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Abstract

Lignocellulosic biomass is a sustainable source of renewable substrate to produce low carbon footprint energy and materials. Biomass conversion is usually performed in two steps: a biomass pretreatment for improving cellulose accessibility followed by enzymatic hydrolysis of cellulose. In this study we investigated the efficiency of a bioextrusion pretreatment (extrusion in the presence of cellulase enzyme) for production of reducing sugars

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from corn crop agricultural residues. Our results demonstrate that bioextrusion increased the reducing sugar conversion yield by at least 94% at high solid/liquid ratio (14% to 40%). Monitoring biomass surface with carbohydrate-binding modules (FTCM-depletion assay) revealed that well known negative impact of high solid/liquid ratio on conversion yield is not due to lack of exposed cellulose, which was abundant under such conditions. Bioextrusion was found to be less efficient on alkaline pretreated biomass but being a mild and solvent limiting pretreatment, it might help to minimize the waste stream.

Keywords

Lignocellulosic biomass, bioextrusion, carbohydrate-binding modules, enzymatic hydrolysis, FTCM, FTCM-depletion assay

1. Introduction

Lignocellulosic biomass (LCB) is a sustainable, renewable, abundant and inexpensive substrate. Its valorization can lead to the production of energy and materials with a low carbon footprint. LCB has been identified as a possible solution to the current energy crisis, characterized by depletion of fossil resources and a pressing need for reduced CO_2 emissions (Lynd, 2017). However, the use of LCB as a substrate for 2nd generation bioethanol production is hampered by its complex structure and recalcitrance to enzyme actions (Himmel *et al.*, 2007). To overcome this limitation, biomass conversion is usually performed in two steps: the first step involves a biomass pretreatment for improving cellulose accessibility, which is followed by enzymatic hydrolysis of cellulose (Himmel *et al.*, 2007; Sun and Cheng, 2002; Khatri *et al.*, 2018a).

The main objective of pretreatments for subsequent biochemical conversion is to increase access to cellulose (also known as cellulose accessibility), which can later be hydrolyzed by enzymatic hydrolysis processes (Khatri *et al.*, 2018a). Twin screw extrusion has been frequently used as the pretreatment of LCB (Karunanithy and Muthukumarappan, 2013).

Extrusion pretreatment has many advantages including high shear, rapid heat transfer, and effective and rapid mixing afforded by good modulation of treatment steps (Vandenbossche et al., 2014). The use of biocatalyst during the extrusion process could improve the biocatalysts impregnation to biomass and boost the subsequent batch saccharification step. Therefore, in an attempt to improve the biocatalysts impregnation, the enzymes were injected during the extrusion process (Duque et al., 2014; Vandenbossche et al., 2015, 2014). This novel and short bioreactive process, named bioextrusion (a mechano-enzymatic pretreatment), has been proven to enhance the enzymatic hydrolysis of the LCB, as summarized by Gatt et al., 2018; Vandenbossche et al., 2015.

One of the key advantages of the bioextrusion process is its ability to work with high substrate loading. This is a critical parameter in order to decrease water consumption and the costs associated with its removal (Lin and Tanaka, 2006). However, high solid loading often decreases hydrolytic efficiency (Ramachandriya *et al.*, 2013). In-depth studies about high solid loadings suggest that low agitation efficiencies, reduction of the contact area between enzymes and substrates, loss of enzymatic activities and non-specific adsorption are some of the factors associated with decrease in hydrolytic efficiency (Ramachandriya *et al.*, 2013). Other conditions can also decrease biomass hydrolysis, such as inhibition of enzymes by reaction products and mass transfer limitations (Hodge *et al.*, 2008; Kim *et al.*, 2008). Therefore, it is important to study the bioextrusion process parameters in great detail in order to cost-effectively maximize the saccharification of the cellulose and hemicellulose components to fermentable sugars.

The main objective of any pretreatment, including bioextrusion, is to improve the subsequent enzymatic hydrolysis of the holocellulose fraction by increasing the access of enzymes to cellulose (also known as cellulose accessibility to enzymes) (Lynd *et al.*, 1999; Wyman, 2013, Khatri *et al.*, 2018a). However, pretreatments vary greatly in the way they help

to expose cellulose and other components. Contrary to other pretreatment methods which uses intensive physical and/or chemical conditions, bioextrusion is a mild mechano-enzymatic pretreatment technique because it requires less energy and water consumption (Vandenbossche et al., 2014). The combination of mechanical and biochemical constraints is believed to overcome limitations associated with high solid and enzymes loadings (Gatt *et al.*, 2018). Currently, our incomplete understanding about the impact of pretreatment (on microstructure) on a particular biomass is believed to be a key issue for reducing costs associated with bioenergy production (Rollin *et al.*, 2011; Zhang and Lynd, 2006; Khatri *et al.*, 2018a). Therefore, is it important to study the effectiveness and impact of pretreatments on a biomass substrate that may play a significant role in a commercial viability of bioenergy production. One of the major difficulties in studying pretreatment and process parameters is the lack of rapid, high throughput and reliable tools for monitoring and/or tracking lignocellulosic polymers at the biomass surface (DeMartini *et al.*, 2013; Khatri *et al.*, 2016). Recently, a rapid and low-cost method has been developed to directly and precisely monitor the surface of wood fibers and agricultural LCB using selected carbohydrate-binding modules (CBMs). Named "fluorescent protein-tagged carbohydrate-binding modules method" (FTCM) this method, and its adaptation FTCM-depletion assay (Khatri *et al.*, 2018a), relies on the use of four specific ready-to-use probes made of recombinant CBMs genetically linked to a designated fluorescent protein of the green fluorescent protein (GFP) family (Hébert-Ouellet *et al.*, 2017; Khatri *et al.*, 2018a, 2018b, and 2016). The FTCM and FTCM-depletion assay have been extensively studied and shown as robust, rapid, easy to use, unambiguous and cost-effective surface characterization methods (Khatri *et al.*, 2018a, 2018b, and 2016; Hébert-Ouellet *et al.*, 2017; Bombeck *et al.*, 2017). Therefore, the use of this surface characterization method for studying the impact of various pretreatments (bioextrusion and alkaline pretreatment) on fiber microstructure provokes substantial interest.

The aim of this work is to study the influence of bioextrusion on a subsequent batch hydrolysis of LCB at high solid to liquid (S/L) ratios. In addition, this study also describes the use of carbohydrate-binding modules to understand the influence of bioextrusion on cellulose accessibility. Raw (RC) and alkaline pretreated corn crop residues (PC) were hydrolyzed in batch conditions for 48 h using a cellulolytic cocktail and different substrate concentrations (S/L ratios from 14 to 40%), with or without a previous bioextrusion step. A number of analyses were performed in order to further our understanding of the impact of bioextrusion and various experimental parameters on corn crop residues and its subsequent enzymatic hydrolysis.

2. Materials and methods

2.1. Biomass: preparation and pretreatments

Raw corn (RC) crop residues (*Zea mays L.*) provided by Ferme Olivier and Sebastien Lépine of Agrosphere Co. (Québec, Canada) were used in this study. Corn crop residue was a mixture of lightly ground cobs, stover and leaves. The entire mixture was milled together to produce a working sample with particle sizes lower than 5 mm.

Raw corn (RC) stover residues were also pretreated by cooking in NaOH solution under pressure in a laboratory digester provided by M/K Systems Inc. (Danver, MA, USA). The digestion conditions were as follows: a liquor ratio of 5 (12% NaOH/g of residue); 175 °C; 60 min digestion period as described by Adjalle *et al.*, 2017. The alkaline pretreated corn (PC) was later washed with a pH 4.5 citrate buffer (50 mM) and dried at 50°C for 48 h. This pretreatment removes a significant portion of the hemicelluloses and lignins, producing a less complex starting material that exhibits an increased enzymatic accessibility (Khatri *et al.*, 2018a).

2.2. Enzyme cocktail

Enzymatic saccharification was carried out using the ACCELLERASE® DUET cocktail from DuPont Industrial Biosciences in a citrate buffer (50 mM, pH 4.5). The enzymatic

cocktail had a protein content of 87.8 mg/ml and a cellulases activity of 115.6 FPU/mL. According to the supplier's specification the ACCELERASE enzyme cocktail is expected to have significant accessory enzymes (xylanase) activity. The protein content was measured with the total protein micro Lowry kit (with Peterson's modification) from Sigma-Aldrich. The cellulase activity was measured according to Ghose, 1987. The enzymatic cocktail had an optimum temperature of 50°C and an optimum pH of 4.5.

2.3. Bioextrusion

Bioextrusion was carried out using a 27 mm twin-screw extruder (Entek, OR, US). Raw or alkaline pretreated corn residues were introduced to the enzymatic cocktail and citrate buffer (to control the S/L ratio) as indicated in Figure 1. Extrusions were performed at 50°C, 125 rpm and the residence time was 5 minutes. The screw configuration was chosen according to the typical bioextrusion screw configuration as described elsewhere (Vandenbossche *et al.*, 2015, 2014; Duque *et al.*, 2014; Gatt *et al.*, 2018). Alternating between transport areas (T) (conveying elements) and intensive mixing areas with kneading blocks (M) promoted both good mixing conditions and isolated areas for reactions to occur (Figure 1). All the operating conditions used here have previously been optimized by Duque et al., 2014; Vandenbossche et al., 2015, 2014. The temperature used here was optimum for the enzymatic hydrolysis. The screw rotation speed of 125 rpm was used to provide the longest residence time possible for the enzyme incubation. The original objective of the bioextrusion was to start the enzymatic hydrolysis in the extrusion pretreatment step, taking advantage of the good impregnation capacities that extrusion has to offer.

Figure 1: Bioextrusion screw configuration. T corresponds to transport areas with conveying elements and M are mixing zones with kneading blocks. Ex corresponds to the bioextrudated biomass. Numbers in first row indicate the pitch of the screws or the angle between the kneading blocks. Numbers in the second row indicate the length of the screw in mm.

2.4. Enzymatic hydrolysis

Enzymatic hydrolysis were performed in 50 mL tubes with 50 mM citrate buffer (pH 4.5), 0.1% sodium azide, and 0.5 mL enzymatic cocktail per g of cellulose (Danisco US Inc., 2007). Tubes were placed in an Ecotron® incubation shaker at 50°C and 200 rpm. Treatments with raw corn and without previous bioextrusion are noted as RC-E; treatments with raw corn and with previous bioextrusion are named as RC-XE; treatments with alkaline pretreated corn and without previous bioextrusion are titled as PC-E; and treatments with alkaline pretreated corn and with previous bioextrusion are abbreviated as PC-XE. These experimental conditions are schematically represented in Figure 2.

Moisture content is one of the most influencing factors for optimal biomass saccharification as described in most of the previous bioextrusion studies (Chouvel *et al.*, 1983; Gatt *et al.*, 2018; Govindasamy *et al.*, 1997 Vandenbossche *et al.*, 2015, 2014). Therefore, its impact was tested by varying the substrate loading. This parameter is expressed as the ratio of grams of solid per 100 g of liquid (S/L ratio). Non-bioextrudated and bioextrudated raw corn (RC-E and RC-XE) were prepared with four different S/L ratios, 14% (± 0.04%), 23% (± 1.00%), 31% (± 0.72%), and 40% (± 0.35%). Non-bioextrudated and bioextrudated alkaline pretreated corn (PC-E and PC-XE) were prepared with two different S/L ratios, 14% (± 0.50%) and 31% (± 1.91%). For PC-E and PC-XE, the S/L ratio of 14% was selected because it corresponds to the enzymes supplier's recommendations and 31% because it allows to evaluation of the influence of a higher substrate loading, without clear process limitations observed with 40% in RC-E and RC-XE. Reactions were stopped by immersing the tubes in boiling water for 5 min and then cooled in an ice bath for 5 minutes. Each experimental condition was carried out in triplicate in separate tubes.

Figure 2: Schematic representation of the experimental and analytical parts. RC, raw corn; PC, alkaline pretreated corn. XE, batch hydrolysis with previous bioextrusion step; E, batch hydrolysis without previous bioextrusion step; B, batch hydrolysis process. Path 1: samples diluted to 5% of dry content. Path 2: Non-diluted samples.

2.5. Analytical methods

2.5.1. Dry matter and parietal compounds

Moisture content was determined according to the French standard procedure NF V 03-903. A relative proportion of each of the three parietal constituents (cellulose, hemicelluloses, and lignins) contained in the solids was measured using the ADF-NDF method (Van Soest and Wine, 1968). All determinations were carried out in triplicate.

2.5.2. Sugars analysis

Tube content (Figure 2, path 1) was diluted with distilled water in order to reach 5% of dry matter and was centrifuged at 5,000 g for 10 min at 20°C. The reducing sugars in the supernatant were determined using the DNS method (Miller, 1959). The quantity of reducing sugars was used to calculate the rate of holocellulose deconstruction (r_{HD}). It is expressed as the ratio of reducing sugars in the sample, measured in terms of glucose equivalents, over the initial mass of holocellulose. Results were corrected with blank values obtained at t = 0 h without bioextrusion. However, as this method titrates all the reducing functions of the different sugars in solution (e.g. oligosaccharides, pentose, hexoses etc.), specific glucose concentration was also measured using high performance liquid chromatography (HPLC). HPLC analysis were carried out with a Rezex RHM-Monosaccharide with deionized water as the eluent in isocratic mode, with a flow rate fixed at 0.6 mL/min. Column was kept at 85°C and RI detector at 50°C (Sluiter et al., 2010).

2.5.3. Particle size distribution

After 48h of enzymatic hydrolysis, the samples were washed with distilled water and filtered through glass fiber Whatman 934 AH filters. These filters have a porosity of 1.5 µm. Retained solids were then dried at 103°C during 12 h prior to mass measurements. Dry matter left after filtration was compared to the initial dry matter in order to calculate the proportion of dry solid with particle size smaller than 1.5 µm ($p_{\emptyset \leq 1.5\ \mu m}$).

$$p_{\emptyset \leq 1.5\ \mu m} = \frac{m_{dt} - m_{df}}{m_{dt}}$$

m_{dt}: dry mass before filtration

m_{df}: residual dry mass after filtration

2.5.4. Cellulose accessibility (surface cellulose exposure) using FTCM-depletion assay

After 48 h of enzymatic hydrolysis, the unhydrolyzed residues were filtered, dried and grounded. FTCM-depletion assay was used on these unhydrolyzed solid residues in order to detect the total cellulose accessibility or surface cellulose (both crystalline and amorphous cellulose) exposure after the various chemical, mechanical and enzymatic treatments described above. The tracking assay was performed as described by Khatri *et al.*, 2018a with two different FTCM probes: eGFP-CBM3a (GC3a) specific to crystalline cellulose and eCFP-CBM17 (CC17) specific to amorphous (non-crystalline) cellulose. Probe production and characterization (spectroscopic maxima, affinity to related substrate, and discrimination among substrates) were described in earlier reports (Hébert-Ouellet *et al.*, 2017; Khatri *et al.*, 2018a, 2018b, and 2016; Bombeck *et al.*, 2017). Fluorescence measurements were recorded at room temperature using a Synergy Mx microplate reader (BioTek). Fluorescence values were further converted into µmol of fluorescent probes per g of dry biomass, using the appropriate fluorescence standard curves for each probe. FTCM-depletion assay allows to specifically measure the crystalline and amorphous cellulose exposure at the surface of the biomass (Khatri *et al.*, 2018a).

3. Results and discussion

3.1. Biomass characterization

Figure 3 represents biomass composition characterization of raw (RC) and alkaline pretreated corn (PC).

Figure 3: Biomass composition before and after alkaline pretreatment step indicated on dry weight basis (dwb). Error bars represent the standard deviation.

The alkaline pretreatment led to the removal of a significant part of the extractives such as proteins, starches, pectins, tannins present in the raw biomass (Sluiter *et al.*, 2010). In addition, the alkaline action also induces the solubilization of most of the lignins and hemicelluloses fractions. The amount of lignin in the RC biomass was 10.9%, which is similar to the earlier published reports on identical biomass (Adjalle *et al.*, 2017, Khatri *et al.*, 2018a). The alkaline pretreatment reduced the amount of lignin by ~ 65% which is compatible with the known effect of alkaline pretreatment (Adjalle *et al.*, 2017, Khatri *et al.*, 2018a). Furthermore, the total cellulose content (detected by ADF-NDF protocol) was significantly increased (~ 78%) by alkaline pretreatment (Figure 3) which is in accordance with the known impact of this widely used pretreatment technique (Kim *et al.*, 2016). The alkaline pretreated biomass was a mixture of relatively less colored, swollen and broken fibers, which is compatible with a decreased content of colored lignin.

The main objective of pretreatment for subsequent biochemical conversion is to increase access to cellulose (also known as cellulose accessibility) by removing most of the lignin and hemicelluloses. From total composition analysis (ADF-NDF analysis; Figure 3), one can reasonably infer that more cellulose will become available at the fiber surface when lignin and/or hemicelluloses are partially removed from biomass. However, such an interpretation of pretreatment impact is indirect as total composition analysis represents a bulk analysis and does not interrogate fiber surface properties (such as cellulose accessibility) (Khatri *et al.*, 2018a).

The surface exposure or surface accessibility of lignocellulosic polymers is an important substrate characteristic that influences the enzymatic hydrolysis rates (Arantes and Saddler, 2010; Hong *et al.*, 2007; Mansfield *et al.*, 1999; Zhang and Lynd, 2004). A recent study using FTCM-depletion assay probes reported that enzyme access to cellulose (total cellulose surface accessibility) was a determinant for saccharification yield (Khatri *et al.*

255 2018a). Therefore, FTCM-depletion assay was used in this study to specifically detect the
256 proportion of the surface exposed crystalline and amorphous cellulose at the fiber surface of
257 raw and pretreated biomasses.

258 Figure 4 represents the surface exposure or surface accessibility of cellulose in raw
259 (RC) and pretreated (PC) corn crop residues (before enzymatic hydrolysis). Results show that
260 the amount of bound FTCM probes (µmol) per gram of biomass (which depends on their
261 cognate lignocellulosic component(s) surface exposure or surface accessibility) has increased
262 after the alkaline pretreatment step. When comparing to RC crop residues, the total cellulose
263 exposure (GC3a + CC17 probes binding) or total cellulose surface accessibility of PC crop
264 residues increased 2-fold (Figure 4A). The increase in total cellulose exposure (GC3a + CC17
265 probes binding) on the fiber surface is accompanied by the 2.01 ± 0.09-fold increase in
266 crystalline cellulose (GC3a probe binding) and 2.30 ± 0.07-fold increase in amorphous
267 cellulose (CC17 probe binding) (Figures 4B and C). This indicates that the surface
268 accessibility of cellulose has increased after the alkaline pretreatment. Furthermore, the
269 increase of the amorphous cellulose exposure was slightly higher than the increase of the
270 crystalline cellulose exposure (Figures 4B and C). One explanation of this difference involves
271 the well-known negative impact of the alkaline pretreatment on cellulose crystallinity (Fan *et*
272 *al.*, 1987; Sun and Cheng, 2002). The increase in the surface accessibility of cellulose or
273 FTCM probe binding can also be relate to the Brunauer-Emmett-Teller (BET) surface area of
274 the biomass. Several studies have suggested that alkaline pretreatment is expected to increase
275 the surface area of fibers (Boonsombuti *et al.,* 2013; Kim *et al.,* 2014; Lee *et al.,* 2015; Song
276 *et al.,* 2016) which might reflect the increased binding of FTCM probes. These results are also
277 compatible with the expected impact of similar alkaline pretreatment on corn crop residues
278 (Khatri et al.*et al.*, 2018a).

279

Figure 4: Surface exposure of A) total cellulose, B) crystalline cellulose and C) amorphous cellulose before and after alkaline pretreatment as monitored by FTCM-depletion assay. Error bars represent the standard deviation.

3.2. Biomass deconstruction

3.2.1. Sugar conversion rates and biomass fractionation

As mentioned previously, the increase in total cellulose accessibility is expected to enhance the subsequent hydrolysis efficiency. The impact of different treatments on production of reducing sugars (Figure 5A), glucose production yield (Figure 5B) and biomass fractionation (Figure 5C) are presented in Figure 5.

Figure 5: A) impact of S/L ratio on reducing sugar conversion rates after 48 h of enzymatic hydrolysis. r_{HD} is the rate of holocellulose deconstruction (or reducing sugars conversion), expressed as grams of glucose equivalents per 100 grams of holocellulose into the initial biomass. B) glucose production yield (% of theoretical), after 48 h enzymatic hydrolysis. C) fraction of total dry biomass smaller than 1.5 µm after 48 h of enzymatic hydrolysis. RC-E, raw corn without a previous bioextrusion step; RC-XE, raw corn with a previous bioextrusion step; PC-E, alkaline pretreated corn without bioextrusion; PC-XE alkaline pretreated corn with previous bioextrusion. Error bars represent the standard deviation.

3.2.2.Influence of solid loadings.

In all cases, increasing solid concentration led to a gradual decline in the holocellulose hydrolysis. In particular, increasing the S/L ratio from 14% to 40% led to a 42% and 25% decrease in the reducing sugars production for non-bioextrudated (RC-E) and bioextrudated (RC-XE) raw corn, respectively (Figure 5A). In addition, the S/L ratio increment led to a 47% and a 52% decrease in the final glucose production yield for RC-E and RC-XE, respectively (Figure 5B). In the case of PC, no significant differences between non-bioextrudated (PC-E) and bioextrudated (PC-XE) biomasses were observed. Reducing sugar production and glucose production yield dropped by at least 36% and 35%, respectively, when increasing the S/L ratio from 14% to 31%. Due to the resource limitations only two conditions of the S/L ratio were used in the case of PC. Therefore, one has to be careful while making any strong and critical interpretation in case of the PC biomass. Regardless, these results clearly demonstrate the negative influence of solid loadings on reducing sugar production and glucose production yield, despite of using alkaline pretreatment or bioextrusion. The negative influence of the solid loading factor on the hydrolysis has been observed with other biomasses and batch conditions (Du *et al.*, 2017; Geng *et al.*, 2015).

3.2.3.Influence of the alkaline pretreatment step

Alkaline pretreatment of the biomass led to a significant increase of the final sugar conversion rates. The highest total reducing sugars production (~ 68%) and glucose production yield (~ 53%) were obtained with the alkaline pretreated biomass (Figures 5A and B). These results are also fully compatible with the higher surface cellulose exposure (both crystalline and amorphous) detected for PC biomass by FTCM-depletion assay (Figure 4). Alkaline pretreatment has been shown to improves the enzymatic hydrolysis of biomass (Sun and Cheng, 2002). Furthermore, there was no significant difference in the reducing sugar production and glucose production yield between the non-bioextruded (PC-E) and bioextruded (PC-XE) alkaline pretreated corn (Figures 5A and B). The efficiency of the

pretreatment used in this study could explain the absence of impact from the additional bioextrusion step. The efficient alkaline pretreatment highly improves the enzymatic accessibility thus strongly reducing the need for an additional biocatalyst impregnation step (bioextrusion).

3.2.4.Influence of the bioextrusion step.

In RC biomass, bioextrusion increased the total reducing sugar conversion by at least 94% (in the case of 14% S/L ratio) for all S/L ratio conditions (Figure 5A). The highest increase of 149% was seen in the case of 40% S/L ratio. In the case of glucose production yield, bioextrusion increased the glucose production yield rates by at least 31% (in the case of 40% S/L ratio) and the highest increase of 49% was observed for S/L ratio of 31% (Figure 5B). The bioextrusion's positive influence on glucose production yield was less pronounced than on total reducing sugar conversion (Figures 5A and B). However, one must be aware that reducing sugar measurements are non-specific. DNS analysis also titrates poly/oligosaccharides and monosaccharides reducing ends obtained from hemicellulose hydrolysis. This may explain the observed differences between glucose conversion yield and total reducing sugars conversions. It should be noted that the maximal glucose conversion yield achieved with bioextrusion (RC-XE) was 23% (in the case of 14% S/L ratio) compared to the mere 16% (in the case of 14% S/L ratio) conversion yield measured for non bioextrusion (RC-E) residues (Figure 5B), indicating that it did improve hydrolysis, although much less efficiently than alkaline pretreatment (~ 50% glucose conversion yield). The positive influence of bioextrusion on glucose production yield is clearly negatively influenced by the solid loading but to a lesser extent than on reducing sugar conversion (Figures 5A and B).

Mixing the enzymes with biomass during mechanical treatment using the bioextrusion technique appears to significantly improve the enzymatic hydrolysis of RC. These enhanced

hydrolysis results can be associated with good bioextrusion dispersive and distributive mixing conditions, especially by means of the kneading blocks. These characteristics can improve mass transfer, agitation efficiency and allow higher contact between enzymes and substrate. These significant improvements are obtained without high temperatures or strong chemicals and with relatively very high solid loadings (up to 40 % of S/L ratio). Bioextrusion positive influence can be used as a way to increase the sugar production for a given substrate loading, or as a way to retain good hydrolysis outcomes with higher substrate loadings and reduced water consumption.

In the case of PC, comparable conversion rates were obtained with or without the bioextrusion step (Figures 5A and B). This information highlights that the good mixing conditions of the bioextrusion step did not afford any additional advantage when the intensive alkaline pretreatment was used.

3.2.5. Impact on biomass fractionation

The proportion of dry mass loss after 48 h of enzymatic hydrolysis was measured and analyzed for biomass fractionation using particle size distribution for all reaction conditions (Figure 5C). The changes in proportion of particles under 1.5 µm should corroborate the sugar conversion rates discussed above (Figures 5A and B).

It was apparent that increasing the S/L ratio led to a diminished production of small particles (dry biomass smaller than 1.5 µm) (Figure 5C). This suggests that the deconstruction of biomass by enzymes is relatively hindered at higher substrate loadings, which also explains the negative influence of solid loadings on sugar conversion rates (Figures 5A and B). Sugar production is a result of holocellulose deconstruction by enzymatic hydrolysis that is dependent on biomass fractionation (Samaniuk *et al.*, 2011). However, the correlation between size and hydrolysis varies depending on the initial biomass and the process conditions. In raw biomass, bioextrusion (RC-XE) promoted particles size reduction when

372 compared to RC-E. Indirectly, this explains the impact of bioextrusion on increased
373 enzymatic hydrolysis which is in agreement with reducing sugar conversion rates and glucose
374 production yield (Figures 5A and B).

375 Furthermore, alkaline pretreated biomass exhibited maximum biomass fractionation when
376 compared to raw biomass. Figure 5C shows that for PC-E and PC-XE the difference in terms
377 of particle size distribution between 14 and 31% of S/L ratios is relatively less pronounced
378 than the difference in terms of conversion rates (Figures 5A and B). Moreover, a slight
379 increase (~ 9%) of the proportion of particles under 1.5 µm is observable for 31% of S/L ratio
380 for the PC-XE (Figure 5C). This may indicate a small improvement of the fractionation using
381 the bioextrusion step even when an intense alkaline pretreatment step was used.

382 ### 3.2.6. Surface exposed cellulose after enzymatic hydrolysis.

383 The maximum observed glucose production yield of ~ 50% suggests that there must be
384 a significant amount of unhydrolyzed biomass retained. Therefore, after hydrolysis (48 h) of
385 various pretreated corn crop-residues, the unhydrolyzed components were tracked by FTCM-
386 depletion assay. Figures 6 A-D represent crystalline and amorphous cellulose accessibility
387 profiles before and after hydrolysis, for both RC and PC biomass, as detected by FTCM-
388 depletion assay. Equivalent quantities (*i.e.* 25 mg) of biomass residues were used for all the
389 FTCM tests presented in Figure 6.

390

391 *Figure 6. Surface accessibility profile of lignocellulosic components in corn crop residues after enzymatic*
392 *hydrolysis at various S/L ratios. A) FTCM probes signals for RC-E, compared to raw biomass (RC) B) RC-XE*
393 *samples compared to RC C) PC-E samples compared to pretreated corn (PC) D) PC-XE samples compared to*
394 *PC. RC-E, raw corn without a previous bioextrusion step; RC-XE, raw corn with a previous bioextrusion step;*
395 *PC-E, alkaline pretreated corn without bioextrusion; PC-XE, alkaline pretreated corn with previous*
396 *bioextrusion. Green and cherry color represents GC3a and CC17 FTCM probe, respectively. RC represents the*
397 *surface accessibility profile unhydrolyzed raw corn crop residues (from Figure 5). PC represents the surface*
398 *accessibility profile of unhydrolyzed alkaline pretreated corn crop residues (from Figure 5). The RC and PC*
399 *surface accessibility profiles are used here for comparison purposes. Error bars represent the standard deviation.*

400 The fiber surface exposure of raw biomass after batch hydrolysis was dominated by
401 crystalline cellulose (2.5-3 fold higher than amorphous cellulose) under all S/L ratio (Figure
402 6A). This indicates that in all cases, the exposed outer surface is mostly composed of
403 crystalline cellulose and that S/L ratio did not lead to preferential hydrolysis of either
404 cellulose form. The dominance of crystalline cellulose in RC biomass is fully compatible with
405 previously measured crystallinity indexes for raw corn crop residues (Kumar et al., 2009).

406 Cellulose accessibility profile of RC-E exhibited a gradual increment in both
407 crystalline and amorphous cellulose with respect to the S/L ratio (Figure 6A). This
408 comparatively higher cellulose surface exposure is due to the inherent nature of enzymatic
409 hydrolysis. Enzymatic hydrolysis is a layer by layer process *i.e.* after the complete hydrolysis
410 of an exposed polysaccharide layer, a new, previously buried or hidden, layer with different
411 surface characteristics will expose. In this case, after 48 h of enzymatic hydrolysis, it seems
412 that in all the S/L ratios a new polysaccharide layer with higher amount of both crystalline
413 and amorphous cellulose is exposed. Furthermore, this increase in the surface cellulose
414 exposure could also be related to the increase in the substrate loading. Higher substrate
415 loading with limited enzyme supplementation or non-optimum reaction conditions is expected
416 to leave behind a significant amount of unhydrolyzed substrate, thus increasing the detection
417 of both crystalline and amorphous cellulose.

418 This suggests that enzymatic hydrolysis of RC biomass became more and more
419 inefficient at higher S/L ratio. The higher content in exposed cellulose left after hydrolysis is
420 fully compatible with the decreased sugar conversion yield observed for higher S/L ratio
421 (Figures 5A and B). The detection of larger content of exposed cellulose in higher S/L ratio
422 appears to be a direct consequence of inability of enzyme to achieve efficient cellulose
423 degradation in low water biomass. It also suggests that inefficient conversion at high S/L ratio
424 is not a result of lack of exposed cellulose, which was abundant under such conditions.

Similar trends were observed regarding the cellulose accessibility profiles of RC-XE biomass (Figure 6B). The amount of available cellulose at surface grew as the S/L ratio increased, corroborating sugar conversion yield indicating the negative impact of S/L ratio on enzymatic efficiency (figures 5A and B). Further, comparing results from panel A to panel B allows to emphasize the positive impact of bioextrusion on following batch hydrolysis. Comparing to RC-E cellulose accessibility profile, both crystalline and amorphous cellulose accessibility in RC-XE biomass was at least 1.25-fold lower at any given S/L ratio. This is due to an additional bioextrusion pretreatment step which led to enhanced enzymatic hydrolysis of the corn crop residues biomass as discussed earlier (Figures 5A and B). Again, in the RC-XE biomass, the fiber surface exposure was dominated by crystalline cellulose. The amorphous cellulose exposure was 2.5-3-fold lower than crystalline cellulose, suggesting that S/L ratio did not change cellulose populations (crystalline versus non-crystalline).

Figure 6C represents the cellulose accessibility profile of PC-E biomass. The result exhibits an important decrement in both crystalline and amorphous cellulose with respect to the S/L ratio. The improved enzymatic hydrolysis is likely to have hydrolyzed most of the biomass, thus leaving behind significantly lower amount of unhydrolyzed cellulose. These observations are fully compatible with the results presented in Figures 5A and B showing that alkaline pretreatment lead to the highest sugar conversion yield. The fiber surface exposure in PC-E biomass was dominated by crystalline cellulose. On the other hand, very small amount of amorphous cellulose was detected by FTCM probes for PC-E which is due to the preferential vulnerability of disordered cellulose. In the case of PC-XE biomass (Figure 6D), FTCM probes indicate a further degradation of cellulose afforded by the addition of bioextrusion to alkaline pretreatment. This additional degradation corroborates results on particle size reduction where a slight increase (~ 9%) in percentage of small particles was observed for PC-E vs PC-XE (Figure 5C). However, the additional degradation suggested

here remains small, and did not lead to significant increase in sugar production yield. In summary, the FTCM-depletion assay strongly suggests that after 48 h of enzymatic hydrolysis of RC biomass, there was still a substantial amount of unhydrolyzed cellulose left which could be hydrolyzed by improving the enzyme concentration and/or hydrolysis time. This also suggests that the FTCM-depletion assay could be used to characterize the unhydrolyzed biomass for optimum enzymatic hydrolysis. Bioextrusion significantly reduced the unhydrolyzed total cellulose amount which may be linked with good dispersive and distributive mixing conditions, overcoming some limitations associated with high solid loadings (Ramos *et al.*, 2015) and biomass microstructure. Alkaline pretreatment led to a strong reduction of unhydrolyzed total cellulose on the surface, which is compatible with extended biomass deconstruction (Figure 5C) and hydrolysis (Figures 5A and B). The strong alkaline pretreatment conditions led to a very limited influence of the previous bioextrusion step. However, this finite influence was only detectable via FTCM-depletion assay.

4. Industrial perspectives

Bioextrusion demonstrated a significant improvement for the raw LCB valorization. In raw LCB, this mild condition pretreatment proved to be efficient with very high substrate loadings (up to 40% of solid to liquid ratio) and allowed good hydrolysis conditions while increasing the substrate concentration. In addition, extrusion is a continuous process that is easily adaptable to industrial scale. Therefore, bioextrusion would be a viable option for industries to obtain concentrated sugars, especially for second generation bioethanol production, while keeping mild pretreatment conditions with a very limited impact of water consumption and removal.

However, the best hydrolysis outcomes were obtained with severe alkaline pretreatment, which seems to strongly reduce the positive influence of the bioextrusion step. This result supports the idea that the removal of the ligno-hemicellulosic barrier allows good enzymatic

impregnation of the biomass even without bioextrusion. Nevertheless, LCB pretreatment is one of the costliest step in the biological production of cellulosic ethanol (about 20% of the total cost) (Yang and Wyman, 2008). Such a severe alkaline pretreatment, close to the conditions used in the paper industry, is not economically viable. This suggests that there is a challenge to find a milder alkaline pretreatment conditions which could go along with bioextrusion to improve the LCB hydrolysis with high solid loadings. Existing mild reactive extrusion pretreatments (Duque *et al.*, 2017; Vandenbossche *et al.*, 2016) could be a solution to limit the inhibiting side-effects of strong physico-chemical pretreatments, while allowing process continuity with bioextrusion.

Furthermore, FTCM-depletion assay allowed inspection of the cellulose accessibility profile for both raw and alkaline pretreated corn crop residues. Total cellulose accessibility was found to be higher for alkaline pretreated biomass. To our knowledge, this is the first time the FTCM methodology has helped to characterize the unhydrolyzed materials (after 48h of enzymatic hydrolysis). Compare to alkaline pretreated biomass, the raw biomass exhibited significantly higher total cellulose accessibility in the unhydrolyzed biomass after 48 h of enzymatic hydrolysis. Moreover, it demonstrated that surface exposed cellulose was mainly in its crystalline (most resistant) form. The FTCM-depletion assay findings supported the information obtained with classical analytical techniques. FTCM-depletion assay may offer considerable potential to optimize and increase the overall performance of the biomass pretreatment strategies for biofuel production technologies.

5. Conclusions

The bioextrusion pretreatment enhanced the subsequent batch hydrolysis of raw corn crop residues at high solid loadings (solid/liquid ratio up to 40%). The positive influence of bioextrusion was negatively influenced by the solid loading(s) The surface cellulose

accessibility analysis via FTCM-depletion assay supported classical analyses and exhibited that the negative impact of high solid/liquid ratio on conversion yield were not associated with a lack of exposed cellulose. Bioextrusion was found to be less efficient on severe alkaline pretreated biomass but being a mild and solvent limiting pretreatment technique, it might help to decrease the pretreatment waste stream.

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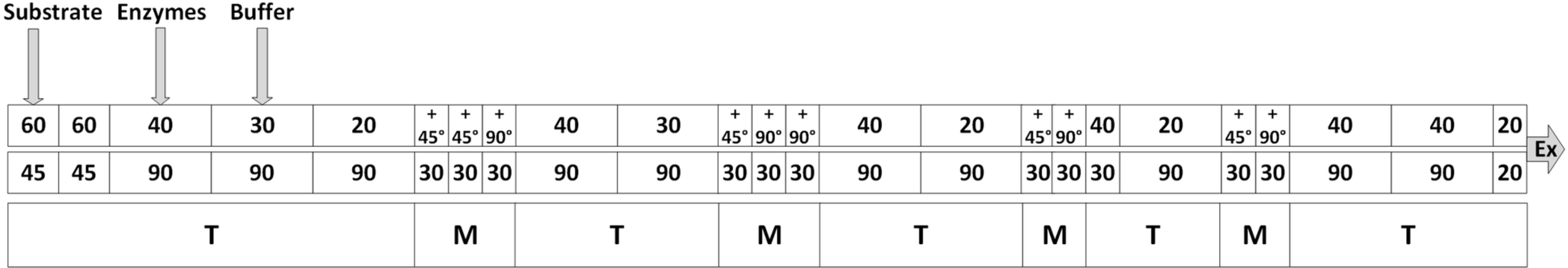
Substrate
Enzymes
Buffer
60 60 40 30 20 + 45° + 45° + 90° 40 30 + 45° + 90° + 90° 40 20 + 45° + 90° 40 20 + 45° + 90° 40 40 20
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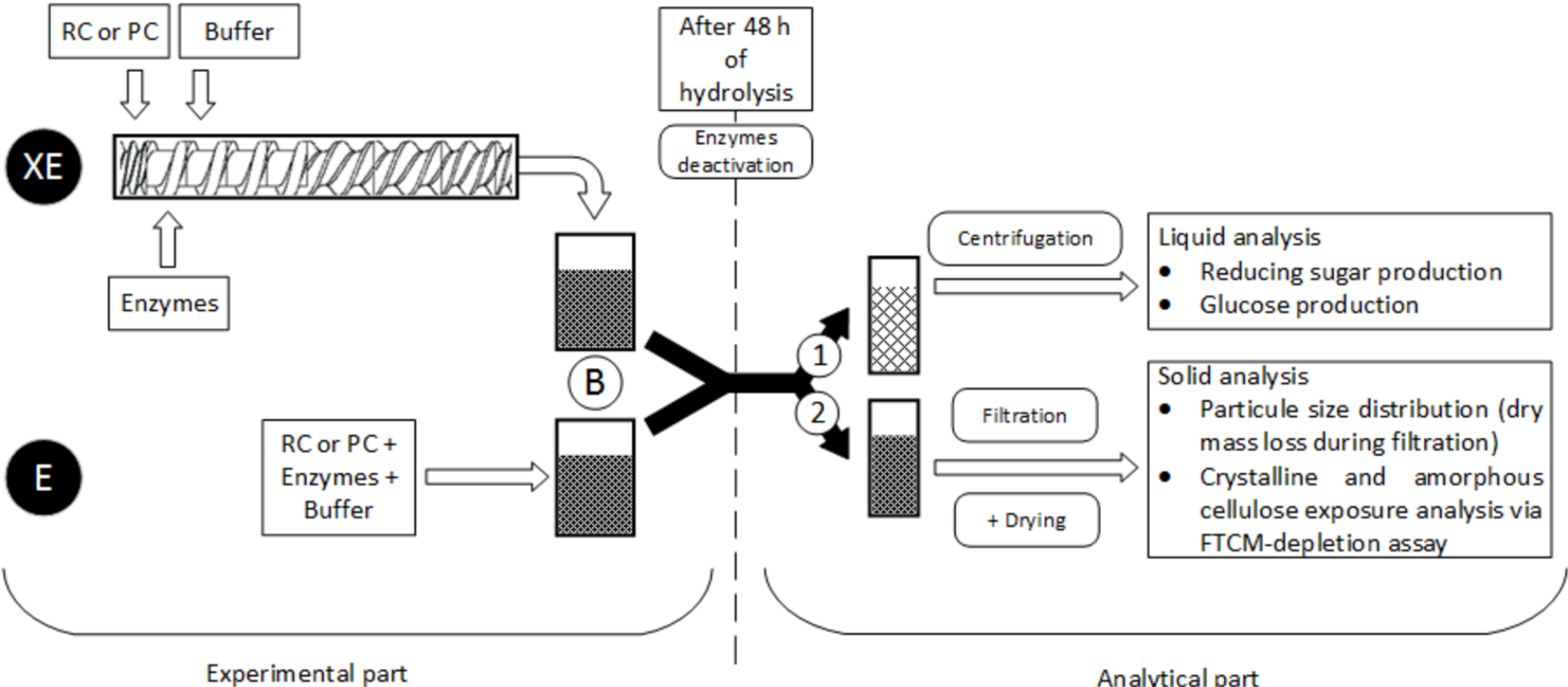
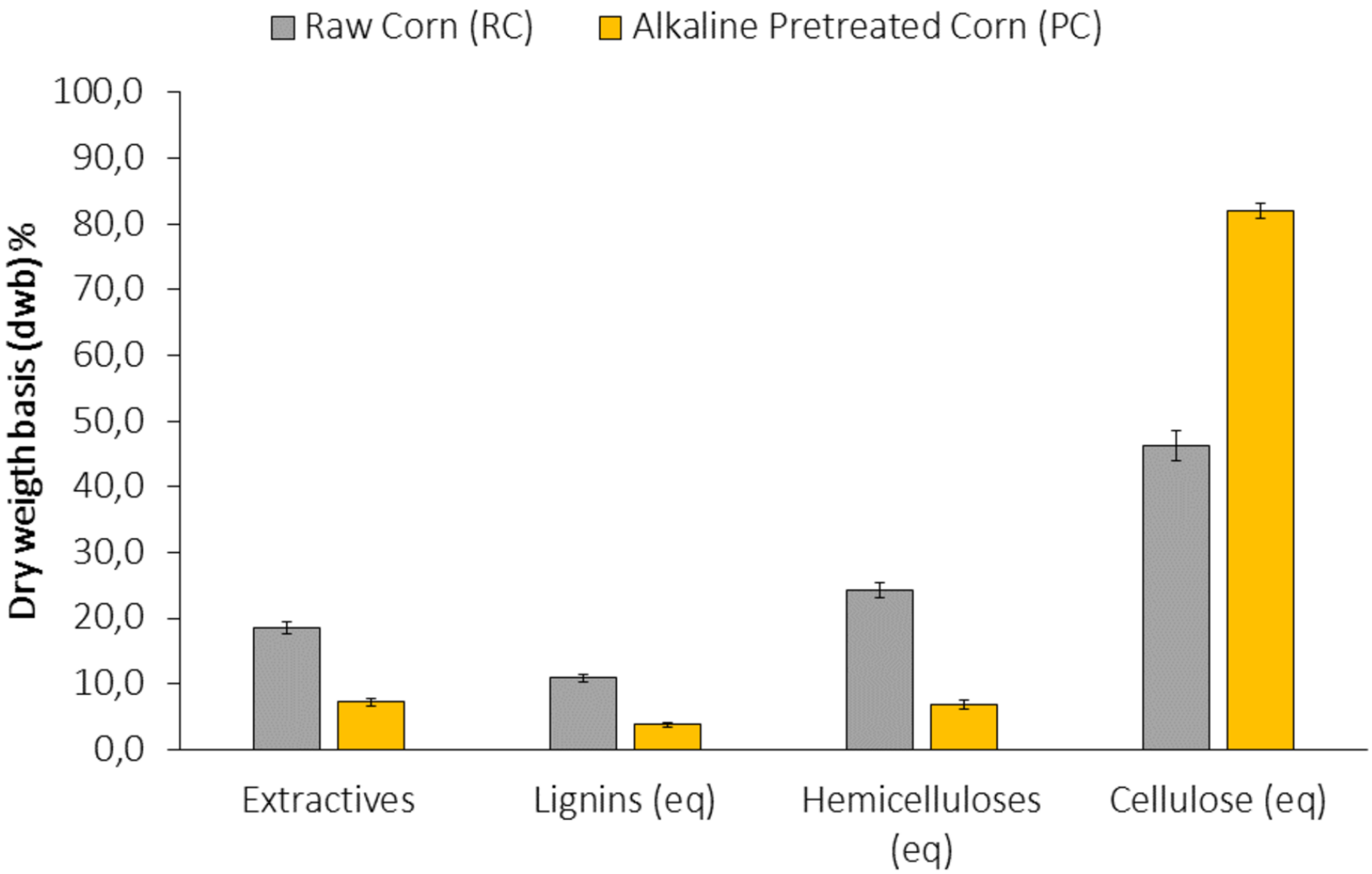
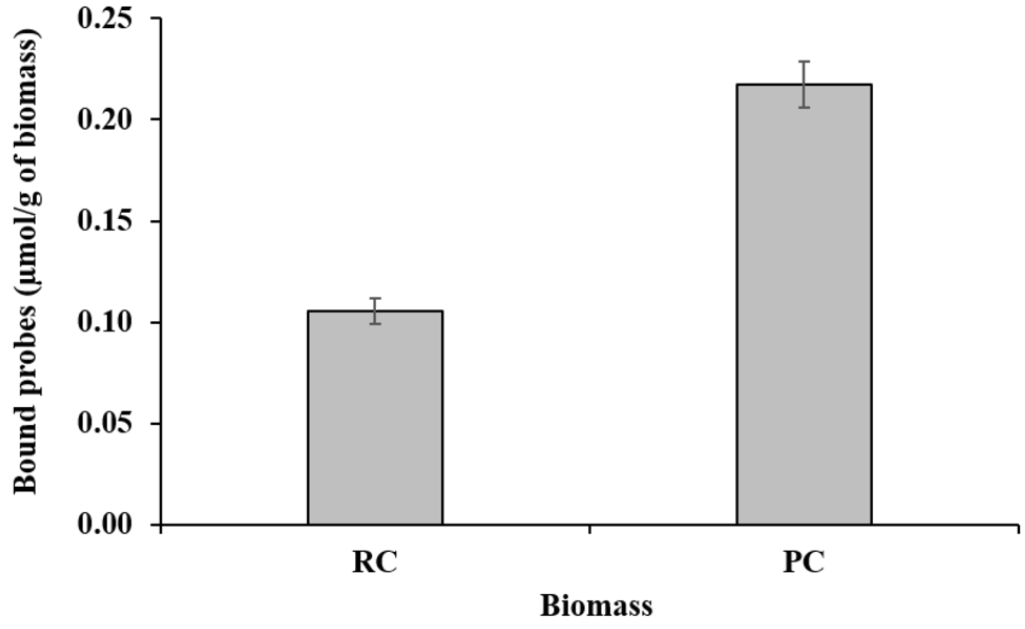
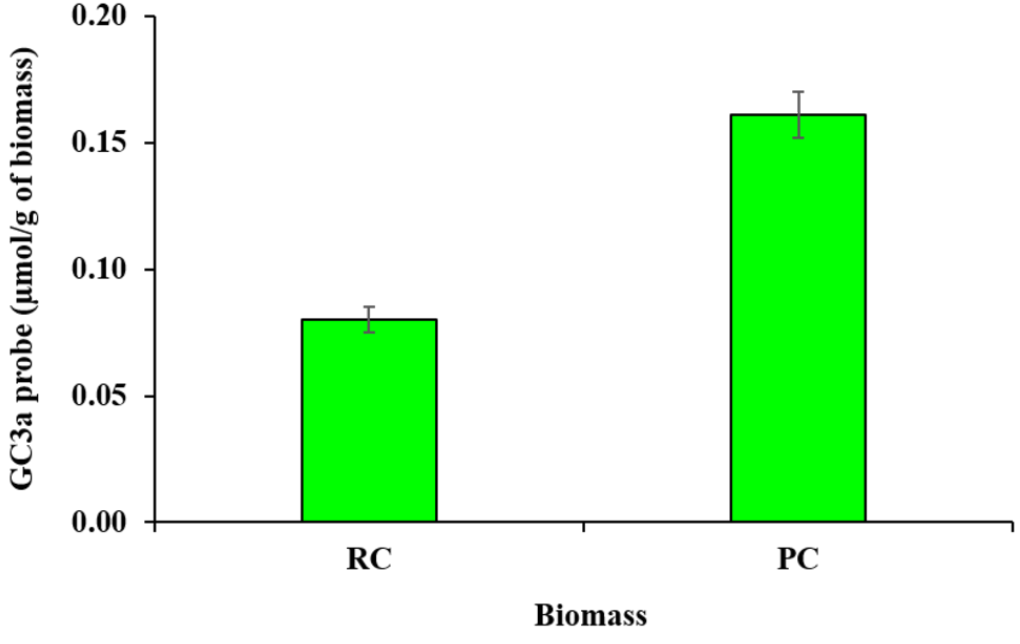

RC or PC
Buffer
XE
Enzymes
After 48 h of hydrolysis
Enzymes deactivation
B
E
RC or PC + Enzymes + Buffer
1
2
Centrifugation
Liquid analysis
Reducing sugar production
Glucose production
Filtration
+ Drying
Solid analysis
Particule size distribution (dry mass loss during filtration)
Crystalline and amorphous cellulose exposure analysis via FTCM-depletion assay
Experimental part
Analytical part


Raw Corn (RC)
Alkaline Pretreated Corn (PC)
Dry weigth basis (dwb) %
100,0
90,0
80,0
70,0
60,0
50,0
40,0
30,0
20,0
10,0
0,0
Extractives
Lignins (eq)
Hemicelluloses (eq)
Cellulose (eq)

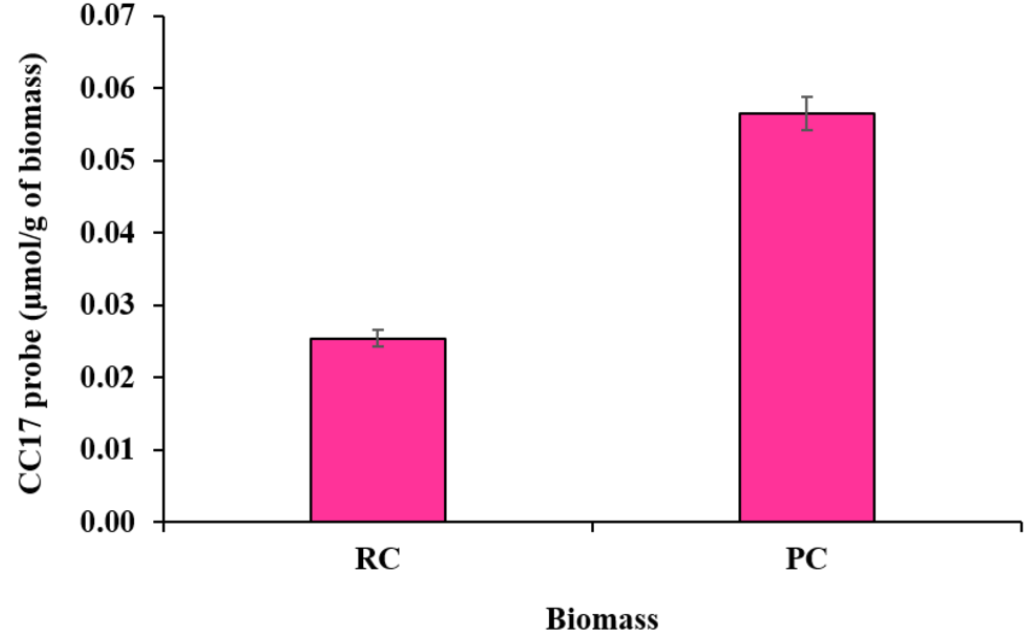
A

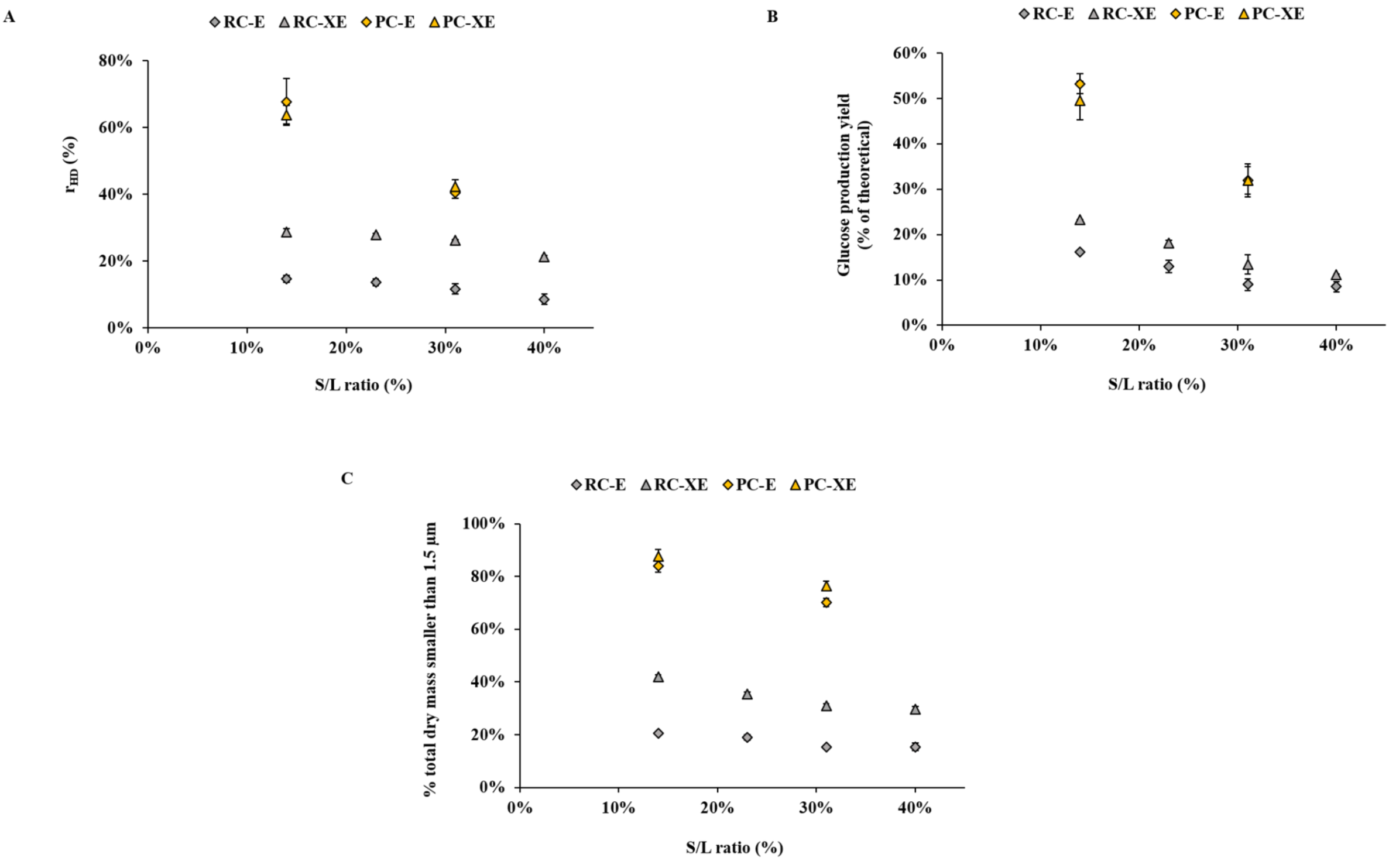

Bound probes (µmol/g of biomass)
0.25
0.20
0.15
0.10
0.05
0.00
RC
PC
Biomass

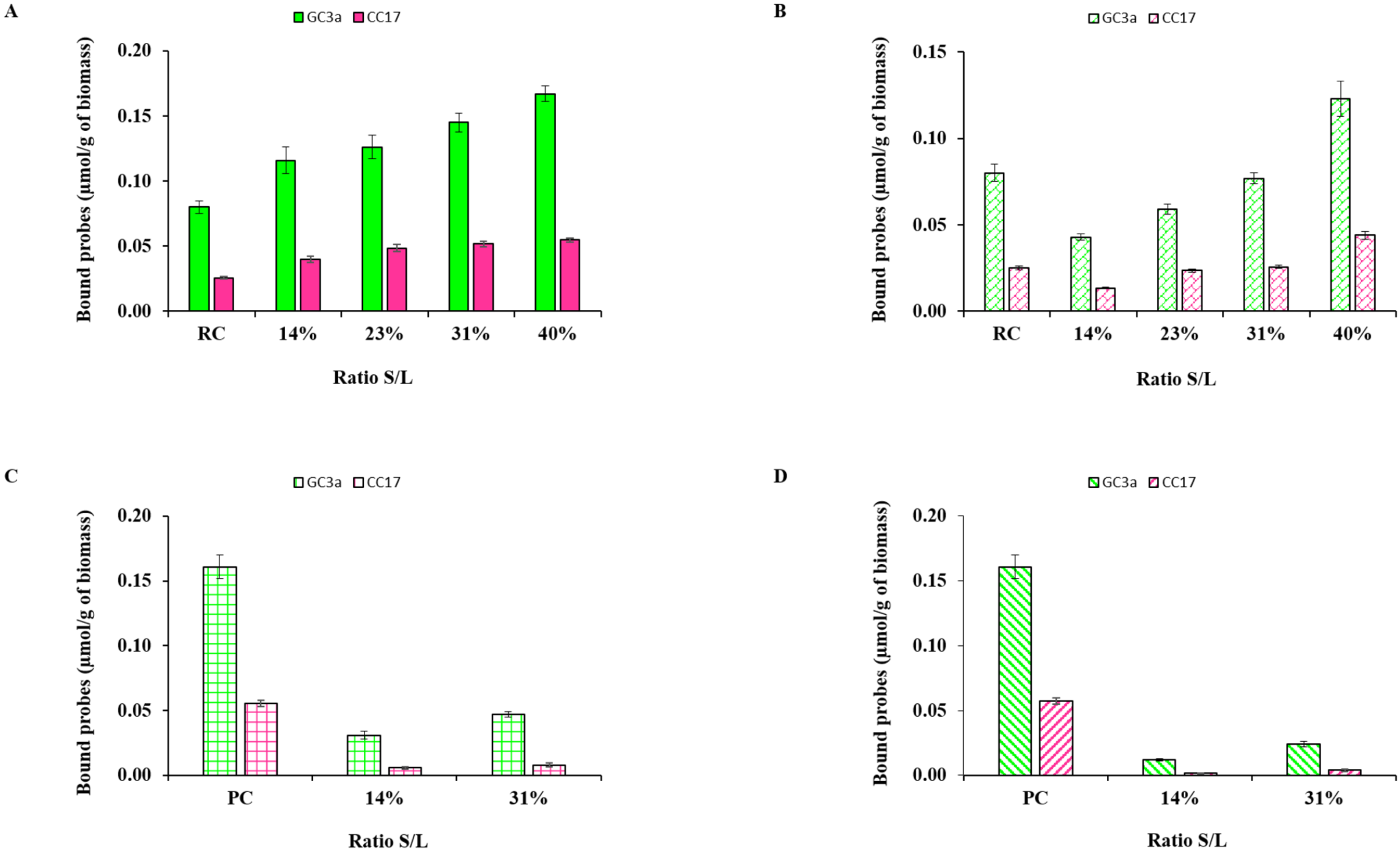
B


GC3a probe (µmol/g of biomass)
0.20
0.15
0.10
0.05
0.00
RC
PC
Biomass

C


CC17 probe (µmol/g of biomass)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
RC
PC
Biomass


A
RC-E
RC-XE
PC-E
PC-XE
80%
60%
40%
20%
0%
r_{HD} (%)
0%
10%
20%
30%
40%
S/L ratio (%)
B
RC-E
RC-XE
PC-E
PC-XE
60%
50%
40%
30%
20%
10%
0%
Glucose production yield
(% of theoretical)
0%
10%
20%
30%
40%
S/L ratio (%)
C
RC-E
RC-XE
PC-E
PC-XE
100%
80%
60%
40%
20%
0%
% total dry mass smaller than 1.5 µm
0%
10%
20%
30%
40%
S/L ratio (%)


A
GC3a
CC17
Bound probes (µmol/g of biomass)
0.20
0.15
0.10
0.05
0.00
RC
14%
23%
31%
40%
Ratio S/L
B
GC3a
CC17
Bound probes (µmol/g of biomass)
0.15
0.10
0.05
0.00
RC
14%
23%
31%
40%
Ratio S/L
C
GC3a
CC17
Bound probes (µmol/g of biomass)
0.20
0.15
0.10
0.05
0.00
PC
14%
31%
Ratio S/L
D
GC3a
CC17
Bound probes (µmol/g of biomass)
0.20
0.15
0.10
0.05
0.00
PC
14%
31%
Ratio S/L