heights at
272 1429 cm^{-1} and 894 cm^{-1} has been used as a relative measure for crystallinity of cellulose
273 (O'Connor et al., 1958). Using this approach it was found that ball milling decreased the
274 relative cellulose crystallinity drastically with increasing milling time up to 6 h (Fig. 3). No
275 further change was, however, observed with prolonged milling as indicated by similar band
276 height ratios from 6 h and 12 h ball-milled samples. Data in Table 1 showed highest cellulose
277 conversions as a result of long duration ball milling for 3 h to 12 h (88% to 92%) compared
278 to cellulose conversion with 1 h milling (72%). Linear correlation between cellulose
279 crystallinity and cellulose conversion was found (statistically significant coefficient of
280 determination; $R^2=0.81$, $p=0.003$), suggesting that cellulose crystallinity could be a factor
281 limiting enzymatic conversion.

282

283 When analyzed from the solid residues after hydrolytic treatment of the milled samples with
284 Onozuka R-10, the infrared spectra showed decreasing intensity in the broad band at 950 cm^{-1}
285 1 - 1100 cm^{-1} with increasing milling time (Fig. S1 in the supplementary material). Decreased
286 intensity in this wavenumber region can be explained by decreasing relative amount of
287 carbohydrates as a result of Onozuka R10 treatment of the solids obtained as a function of
288 increasing milling time. Relative crystallinity of cellulose in the solid residues from
289 enzymatic hydrolysis was lower than before hydrolysis, probably as a result of enrichment of
290 lignin on particle surfaces due to hydrolysis of cellulose from the surfaces (Fig. 3). This is
291 consistent with the suggested penetration depth of ATR-FTIR between $0.5\text{ }\mu\text{m}$ and $1\text{ }\mu\text{m}$ in
292 wheat (*Triticum aestivum* L.) straw with the signal intensity decreasing exponentially with
293 depth of penetration (Kristensen et al., 2008). While changes in cellulose structure could be
294 elucidated from the ATR-FTIR spectra, little information on lignin structure was obtained.
295 Therefore, analytical procedures targeted to providing specific information on changes in
296 lignin structure were used.

297

298 **3.2.3 Reactivity of lignin towards thioacidolysis**

299 Lignin has been said to be detrimental to enzymatic hydrolysis for instance because it hinders
300 access of enzymes on carbohydrates (Mooney et al., 1998), but the effect of lignin structure
301 on enzymatic hydrolysis has not been fully elucidated. Although ball milling did not change
302 the lignin content of maize stem material, changes in lignin structure were taken under study.
303 Thioacidolysis is an analytical degradation based on selective solvolytic cleavage of aryl
304 ether linkages of lignin in presence of ethanethiol. Analysis of the C₆C₃ trithioethyl
305 phenylpropane monomers provides information on the amount of lignin units only involved
306 in aryl ether bonds (Rolando et al., 1992). When thioacidolysis was performed directly on
307 ball-milled samples, yield of trithioethyl derivatives of S, G, and H monomers decreased
308 linearly with increasing ball milling duration, showing up to 86% decrease with 12 h milling
309 (Fig. 4). This severe decrease in thioacidolysis yield would suggest that ball milling induced
310 extensive cleavage of β -O-4 bonds and formation of C-C bonds resistant to thioacidolysis.
311 However, thioacidolysis yields from solid residues after enzymatic hydrolysis were
312 systematically substantially higher than that obtained directly from the ball-milled samples
313 (Fig. 4). Compared to the the ratio of syringyl to guaiacyl monomers from thioacidolysis
314 without ball milling (S/G=0.89), the ratio was similar with the solid residue from 8 h milled
315 material after enzymatic hydrolysis (S/G=0.90). Moreover, similar thioacidolysis yields were
316 obtained from the untreated maize stem material (840 μ mol/g) and the enzymatic residue
317 from 8 h ball-milled sample (838 μ mol/g). It can be thus unambiguously concluded that only
318 negligible degradation in lignin units that are linked only by aryl ether linkages could have
319 occurred. Therefore, enzymatic carbohydrate conversion restored the normal thioacidolysis
320 yield. These results suggest that some kind of physical barrier formed by lignin and
321 carbohydrate fragments affected the progression of the thioacidolysis reaction. When

322 thioacidolysis of 4 h ball-milled maize stem sample was carried out with prolonged reaction
323 time (16 h or 22 h), total thioacidolysis yield (594 $\mu\text{mol/g}$ or 600 $\mu\text{mol/g}$) was 1.3-times
324 higher compared to the yield (470 $\mu\text{mol/g}$) from the standard 4 h reaction. It is not completely
325 understood at present whether ball milling-induced lignin-carbohydrate barriers could also
326 lead to lower yields in other analytical degradations of lignin.

327

328 **3.3 Effect of lignin-carbohydrate interactions and structure of lignin on enzymatic** 329 **hydrolysis**

330 It was recently shown that two structurally distinct lignin-carbohydrate fractions can be
331 isolated from extractive-free maize stems material by sequential alkaline extraction before
332 and after endoglucanase treatment (Sipponen et al., 2013). The first alkali-soluble fraction
333 (LC1) contained lignin with 12-times more units only linked by aryl ether bonds than the
334 second fraction (LC2). To study the effect of lignin-carbohydrate interactions on enzymatic
335 hydrolysis independently of crystalline cellulose, LC1 and LC2 were used as model materials
336 in enzymatic hydrolysis. Based on the ATR-FTIR analysis the relative crystallinities
337 (A_{1429}/A_{894}) of LC1 and LC2 were 2.5 and 3.7, respectively, indicating low crystallinity
338 comparable to the value of 12 h ball-milled maize stem material ($A_{1429}/A_{894} = 3.4$). LC1
339 and LC2 were treated with purified endoxylanase PaXyn11a, Onozuka R-10, or with buffer
340 solution only. These treatments resulted in different levels of carbohydrate conversions. The
341 results show that LC2 was easier material to hydrolyze than LC1 by both PaXyn11a and
342 Onozuka-R10 (Table 2). The latter enzyme gave total carbohydrate conversion from LC2,
343 while the carbohydrate conversion of LC1 was incomplete (83%). Moreover, lignin content
344 of LC2 (18.3%) was comparable to the lignin content of the ball-milled maize stem materials
345 (17.1%). Based on this information it is evident that presence of lignin does not alone restrict
346 complete hydrolysis of carbohydrates (hemicelluloses) with the enzyme dosage used. The

347 lower carbohydrate conversion from LC1 could be explained by both its higher lignin content
348 and less condensed lignin structure compared to LC2. Moreover, Onozuka R-10 released
349 higher relative proportion of non-monomeric carbohydrates from LC1 (39%) than from LC2
350 (27%). This suggests that LC1 was overall more difficult material to hydrolyze by the
351 enzymes, perhaps additionally due to its less condensed lignin structure and the presence of
352 12-times more hydroxycinnamic acids compared to LC2. Indeed, Zhang et al. (2011) found
353 that at similar lignin contents, lignin condensation degree and content of *p*-coumaric acid
354 (CA) esters statistically significantly correlated with maize cell wall *in vitro* degradability. In
355 addition to presence of esterified CA in lignins, enzymatic hydrolysis might be also
356 constrained by ferulic acid that cross-links adjacent arabinoxylan chains and also
357 arabinoxylans and lignins (Grabber et al., 1998).

358

359 To study changes in the lignin-carbohydrate interactions as a result of the enzymatic
360 treatments of LC1 and LC2, solubility in pyridine of the original fractions and the
361 corresponding hydrolysis residues was studied. This enabled to indirectly estimate the role of
362 lignin in restricting enzymatic hydrolysis of associated carbohydrates, because according to
363 solubility parameter, pyridine is a good solvent of various types of lignins (Schuerch, 1952).
364 Solubility was tested both qualitatively (visual observation on solubility) and by measuring
365 absorbance at 317 nm and 380 nm and calculating the respective mass extinction coefficients
366 based on the amount of LC fraction weighed in pyridine. These two wavelengths were
367 selected to measure contribution of associated hydroxycinnamic acids (317 nm) and lignins
368 (380 nm) to the extinction coefficients of the LC samples, given that interference from
369 hydroxycinnamic acids was low at 380 nm (Fig. S2).

370

371 Results show that Onozuka R-10 treatment increased solubility of the LC1 and LC2 residues
372 as compared to the negligible effect of PaXyn11a or buffer solution only (Table 3).

373 Interestingly, PaXyn1 1a did not affect solubility of the LC residues despite 21.8% and 43.0%
374 carbohydrate conversion from LC1 and LC2, respectively (Table 2). In contrast, the LC1-
375 Onozuka R-10 residue was fully soluble, despite the 17% lower carbohydrate conversion
376 compared to the partially soluble LC2-Onozuka R-10 residue (Table 2). The measured
377 extinction coefficients at 380 nm of LC1 and LC2 were divided by their Klason lignin
378 content and compared to the extinction coefficients at 380 nm of the Onozuka R-10 residues
379 of LC1 and LC2 with hypothetical total lignin content. Extinction coefficients of 7.81, 6.38,
380 0.42, and 4.08 mL mg⁻¹ cm⁻¹ were obtained for LC1, LC1-Onozuka R-10, LC2, and LC2-
381 Onozuka R-10, respectively. This confirmed that in the case of LC2, increased solubility
382 rather than enrichment of lignin as a result of Onozuka R-10 treatment explains increased
383 extinction coefficient at 380 nm. These results indicate that although the presence of
384 carbohydrates limits solubility of lignin in pyridine, below certain carbohydrate content lignin
385 structure is a more important factor to determine solubility of lignin. With its lower
386 proportion of lignin with condensed interunit linkages, LC1 and its corresponding residues
387 showed better solubility compared to LC2. On the other hand, lignins rich in aryl ether
388 linkages showed more adverse effect on limiting enzymatic hydrolysis, a trend previously
389 suggested for maize cell walls based on *in situ* correlation study (Zhang et al., 2011). In the
390 current study more direct evidence of the effect of lignin structure on enzymatic hydrolysis
391 was obtained because the LC1 fraction did not contain crystalline cellulose that is itself a
392 poor substrate for cellulases. Moreover, in accordance with the lignin structure of LC1,
393 molecular modelling studies indicate that higher proportion of β -O-4 bonds favoring
394 extended linear structure promotes associative forces between cellulose and lignin (Besombes
395 et al., 2005). The lower proportion of β -O-4 bonds in LC2 suggests higher degree of
396 branching in lignins that has been suggested to significantly hinder associative forces
397 between lignins and cellulose (Besombes et al., 2005). In the current investigation, LC1-type
398 less condensed lignins could be released as a result of Onozuka R-10 treatment of ball-milled

399 maize stem samples. According to our hypothesis, the released lignin might then preferably
400 precipitate and/or adsorb on cellulose and other linear carbohydrates and this would hinder
401 enzymatic hydrolysis.

402

403 **4 Conclusions**

404 In this study ball milling was applied to maize stem extractive-free material in order to
405 investigate the impact of this mechanical treatment on both cell wall structure and enzymatic
406 degradability. As for other grasses, ball milling led to severe depolymerization of
407 hemicelluloses and increased enzymatic conversion yield of total polysaccharides to
408 monomeric sugars. The efficiency of the treatment was assigned to the combination of cell
409 wall disruption, decreased cellulose crystallinity and depolymerization of hemicelluloses.

410 Three hour ball milling time turned out to be a plateau ensuring a subsequent 90% enzymatic
411 conversion yield of cellulose into glucose, and among hemicelluloses a 60% release of xylose
412 but only a 20% release of arabinose. Meanwhile, lignin was not significantly affected by ball
413 milling or enzymatic treatment. These results suggested that LCC structures involving lignin
414 and arabinoxylans might be responsible for the recalcitrance of maize cell wall. This
415 hypothesis led us to use two distinct lignin-carbohydrate fractions as models. One of these
416 fractions, associating arabinoxylan-rich hemicelluloses with lignin of lower condensation
417 degree, was found to resist hydrolysis with a total maximum sugar release of 80%. Thus,
418 disruption of lignin-arabinoxylan structures of this type might offer a promising way to
419 improve grass lignocellulose conversion to soluble sugars. Besides results of technological
420 interest towards sugar-platform based lignocellulose biorefinery, this paper showed that ball
421 milling can lead to artificial decrease in thioacidolysis yields, probably by generating
422 physical barrier to the chemical reagents used in the solvolytic cleavage of lignin. Thus,

423 particular prudence should be taken while interpreting thioacidolysis analysis of ball milled
424 samples.

425

426 **Acknowledgements**

427 Mrs. Mireille Haon is acknowledged for providing the PaXyn11a. MHS is grateful for
428 Sylvain Legay for valuable discussions concerning carbohydrate analysis. MHS
429 acknowledges Grants from Foundation of Emil Aaltonen and Finnish Foundation for
430 Technology Promotion.

431

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544

545

546 **TABLES**

547

548

549 **Table 1.** Enzymatic carbohydrate conversions after treatment of 0 h to 12 h ball-milled maize
550 stem material with cellulolytic preparation Onozuka R-10. Conversions are expressed based
551 on anhydrosugars: arabinose (Ara), glucose (glc), xylose (Xyl). Mean values \pm average
552 deviation relative to the mean value are shown.

Ball milling time (h)	Ara (%, w w ⁻¹)	Glc (%, w w ⁻¹)	Xyl (%, w w ⁻¹)	Total (%, w w ⁻¹)
0	15.0 \pm 2.7	32.7 \pm 1.6	14.1 \pm 0.4	25.7 \pm 1.1
1	24.2 \pm 0.9	72.0 \pm 1.2	46.4 \pm 0.0	61.3 \pm 0.8
2	26.1 \pm 1.3	85.6 \pm 1.5	57.5 \pm 0.4	73.5 \pm 0.9
3	23.0 \pm 2.6	88.9 \pm 1.9	60.0 \pm 0.2	76.2 \pm 1.2
4	28.2 \pm 0.5	92.2 \pm 0.2	62.5 \pm 1.9	79.4 \pm 0.7
5	27.5 \pm 0.1	91.8 \pm 0.4	62.1 \pm 2.1	79.0 \pm 0.9
6	25.1 \pm 2.3	88.2 \pm 3.4	59.8 \pm 1.0	75.9 \pm 2.4
8	22.0 \pm 0.3	89.2 \pm 0.6	61.4 \pm 0.3	76.9 \pm 0.5
12	27.7 \pm 0.4	88.6 \pm 0.5	61.9 \pm 2.4	76.8 \pm 1.1

553

554

555 **Table 2.** The amount of monomeric and non-monomeric anhydrosugars released from lignin-
 556 carbohydrate fractions LC1 and LC2 as a result of various treatments. Composition of the
 557 total solubilized carbohydrates.

Treatment	Carbohydrate conversion (% w w ⁻¹) ^a			Composition of total released carbohydrates (%) ^c		
	Total	monomers	non-monomeric ^b	Xyl	Ara	Glc
LC1-buffer	15.0 ± 0.9	0.8	99.2	76.3	15.4	6.5
LC1- PaXyn11a	21.8 ± 4.0	0.5	99.5	84.5	10.4	4.3
LC1- Onozuka R-10	83.1 ± 1.1	61.3	38.7	83.2	9.8	6.3
LC2-buffer	8.85 ± 0.3	3.5	96.5	83.8	12.2	3.1
LC2- PaXyn11a	43.0 ± 4.1	1.0	99.0	89.6	8.5	1.6
LC2- Onozuka R-10	99.5 ± 3.2	73.0	27.0	82.1	5.8	11.7

558 ^a: Calculated on anhydrosugar basis. ^b: Calculated based on the increase in anhydrosugars
 559 after post-hydrolysis of the hydrolysates (121 C, 1 h). ^c: proportion of galactose was below
 560 1.8% (data not shown).

561

562 **Table 3.** Yield of solid residue after different treatments of lignin-carbohydrate fractions LC1
 563 and LC2. Solubility in pyridine of LC1 and LC2 and the corresponding solid residues was
 564 estimated by the mass extinction coefficient (ϵ) calculated from the Beer-Lambert law.

Treatment	Yield of solid residue (% w w ⁻¹)	ϵ (mL mg ⁻¹ cm ⁻¹) ^a	
		317 nm	380 nm
LC1	100	9.60	2.99
LC1-buffer	75.6 ^b	7.65	3.12
LC1- PaXyn11a	68.1 ± 1.0	7.35	2.98
LC1- Onozuka R-10 ^c	31.1 ± 1.9	16.9	6.38
LC2	100	0.08	0.08
LC2-buffer	79.3 ^b	0.05	0.05
LC2- PaXyn11a	46.2 ± 1.2	0.17	0.28
LC2- Onozuka R-10	11.2 ± 0.7	8.17	4.08

565 ^a: Measurements were made on duplicate dilutions from single suspension or solution. The
 566 average deviation from the mean was less than 2%. ^b: single determination. ^c: only sample
 567 that was fully soluble in anhydrous pyridine.

568

569

570

571 **Figure captions**

572

573 **Figure 1.** The effect of ball milling time on carbohydrate conversion as a result of enzymatic
574 hydrolysis of milled samples with Onozuka R-10. Errorbars shown represent \pm average
575 deviation relative to the mean value.

576

577 **Figure 2.** The effect of ball milling time on (A) Monomeric sugar yield calculated as the
578 amount of free anhydrous monosaccharides released from ball-milled maize stem material in
579 aqueous extraction in absence of enzymes; extinction coefficient at 280 nm as a result of
580 extraction of ball-milled maize stem samples with dioxane:water (9:1, v/v) at room
581 temperature for 24 h; (B) UV-absorbance spectra of the dioxane:water (9:1, v/v) extracts
582 from 1 h, 2 h, 3 h, 4 h, 5 h, 6 h and 12 h ball-milled samples. The spectra are normalized at
583 280 nm. Errorbars shown in (A) represent \pm average deviation relative to the mean value.

584

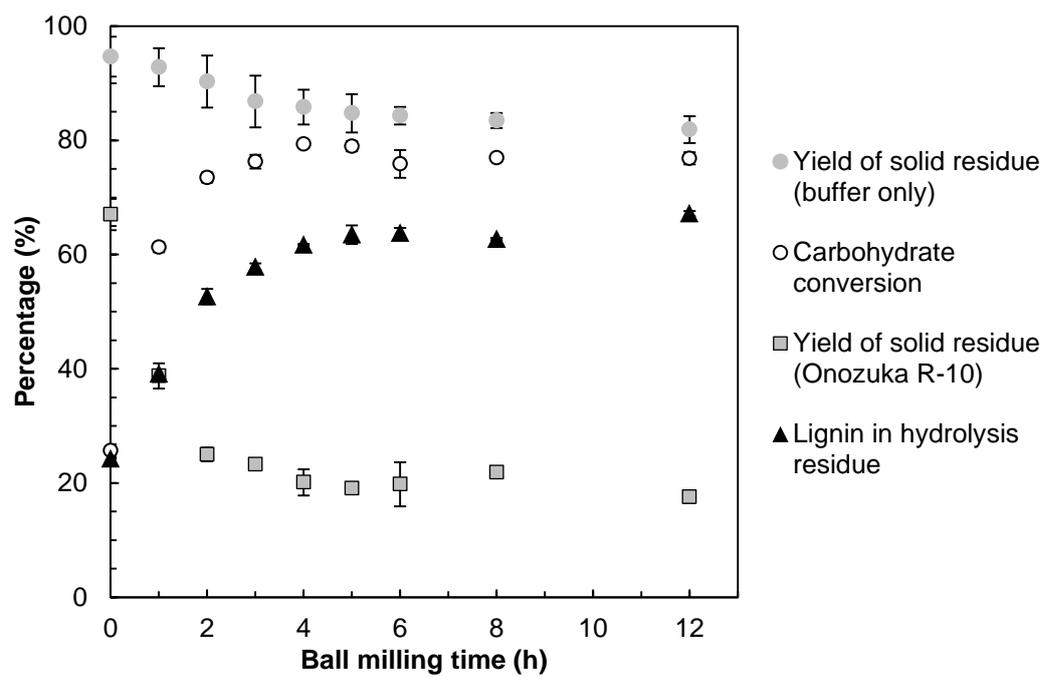
585 **Figure 3.** The effect of ball milling on maize stem samples: (A) fingerprint region of the
586 ATR-FTIR spectra of samples with 0 h to 12 h milling. (B) ratio of absorbance at 1429 cm^{-1}
587 to absorbance at 894 cm^{-1} showing relative changes in cellulose crystallinity in ball-milled
588 maize stem samples (circles, left y-axis) and in solid residues after treatment of ball-milled
589 materials with Onozuka R-10 (triangles, right y-axis).

590

591 **Figure 4.** Yield of trithioethyl derivatives of syringyl (S), guaiacyl (G) and *p*-hydroxyphenyl
592 (H) monomers from (A) maize stem material ball-milled up to 12 h, or (B) solid residues
593 after Onozuka R-10 treatment of ball-milled materials. Errorbars shown represent \pm average
594 deviation relative to the mean value.

595

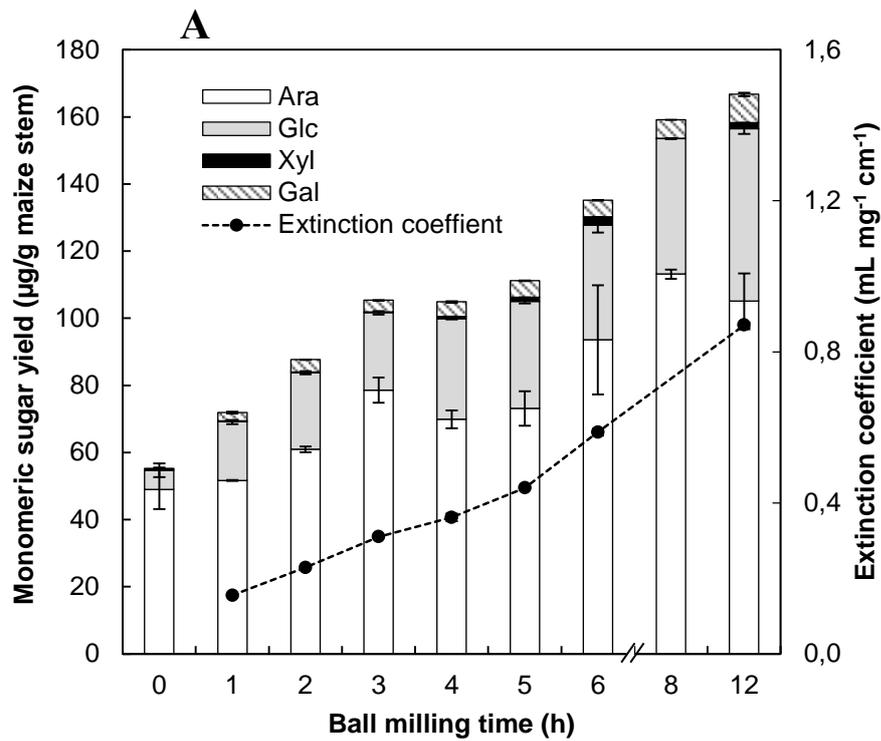
596 **FIGURES**
597



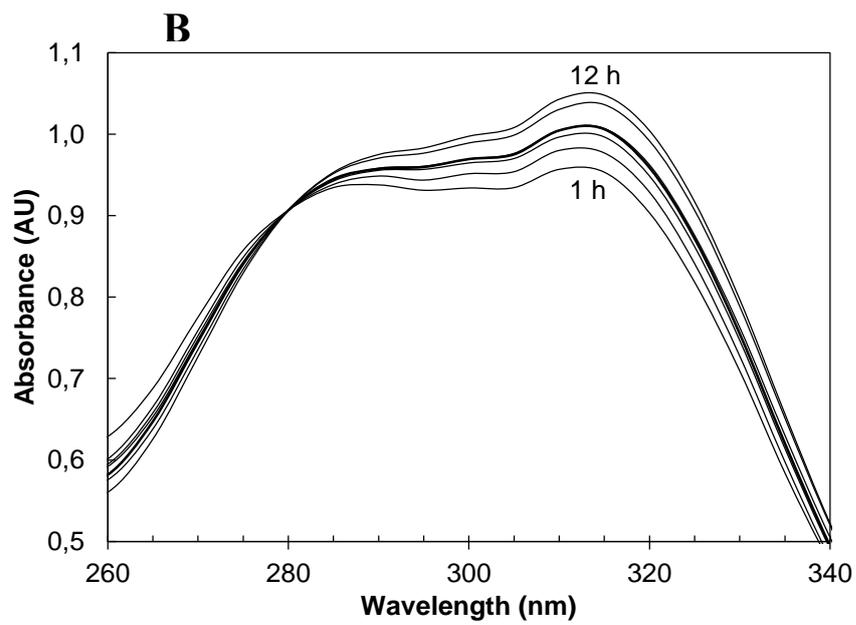
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599 **Figure 1.**

600



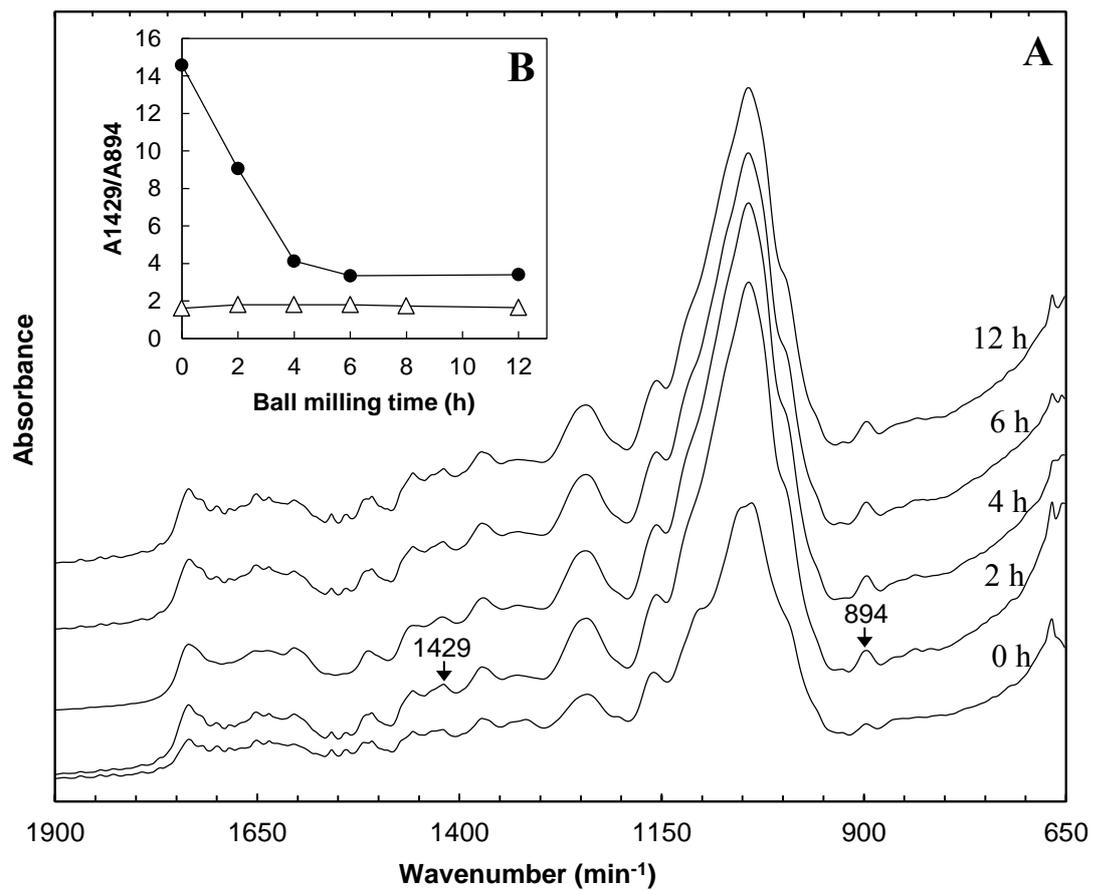
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603 **Figure 2.**

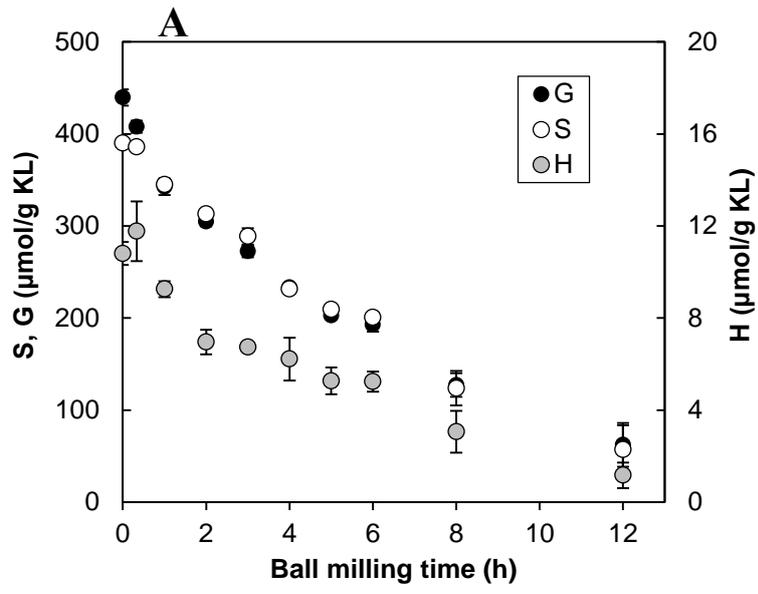
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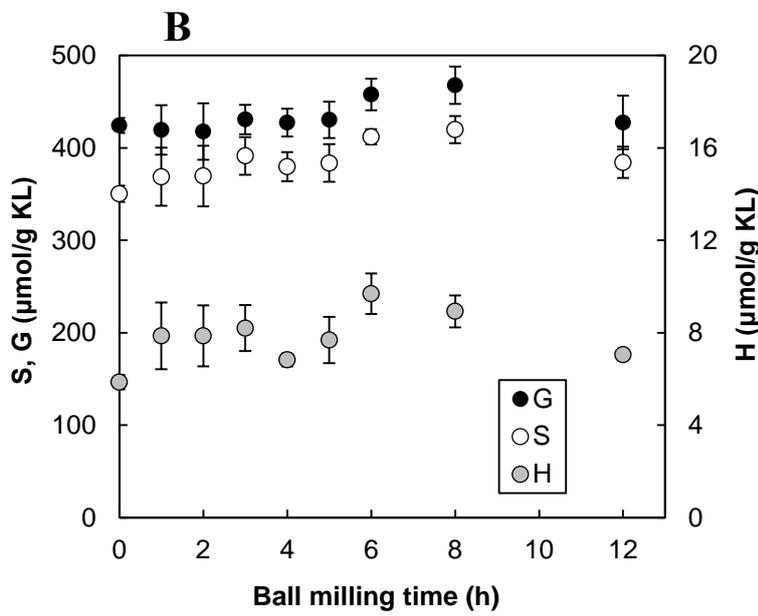
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606 **Figure 3.**

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608



609

610 **Figure 4.**