Author(s): Sipponen, Mika Henrikki & Laakso, Simo & Baumberger, Stéphanie

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.

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

Mika Henrikki Sipponen[a,b]*, Simo Laakso[a], Stéphanie Baumberger[b,c]

[a]Aalto University, School of Chemical Technology, Department of Biotechnology and Chemical Technology, Espoo, Finland

[b]AgroParisTech, UMR 1318 Institut Jean-Pierre Bourgin, F-78000 Versailles, France

[c] INRA, UMR 1318 Institut Jean-Pierre Bourgin, F-78000 Versailles, France

* Corresponding author. Tel.: +358947022547, Fax: +3589462373, Postal address: PO BOX 16100 FI-00076 Aalto, E-mail: mika.sipponen@aalto.fi

Abstract

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

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

1 Introduction

Residues of cereal crops could supply sustainable raw material for biofuel production. Conversion of lignocellulose into valuable products via so-called sugar platform involves four main steps: pretreatment, hydrolysis, fermentation, and product recovery. Initial mechanical treatment, for instance by shredding, precedes pretreatment, and is used to decrease particle size of the lignocellulosic feedstock suitable for mass transfer operations. Enzymatic hydrolysis of lignocellulose is inefficient without further pretreatment that disintegrates the cell wall components and renders carbohydrates susceptible for enzymes.

Mechanical treatments can facilitate enzymatic hydrolysis by disrupting the lignin-carbohydrate network and by increasing accessible surface area of carbohydrates. In addition to the particle size reduction occurring in all mill types, ball milling has been shown to decrease cellulose crystallinity of rice straw, sugarcane bagasse and straw, leading to improved enzymatic digestibility (Hideno et al., 2009; da Silva et al., 2010). While ball milling is typified by high energy consumption, making it too expensive for large scale pretreatment processes, it is an efficient laboratory scale technique used for example in sample preparation prior to extraction (Balakshin et al., 2011; Zoia et al., 2011), size-exclusion chromatography (Salanti et al., 2012) or liquid-state NMR spectroscopy (Lu and Ralph, 2003). In view of the positive effect of ball milling on enzymatic hydrolysis, better understanding of the structural changes induced by ball milling could turn out useful for development of improved hydrothermal or thermochemical pretreatments.

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

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

2 Materials and methods

2.1 Materials

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

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

2.2 Ball milling procedure

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

2.3 Extraction of ball-milled material with aqueous dioxane

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

2.4 Determination of enzyme activities

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

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

2.5 Determination of protein content

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

2.6 Enzymatic treatments

Lignin-carbohydrate fractions (LC1, LC2) or ball-milled extractive-free maize stem samples were mixed to a low consistency (1-2% w/v) suspension in 0.05 M Na-acetate buffer (pH 5) at constant amount of Onozuka R-10 per sample dry matter (4.5 mg protein/g). Hydrolysis reactions were carried out in screw-capped test tubes at 45 °C in continuous free-fall agitation. Sodium azide (0.025%, w/v) was used to prevent microbial growth. Reaction time was 40 h for the LCs and 72 h for the ball-milled samples. Under identical reaction conditions, LC1 and LC2 were treated with PaXyn11A (33 mg protein/g). To obtain enough residual material for lignin analysis, hydrolysis of ball-milled materials was carried out in identical conditions as above, but in stirred suspensions containing 1 g of solids. After reaction, the suspension was centrifuged and 1 mL aliquot was withdrawn from the 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 H_2SO_4 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

2.8 ATR-FTIR spectroscopy

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

2.9 Thioacidolysis

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

2.10 Solubility in pyridine

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

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

3 Results and discussion

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

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

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

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

3.2 Effect of ball milling on maize stem cell wall components

3.2.1 Extractable phenolic and carbohydrate fractions

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

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

3.2.2 Infrared spectra and cellulose crystallinity

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

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

When analyzed from the solid residues after hydrolytic treatment of the milled samples with Onozuka R-10, the infrared spectra showed decreasing intensity in the broad band at 950 cm^{-1}-1100 cm^{-1} with increasing milling time (Fig. S1 in the supplementary material). Decreased intensity in this wavenumber region can be explained by decreasing relative amount of carbohydrates as a result of Onozuka R10 treatment of the solids obtained as a function of increasing milling time. Relative crystallinity of cellulose in the solid residues from enzymatic hydrolysis was lower than before hydrolysis, probably as a result of enrichment of lignin on particle surfaces due to hydrolysis of cellulose from the surfaces (Fig. 3). This is consistent with the suggested penetration depth of ATR-FTIR between 0.5 μm and 1 μm in wheat (*Triticum aestivum* L.) straw with the signal intensity decreasing exponentially with depth of penetration (Kristensen et al., 2008). While changes in cellulose structure could be elucidated from the ATR-FTIR spectra, little information on lignin structure was obtained. Therefore, analytical procedures targeted to providing specific information on changes in 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 C_6C_3 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 μmol/g) and the enzymatic residue
317 from 8 h ball-milled sample (838 μmol/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

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

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

It was recently shown that two structurally distinct lignin-carbohydrate fractions can be isolated from extractive-free maize stems material by sequential alkaline extraction before and after endoglucanase treatment (Sipponen et al., 2013). The first alkali-soluble fraction (LC1) contained lignin with 12-times more units only linked by aryl ether bonds than the second fraction (LC2). To study the effect of lignin-carbohydrate interactions on enzymatic hydrolysis independently of crystalline cellulose, LC1 and LC2 were used as model materials in enzymatic hydrolysis. Based on the ATR-FTIR analysis the relative crystallinities (A1429/A894) of LC1 and LC2 were 2.5 and 3.7, respectively, indicating low crystallinity comparable to the value of 12 h ball-milled maize stem material (A1429/A894 = 3.4). LC1 and LC2 were treated with purified endoxylanase PaXyn11a, Onozuka R-10, or with buffer solution only. These treatments resulted in different levels of carbohydrate conversions. The results show that LC2 was easier material to hydrolyze than LC1 by both PaXyn11a and Onozuka-R10 (Table 2). The latter enzyme gave total carbohydrate conversion from LC2, while the carbohydrate conversion of LC1 was incomplete (83%). Moreover, lignin content of LC2 (18.3%) was comparable to the lignin content of the ball-milled maize stem materials (17.1%). Based on this information it is evident that presence of lignin does not alone restrict complete hydrolysis of carbohydrates (hemicelluloses) with the enzyme dosage used. The

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

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

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

373 Interestingly, PaXyn11a 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

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

4 Conclusions

In this study ball milling was applied to maize stem extractive-free material in order to investigate the impact of this mechanical treatment on both cell wall structure and enzymatic degradability. As for other grasses, ball milling led to severe depolymerization of hemicelluloses and increased enzymatic conversion yield of total polysaccharides to monomeric sugars. The efficiency of the treatment was assigned to the combination of cell wall disruption, decreased cellulose crystallinity and depolymerization of hemicelluloses. Three hour ball milling time turned out to be a plateau ensuring a subsequent 90% enzymatic conversion yield of cellulose into glucose, and among hemicelluloses a 60% release of xylose but only a 20% release of arabinose. Meanwhile, lignin was not significantly affected by ball milling or enzymatic treatment. These results suggested that LCC structures involving lignin and arabinoxylans might be responsible for the recalcitrance of maize cell wall. This hypothesis led us to use two distinct lignin-carbohydrate fractions as models. One of these fractions, associating arabinoxylan-rich hemicelluloses with lignin of lower condensation degree, was found to resist hydrolysis with a total maximum sugar release of 80%. Thus, disruption of lignin-arabinoxylan structures of this type might offer a promising way to improve grass lignocellulose conversion to soluble sugars. Besides results of technological interest towards sugar-platform based lignocellulose biorefinery, this paper showed that ball milling can lead to artificial decrease in thioacidolysis yields, probably by generating physical barrier to the chemical reagents used in the solvolytic cleavage of lignin. Thus,

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

Acknowledgements

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

References

Balakshin, M., Capanema, E., Gracz, H., Chang, H., Jameel, H., 2011. Quantification of lignin–carbohydrate linkages with high-resolution NMR spectroscopy. Planta 233, 1097–1110.

Barrière, Y., Méchin, V., Riboulet, C., Guillaumie, S., Thomas, J., Bosio, M., Fabre, F., Goffner, D., Pichon, M., Lapierre, C., Martinant, J.-P., 2009. Genetic and genomic approaches for improving biofuel production from maize. Euphytica 170, 183–202.

Besombes, S., Mazeau, K., 2005. The cellulose/lignin assembly assessed by molecular modeling. Part 2: seeking for evidence of organization of lignin molecules at the interface with cellulose. Plant Physiol. Biochem. 43, 277–286.

Bradford, M.M., 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. Anal. Biochem. 72, 248–254.

Couturier, M., Haon, M., Coutinho, P.M., Henrissat, P., Lesage-Meessen, L., Berrin, J.-G., 2011. *Podospora anserina* Hemicellulases Potentiate the *Trichoderma reesei* Secretome for Saccharification of Lignocellulosic Biomass. Appl. Environ. Microbiol. 77, 237–246.

da Silva, A.S., Inoue, H., Endo, T., Yano, S., Bon, E.P.S., 2010. Milling pretreatment of sugarcane bagasse and straw for enzymatic hydrolysis and ethanol fermentation. Biores.Technol. 101, 7402–7409.

Dence, C.W., 1992. The determination of lignin. In: Lin, S.Y., Dence, C.W. (Eds.), Methods in Lignin Chemistry. Springer-Verlag, New York, pp. 33–61.

Fujimoto, A., Matsumoto, Y., Chang, H.-M., Meshitsuka, G., 2005. Quantitative evaluation of milling effects on lignin structure during the isolation process of milled wood lignin. J. Wood Sci. 51, 89–91.

Hideno, A., Inoue, H., Tsukahara, K., Fujimoto, S., Minowa, T., Inoue, S., Endo, T., Sawayama, S., 2009. Wet disk milling pretreatment without sulfuric acid for enzymatic hydrolysis of rice straw. Biores.Technol. 100, 2706–2711.

Ghose, T.K., 1987. Measurement of cellulase activities. Pure Appl. Chem. 59, 257–268.

Grabber, J.H., Ralph, J., Hatfield, R.D., 1998. Ferulate Cross-Links Limit the Enzymatic Degradation of Synthetically Lignified Primary Walls of Maize. J. Agric. Food Chem. 46, 2609–2614.

Guerra, A., Filpponen, I., Lucia, L.A., Saquing, C., Baumberger, S., Argyropoulos, D.S., 2006. Toward a Better Understanding of the Lignin Isolation Process from Wood. J. Agric. Food Chem. 54, 5939–5947.

Higuchi, T., Ito, Y., Shimada, M., Kawamura, I., 1967. Chemical properties of milled wood lignin of grasses. Phytochem. 6, 1551-1556.

Ikeda, T., Holtman, K., Kadla, J.F., Chang, H., Jameel, H., 2002. Studies on the Effect of Ball Milling on Lignin Structure Using a Modified DFRC Method. J. Agric. Food Chem. 50, 129–135.

Kristensen, J.B., Thygesen, L.G., Felby, C., Jorgensen, H., Elder, T., 2008. Cell-wall structural changes in wheat straw pretreated for bioethanol production. Biotechnol. Biofuels, 1:5.

Lapierre, C., Pollet, B., Rolando, R., 1995. New insights into the molecular architecture of hardwood lignins by chemical degradation methods. Res. Chem. Intermed. 21, 397–412.

Lu, F.; Ralph, J., 2003. Non-degradative dissolution and acetylation of ball-milled plant cell walls: high resolution solution-state NMR. Plant J. 35, 535–544.

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31, 426–428.

Mooney, C.A., Mansfield, S.D., Touhy, M.G., Saddler, J.N., 1998. The effect of initial pore volume and lignin content on the enzymatic hydrolysis of softwood. Biores. Technol. 64, 113–119.

Nakamura, S., Wakabayashi, K., Nakai, R., Aono, R., Horikoshi, K., 1993. Purification and some properties of an alkaline xylanase from alkaliphilic *Bacillus sp.* strain 41M-1. Appl. Environ. Microbiol. 59, 2311–2316.

O'Connor, R.T., DuPré, E.F., Mitcham, D., 1958. Applications of Infrared Absorption Spectroscopy to Investigations of Cotton and Modified Cottons: Part I: Physical and Crystalline Modifications and Oxidation. Textile Res. J. 28, 382–392.

Rolando, C., Monties, B., Lapierre, C. Thioacidolysis. In: Methods in Lignin Chemistry; Dence, C. W., Lin, S. Y., Eds.; Springer-Verlag: Heidelberg, 1992; pp 334–349.

Salanti, A., Zoia, L., Tolppa, E.-L., Orlandi, M., 2012. Chromatographic Detection of Lignin-Carbohydrate Complexes in Annual Plants by Derivatization in Ionic Liquid. Biomacromolecules 13, 445–454.

Schuerch, C., 1952. The solvent properties of lignins and their relation to the solubility, swelling, isolation and fractionation of lignin. J. Am. Chem. Soc. 74, 5061–5067.

Schwanninger, M., Rodrigues, J.C., Pereira, H., Hinterstoisser, B., 2004. Effects of short-time vibratory ball milling on the shape of FT-IR spectra of wood and cellulose. Vib. Spectrosc. 36, 23–40.

524 Sipponen, M.H., Lapierre, C., Méchin, V., Baumberger, S., 2013. Isolation of structurally
525 distinct lignin-carbohydrate fractions from maize stem by sequential alkaline extractions and
526 endoglucanase treatment. Biores. Technol. 133, 522–528.
527
528 Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., 2008. Determination
529 of sugars, byproducts, and degradation products in liquid fraction process samples.
530 Laboratory Analytical Procedure (LAP), NREL/TP-510–42623, January 2008.
531
532 Van Crayeveld, V., Holopainen, U., Selinheimo, E., Poutanen, K., Delcour, J.A., Courtin,
533 C.M. , 2009. Extensive Dry Ball Milling of Wheat and Rye Bran Leads to in
534 Situ Production of Arabinoxylan Oligosaccharides through Nanoscale Fragmentation J.
535 Agric. Food Chem. 57, 8467–8473.
536
537 Zoia, L., King, A.W.T., Argyropoulos, D.S., 2011. Molecular Weight Distributions and
538 Linkages in Lignocellulosic Materials Derivatized from Ionic Liquid Media. J. Agric. Food
539 Chem. 59, 829–838.
540
541 Zhang, Y., Culhaoglu, T., Pollet, B., Melin, C., Denoue, D., Barrière, Y., Baumberger, S.,
542 Méchin, V., 2011. Impact of Lignin Structure and Cell Wall Reticulation on Maize Cell Wall
543 Degradability. J. Agric. Food Chem. 59, 10129–10135.
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 ± 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 ± 2.7	32.7 ± 1.6	14.1 ± 0.4	25.7 ± 1.1
1	24.2 ± 0.9	72.0 ± 1.2	46.4 ± 0.0	61.3 ± 0.8
2	26.1 ± 1.3	85.6 ± 1.5	57.5 ± 0.4	73.5 ± 0.9
3	23.0 ± 2.6	88.9 ± 1.9	60.0 ± 0.2	76.2 ± 1.2
4	28.2 ± 0.5	92.2 ± 0.2	62.5 ± 1.9	79.4 ± 0.7
5	27.5 ± 0.1	91.8 ± 0.4	62.1 ± 2.1	79.0 ± 0.9
6	25.1 ± 2.3	88.2 ± 3.4	59.8 ± 1.0	75.9 ± 2.4
8	22.0 ± 0.3	89.2 ± 0.6	61.4 ± 0.3	76.9 ± 0.5
12	27.7 ± 0.4	88.6 ± 0.5	61.9 ± 2.4	76.8 ± 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.

	Carbohydrate conversion (%, w w^{-1})[a]			Composition of total released carbohydrates (%)[c]		
Treatment	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.

	Yield of solid residue (% w w^{-1})	ε (mL mg^{-1} cm^{-1})[a]	
Treatment		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 ± 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, 5h , 6 h and 12 h ball-milled samples. The spectra are normalized at
583 280 nm. Errorbars shown in (A) represent ± average deviation relative to the mean value.

584

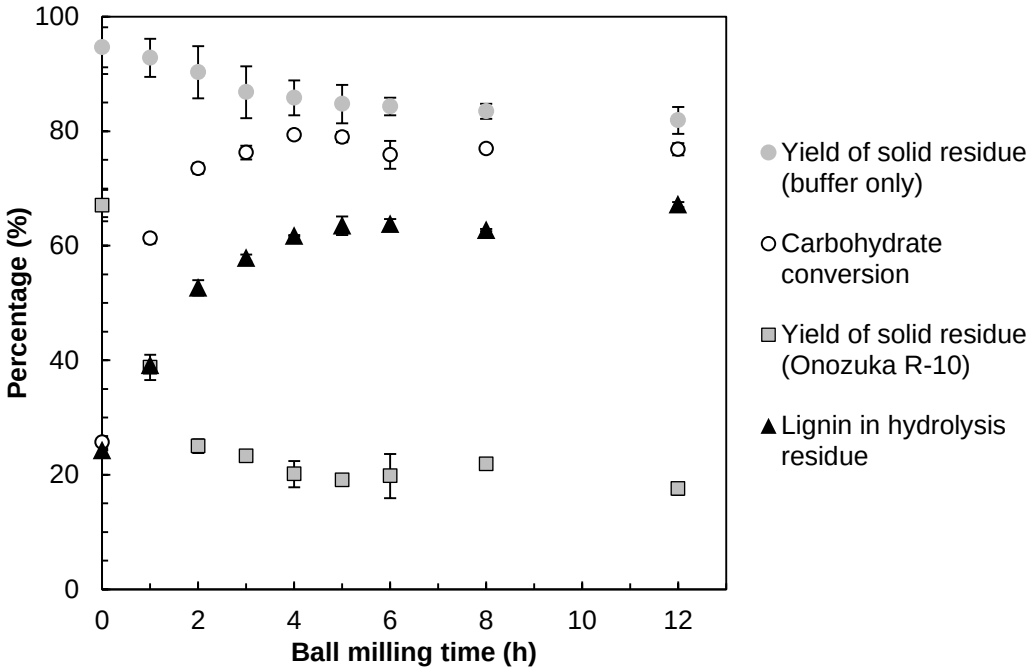
585 **Figure 3.** The effect of ball milling on maize stem samples: (A) fingerprint region of the
586 ATR-FTIR spectra of samples with 0 h to 12 h milling. (B) ratio of absorbance at 1429 cm^{-1}
587 to absorbance at 894 cm^{-1} showing relative changes in cellulose crystallinity in ball-milled
588 maize 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 ± average
594 deviation relative to the mean value.

595

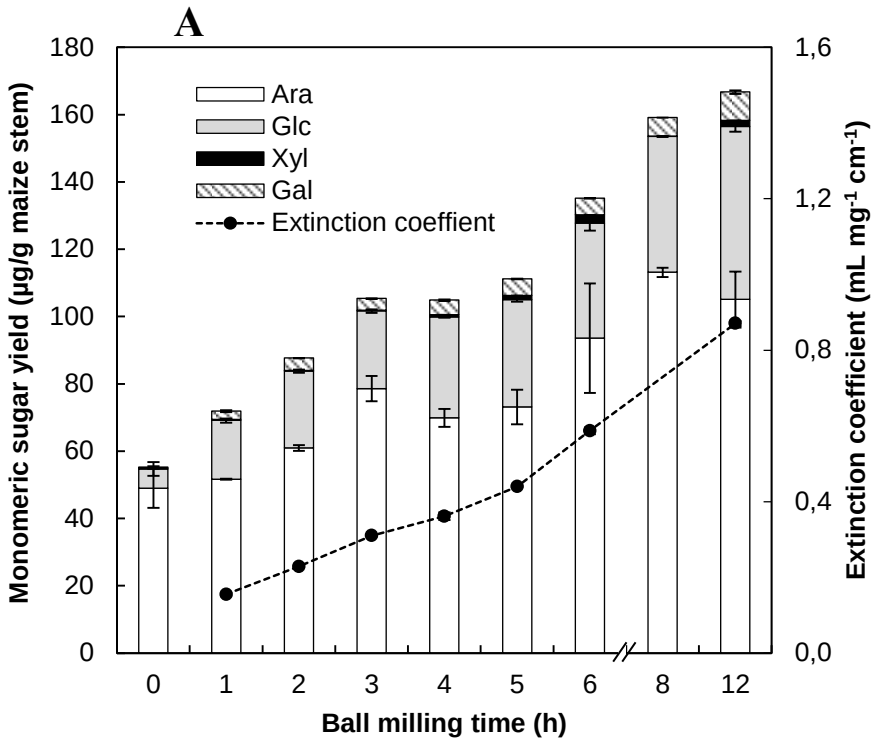
596 **FIGURES**
597



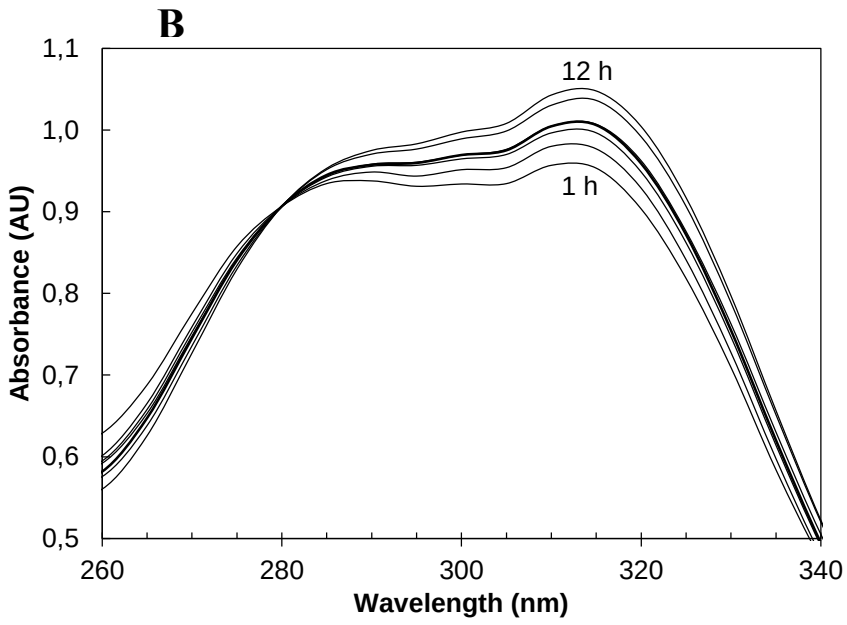
598

599 **Figure 1.**

600



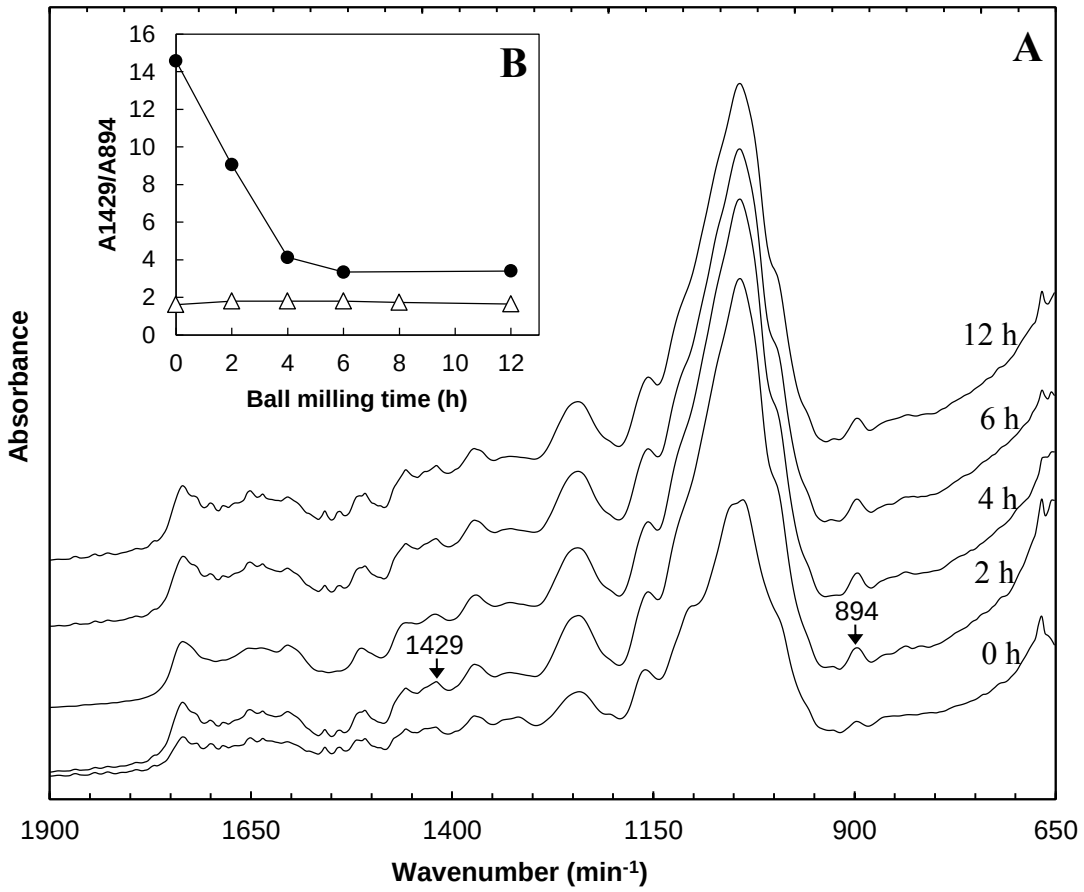
601



602

603 **Figure 2.**

604



605

606 **Figure 3.**

607

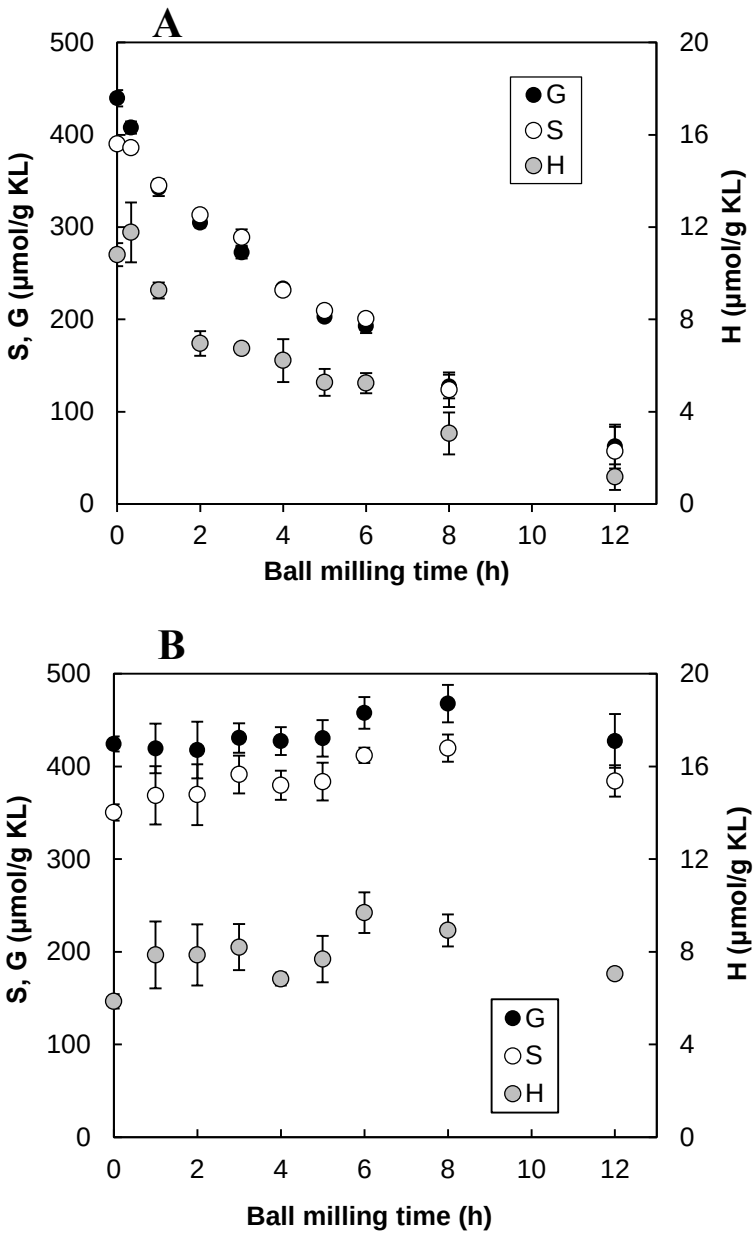

A
B
S, G (µmol/g KL)
H (µmol/g KL)
Ball milling time (h)
G
S
H
0
100
200
300
400
500
0
4
8
12
16
20
0
2
4
6
8
10
12

Figure 4.