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Experimentally designed corn biomass fractionation to obtain lignin nanoparticles and fermentable sugars

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ABSTRACT

Pretreatment steps carefully designed are essential to achieve a more integral and profitable use of plant biomasses in their conversion into building blocks to produce bioethanol and biomaterials. In this work, lignin extraction from corn biomass was optimized using a design of experiments (DOE) applied to a sequential acid-alkali pretreatment, varying temperature, time, and NaOH concentration in the alkali step. Selective extraction of lignin allowed the preparation of lignin nanoparticles, at the same time that it favored glucose release by hydrolysis of the cellulosic matrix. According to DOE results, alkali concentration was the most relevant factor for lignin removal and 2% NaOH, at 90 to 95 °C for 20 to 35 min would be enough to achieve less than 10% of residual lignin in the solid. These conditions were experimentally validated and resulted in a hydrolysis yield of 87.4 ± 7.3% and in a liquor containing approximately 50% of the initial lignin content. A great quantity of this neat lignin (92.9 ± 6.3%) was recovered by non-solvent addition as spherical nanoparticles, with a diameter smaller than 100 nm, and stable in a large pH range (5 to 11). Therefore, the optimization presented herewith resulted in lignin valorization by the suitable fabrication of value-added lignin nanoparticles, concomitantly to improved hydrolysis yields of cellulose aiming at bioethanol production.

1. Introduction

Pretreatment steps contribute to break the recalcitrant cell wall structure of lignocellulosic biomasses. These procedures increase cellulose accessibility to enzymes and improve the hydrolysis yields for bioethanol production (Bondesson et al., 2014). In addition to enhance the porosity and the surface area of the cellulosic matrix (Li et al., 2014), they also allow the fractionation of by-products, such as lignin, hemicellulose or extractives. The selectivity and the performance of the pretreatment depend on biomass intrinsic characteristics and on a variety of experimental conditions such as temperature, reactant concentration, and reaction time.

Several pretreatment methods using various experimental conditions have been studied for corn stover biomass, as a result of its abundance, low cost, and also because of the global importance of this agricultural crop. Some examples include ball milling (Lin et al., 2010; Liu et al., 2018), biological treatments (Saha et al., 2016; Zhuo et al., 2018), and diluted acids or alkalis (Avci et al., 2013; Lee et al., 2015; Li et al., 2012; Xu et al., 2010, 2009). Among these different methods, acid-alkali sequential pretreatments are promising approaches due to their efficiency and low cost (Lee et al., 2015). The idea of combining these two methodologies in series is to ally their advantages and to overcome their drawbacks. When the alkali step is carried out first, a mixed liquor containing lignin residues and xylose is obtained, which requires an extra separation step for a more integral and profitable use of the extracts. On the other hand, the application of a first acid step allows the recovery of a xylan-rich liquor, making possible the use of this hydrolysed sugar in specific applications, including additional ethanol production through xylose fermentation (Bondesson et al., 2014; Lee et al., 2015; Xu et al., 2010). Alkali pretreatments performed

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Abbreviations: CB, corn biomass; CBL, Corn biomass lignin; CBLN, Corn biomass lignin nanoparticles; CBIN, Corn biomass *in natura*; CB1, corn biomass treated with H_2SO_4 1%; CB3, corn biomass treated with H_2SO_4 3%; CB105, corn biomass treated with H_2SO_4 1% and NaOH 0.5%; CB305, corn biomass treated with H_2SO_4 3% and NaOH 0.5%; CB15, corn biomass treated with H_2SO_4 1% and NaOH 5%; CB35, corn biomass treated with H_2SO_4 3% and NaOH 5%

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after an acid step will remove lignin due to its solubilization as a result of the breaking of ester crosslinking bonds (Li et al., 2012) and the deprotonation of ionizable groups at high pH values. Moreover, an initial acid pretreatment improves lignin removal in a second alkali step, resulting in a lignin-rich liquor, when compared to the alkali pretreatment applied directly to the raw untreated biomass (Rezende et al., 2011). Notwithstanding the selectivity of these fractionation methods, most of them are still focused on cellulose isolation for bioethanol production only.

Besides the environmental benefits of replacing the traditional fossil fuels by ethanol, the production of cellulosic ethanol from corn stover is relevant as it allows a substantial increase in ethanol productivity per cultivated hectare due to improvements in land and residue use (Himmel et al., 2007; Ragauskas et al., 2006). However, the approach focusing in a single product should not be suitable in terms of economic viability and sustainability, since important by-products such as lignin, which could be used to produce biomaterials and biochemicals, are wasted (Cherubini and Ulgiati, 2010; Maity, 2015; Stuart and El-Halwagi, 2013). This integrated valorization of corn stover lignin within the bioethanol production chain is still poorly addressed in literature.

Lignin is the most important renewable resource of aromatic structures found on Earth (Duval and Lawoko, 2014). This macromolecule has interesting properties that enable its use in a wide range of applications, allying economic and environmental benefits (Feldman, 2016), for example, the development of advanced composites, polymer blends, carbon fibers, plastics, and nanomaterials. In fact, several characteristics of bulk lignin can be improved in the nanoscale range, such as antioxidant capability, UV-shielding efficacy, thermal stability, and antibacterial activity (Gonzalez et al., 2017; Tian et al., 2017a; Yang et al., 2016; Yearla and Padmasree, 2016). Lignin nanoparticles or nanolignin may present several morphologies (solid or hollow spherical shape, core-shell structures, among others) and colloidal stability parameters. These nanoparticles can be obtained by simple methods, such as the addition of water as non-solvent to a lignin solution (Gilca et al., 2014; Li et al., 2016; Yearla and Padmasree, 2016), ultrasound-assisted procedures (Gilca et al., 2015; Gonzalez et al., 2017; Zhai et al., 2015), or mechanical treatments by high shear homogenization (Nair et al., 2014). Valorization of corn biomass lignin by nanoparticle preparation is thus a promising approach to improve bioethanol production and should be further explored.

In this work, corn biomass was pretreated with a two-step acid-alkali method aiming at the valorization of lignin simultaneously to the improvement of cellulose enzymatic hydrolysis. A design of experiments (DOE) was used to determine the optimal pretreatment conditions for maximum lignin removal. DOE is a valuable approach to optimize pretreatments, since it allows to define the simultaneous influence of different experimental factors and to identify interactions among them with a reduced number of experiments (Box et al., 2005). The factors evaluated here were temperature, reaction time, and NaOH concentration for the alkali step. In the optimal condition proposed by the model, the solid substrate presented low content of residual lignin and high hydrolysis yields. In turn, most of the extractable lignin was recovered from the alkaline liquor as highly pure bulk lignin and could be converted into lignin nanoparticles stable in aqueous dispersions. Preparing value-added lignin nanoparticles can be a profitable way to optimize the production chain of cellulosic ethanol from corn biomass.

2. Materials and methods

2.1. Feedstock

Corn plants of the simple hybrid DKB 340 PRO (Dekalb® Monsanto) of the species *Zea mays* L. were kindly supplied by local farmers from the vicinities of Casa Branca (SP, Brazil, latitude 21°46′26″S and longitude 47°05′11″W). This maize variety has middle-early ripening and grew during summer in Brazil (December and January). Plants without roots were harvested at the milk-dough growing stage, ca. 60 days after sowing. In the laboratory, they were cut and separated into stalks, leaves and husks, before drying at 60 °C for 24 h in a convection oven (Tecnal TE-394/3, Brazil). Corn parts were then separately weighted and the mass ratio 5:3:2 for stalks:leaves:husks was determined. For microscopy assays, part of the samples was stored in pieces (without milling) for a better identification of morphological features. For pretreatments and other characterization assays, dried stalks, leaves and husks were ground separately by knife milling (Solab SL-31, Brazil), until passing through a 2 mm sieve, and were then mixed according to the 5:3:2 dry weight ratio. Samples were stored in plastic bags at room temperature and humidity until used.

2.2. Pretreatments of corn biomass (CB)

CB was initially pretreated using diluted H_2SO_4, followed by an alkaline step with NaOH, as previously described to sugarcane bagasse and elephant grass leaves (Rezende et al., 2018, 2011). Each pretreatment step was carried out in an autoclave (AV-75 Phoenix, Brazil) at 120 °C and 1.05 bar for 40 min, using a 1:10 solid to liquid ratio (grams of corn biomass/mL of solution). Heating to pretreatment temperature and cooling to room temperature took 20 and 60 min, respectively. Solid fraction was separated from the hydrolysate by filtration, abundantly rinsed with tap water until reaching a neutral pH and oven dried at 60 °C for 12 h. In the initial pretreatment steps, acid concentration was set at 1% or 3% (v/v), while NaOH was used at 0.5% or 5% (w/v). Samples were named as follows: CBIN (corn biomass *in natura*); CB1 or CB3 (treated with H_2SO_4 1 or 3%), CB105 (treated with H_2SO_4 1% + NaOH 0.5%); CB305 (treated with H_2SO_4 3% + NaOH 0.5%); CB15 (treated with H_2SO_4 1% + NaOH 5%) and CB35 (treated with H_2SO_4 3% + NaOH 5%).

2.3. Chemical composition of CB before and after pretreatments

The Laboratory Analytical Procedures (LAP) established by the National Renewable Energy Laboratory (NREL) were used to determine extractives, ash, structural carbohydrates, soluble and insoluble lignin in the biomass samples (Sluiter and Sluiter, 2011). Humidity contents were determined in a HG63 Halogen heating balance (Mettler, USA) in triplicate. CB *in natura* and treated with H_2SO_4 1% or 3% (CB1 and CB3) had their extractive content determined by Soxhlet extraction. For the other samples, this extraction step was suppressed as extractives were removed during pretreatment steps.

Carbohydrate, lignin, and ash amounts on extractive-free samples were determined by hydrolysis in H_2SO_4 72% (w/w). The solid fraction was dried at 105 °C until constant mass and calcined in a muffle furnace. The hydrolysate was analysed by high performance liquid chromatography (HPLC) to determine carbohydrates, organic acids, furfural, and hydroxymethylfurfural (HMF) contents. HPLC determinations were carried out in an Agilent (Santa Clara, CA, USA) series 1200 chromatograph equipped with refractive index detector (model G1322A). An Aminex column (HPX-87H, 300.0 x 7.8 mm, Bio-Rad, USA) was used for carbohydrate and organic acid analysis, with diluted H_2SO_4 as a mobile phase (5.10^{-3} mol/L, flow rate of 0.6 mL/min and temperature of 45 °C). Prior to injection, samples were filtered through nylon syringe filters (Millex GN, 0.2 μm pore size, Millipore, USA). Soluble lignin was determined in the hydrolysate by absorbance measurements at 280 nm using a UV-vis Agilent (Santa Clara, CA, USA) spectrophotometer (model Cary 50 Probe Varian). Analyses were carried out in duplicates (at least) and results were expressed as an average (± standard deviation).

2.4. Enzymatic hydrolysis of CB before and after pretreatments

Enzymatic hydrolysis was evaluated in dry and milled CB samples, using a solid to liquid ratio of 4.3% (0.010 ± 0.001 g in 230.0 µL of a 0.050 mol/L sodium citrate buffer (pH = 4.8)). Substrates were let to hydrate in the buffer for 24 h before the enzyme addition. The enzyme cocktail applied consisted of 20 filter paper units (FPU) of Accellerase 1500 (Genencor, USA) and 30 beta-glucanase units (BGU) of Novozyme 188 (Novozymes, Denmark) per gram of biomass. Samples were incubated at 50 °C for 24 h under constant stirring (1500 rpm), using an Accutherm incubator (I-4001 – HCS, Labnet, USA). The amount of released glucose was determined by HPLC, using the same conditions previously described for compositional analysis. D-(+) glucose (Sigma Aldrich, USA) was used as a standard. Analyses were carried out in triplicates and glucose values were expressed as an average (± standard deviation). Hydrolysis yields were calculated using a method previously reported (Rezende et al., 2011).

2.5. Morphological analysis of CB before and after pretreatments

Morphological changes on the corn biomass surface before and after chemical pretreatments were analysed in a FESEM (Quanta 650 F, FEI, USA). Samples (leaves, stalks, and husks separately) were cut into small pieces (< 1 cm) and pretreated without milling to facilitate the identification of their morphological structures. Dried samples were placed in metal stubs and coated with a thin gold layer (sputtering time: 60 s and current: 40 mA) prior to analysis, using a SCD 050 Sputter (Oerlikon-Balzers, Switzerland). At least 20 images per sample were obtained by Secondary Electron Imaging (SEI) to guarantee the reproducibility of the results, using a 5 kV accelerating voltage and a 10 mm working distance.

2.6. Experimental design

The second pretreatment step (alkali) was designed under different factor arrangements, assuming the existence of effects, individual or combined, among three independent factors: 1. Temperature (80 to 120 °C); 2. Reaction time (20 to 60 min); and 3. NaOH concentration (0 to 2.5% w/v). The residual content of lignin in the samples was taken as the response, since low lignin contents in the solid will favor both the hydrolysis yields and the recovery of lignin from the pretreatment liquor. The experimental design involved two steps: (i) an initial screening of the data applying a two-level Full Factorial (FF) design and a Multiple Linear Regression (MLR) fitting model and (ii) the optimization of the data by means of a Response Surface Model (RSM) based on a full quadratic Central Composite Face (CCF) design, and, after that, a model prediction by a Partial Least Square (PLS) projection. The screening in step (i) determined all single and interaction effects among the factors and indicated the most and least relevant factors of the data set, given small variances for the coefficients and small prediction errors. In step (ii), a small set of linear combinations of the factors approximated the response by a quadratic polynomial model, in order to understand further the influence of each factor in the response and to find the optimal region of process operability. Both modelling procedures were carried out using Modde 11.0.1 software (Umetrics, Sweden).

2.7. Isolation and characterization of corn biomass lignin (CBL)

Lignin was isolated from the liquor of the alkali pretreatment carried out under the conditions optimized to minimize the lignin content in the solid. The alkaline liquor was acidified until pH decreased to 2 by adding H_2SO_4 22% (w/w) (Kim et al., 2017) under vigorous agitation (700 rpm) in a magnetic stirrer for 15 min at room temperature. A color change from black to brown was observed in the system, as well as the formation of a precipitate. The suspension was then filtered under vacuum, the remaining solid was rinsed with deionized water until pH 6–7, collected in a Petri dish, and dried at 60 °C for 8 h in a convection oven (Tecnal TE-394/3, Brazil). The liquor of the acid step was filtered under vacuum to separate the acid insoluble lignin. The remaining solid was washed and dried, as previously described for the alkaline liquor. Recovered bulk lignins were characterized by the absorption spectra of lignin solutions (NaOH 2 mol/L) at several weight concentrations using a UV–vis Agilent (Santa Clara, CA, USA) spectrophotometer (model Cary 50 Probe Varian). Commercial alkali lignin with low sulfonate content (Sigma-Aldrich, USA) was analysed as a reference to lignin UV–vis absorption pattern.

2.8. Preparation and characterization of corn biomass lignin nanoparticles (CBLN)

Based on previously reported methods of synthesis (Richter et al., 2016), lignin nanoparticles were prepared by non-solvent addition: 1 mL of a 0.4% (w/v) CBL solution in acetone/water 9:1 (v/v) was gradually diluted with 99 mL of deionized water, while stirring at room temperature. Characterization of CBLN dispersions (0.05% w/v) at several pH conditions was carried out by dynamic light scattering (DLS) using a Zetasizer® 300HS (Malvern, UK) for particle size and zeta potential analyses. All measurements were performed in triplicates of at least 11 scans each. Transmission electron microscopy (TEM) analysis of CBLN dispersions was carried out in a Carl Zeiss LIBRA 120 microscope (Oberkochen, Germany). Dispersions of nanoparticles (20 µg/mL) at pH 2, 5, and 7 were deposited on 400 mesh copper grids with carbon film and dried in a desiccator for 2 h at room temperature.

3. Results and discussion

3.1. Effects of Pretreatments in Corn Biomass (CB): compositional and morphological changes and hydrolysis yields

Acid and alkali pretreatments were quite effective to remove hemicellulose and lignin from corn biomass, as can be observed in Table 1. Corn biomass *in natura* (CBIN) presented component contents within the expected ranges reported in literature, considering that its composition varies significantly with sample source, hybridization of the species, environmental conditions and plant maturity (Chen et al., 2013; Peng et al., 2014; Templeton et al., 2009; Zhang et al., 2015). A

Table 1
Chemical composition of corn biomass samples *in natura* (CBIN) and after different acid and alkali pretreatment conditions. Values refer to percentages determined in the solid samples, expressed on a sample dry weight basis as an average ± standard deviation of two or more replicates.

Sample	Extractives	Cellulose	Hemicellulose	Lignin	Ash	Total
CBIN	23.3 ± 0.4	39.3 ± 0.9	7.2 ± 0.5	28.0 ± 0.5	0.65 ± 0.02	98.5 ± 1.2
CB1	8.3 ± 0.2	52.9 ± 0.1	2.9 ± 0.2	34.2 ± 0.1	1.35 ± 0.01	99.7 ± 0.3
CB3	7.8 ± 0.1	54.0 ± 0.1	1.8 ± 0.2	34.9 ± 0.8	1.49 ± 0.05	100.0 ± 0.8
CB105	**	72.8 ± 1.0	2.1 ± 0.1	23.1 ± 0.2	0.57 ± 0.03	98.6 ± 1.0
CB305	**	73.2 ± 0.5	1.3 ± 0.2	25.1 ± 0.3	0.41 ± 0.01	100.0 ± 0.6
CB15	**	87.1 ± 1.7	1.3 ± 0.1	9.6 ± 0.1	0.24 ± 0.02	98.2 ± 1.7
CB35	**	87.9 ± 0.9	1.2 ± 0.2	9.3 ± 0.9	0.33 ± 0.01	98.7 ± 1.3

** Not measured, because extractives were removed in the pretreatment.

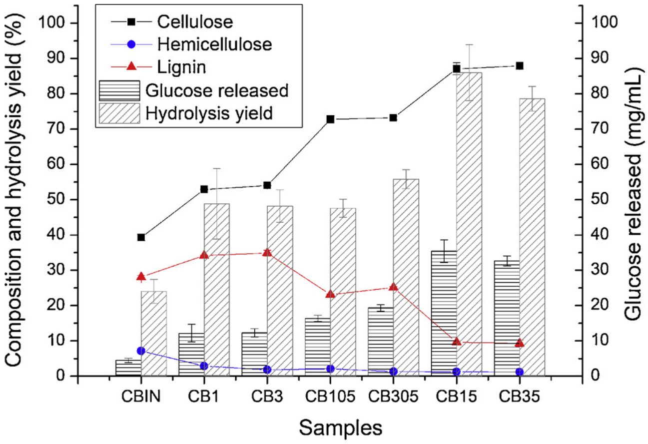

Fig. 1. Cellulose, hemicellulose, lignin percentages and hydrolysis yields (left axis) in corn biomass *in natura* (CBIN) or pretreated under different conditions, associated with the corresponding glucose amount released by enzymatic hydrolysis in each sample (right axis).

large amount of extractives, including soluble sugars, can be noticed in CBIN, and they were partially removed by the one-step acid treatments (CB1 and CB3) and completely removed after the two-step pretreatments (CB105 to CB35).

Results in Table 1 indicate that the acid treatment using H_2SO_4 1% (CB1) or 3% (CB3) was very effective in its role of removing hemicellulose, whose yield decreased from 7.2% in CBIN to ca. 2.9% in CB1 and 1.8% in CB3. A significant percentage of CBIN weight was hydrolysed in the acid step (43.5 ± 0.1% in CB1 and 48.5 ± 0.1% in CB3), and this weight corresponds mainly to extractives and to the hemicellulose fraction. After intensive hemicellulose removal from the solid by the first acid step, hemicellulose amounts remained almost unchanged after the second alkali step using NaOH 0.5 or 5% (Table 1). This result shows that this approach using acid-alkali pretreatments in sequential steps is very suitable to concentrate the first acid liquor in hemicellulose sugars, thus avoiding their presence in the liquor from the following alkali step.

Acid hydrolysis has been reported as a powerful method to promote hemicellulose extraction and the recovery of xylose-rich hydrolysates in grass samples, which can be subsequently hydrolysed by specific enzymes to produce ethanol (Rezende et al., 2011; Xu et al., 2010). Under acid conditions, xylan is more easily removed than glucan from lignocellulosic biomass, due to its low degree of polymerization and its amorphous nature (Lee et al., 2015).

It is also important to highlight that the difference in hemicellulose extraction was not linearly proportional to the H_2SO_4 concentration used. The pretreatment using acid 1% (w/v) reduced the hemicellulose amount to ca. 40% of its initial value, while a 3% (w/v) acid concentration decreased it to 25% of the initial amount. This is consistent with the fact that the hemicellulose present in the raw biomass has various levels of recalcitrance and the most exposed fractions are more easily removed. Furthermore, there is an influence of a kinetic factor, since the reaction time was kept the same but the concentration has increased.

In turn, the lignin content had a much more pronounced reduction after the alkali pretreatment step (Table 1). After the acid pretreatment, lignin percentages slightly increased in CB samples, most likely due to the removal of other cell wall components, while maintaining the lignin weight almost constant. After treating the samples with NaOH, the reduction in lignin amounts was small for samples treated with NaOH 0.5% and more pronounced after NaOH 5%, thus indicating an important contribution of NaOH concentration to lignin extraction. Alkali pretreatments resulted in a reduction of ca. 27% of the solid total weight when a NaOH concentration equal to 0.5% (w/v) was used, and of 54 and 56% when NaOH concentrations of 5% (w/v) were used after a first acid step with H_2SO_4 1% and 3%, respectively. Table 1 shows that this weight loss was mainly due to lignin extraction. This fractionation method resulted thus in a second alkaline liquor basically composed by lignin fragments. In fact, more than 65% of the initial total content of lignin was extracted with the addition of the alkali step for both CB15 and CB35. Therefore, the selectivity of the sequential pretreatments will facilitate lignin isolation in future steps.

In terms of morphology, important differences concerning pretreatment effects on the different CB anatomical fractions were observed. In general, CB leaves are the most recalcitrant structures, followed by the husks and the fibrous parts of stalks. Morphological changes are described in detail in the Supplementary Material (Figs. S1 and S2). The acid step had a milder action in CB morphology than the alkaline one, which can be associated to hemicellulose removal by the acid and to lignin removal by the alkali, according to Table 1. In fibrous samples, the first acid step removed the protecting layer covering the fiber surface (Figs. S1(d), S2(a) and S2(c)), but it maintained the cohesion within the fiber bundle (Fig. S1(d)). After the alkali step, however, fiber bundle structure was partially or completely lost (Fig. S1(g)) and as more severe alkali treatments were applied to CB, it was possible to observe a loosening of the cell wall tight tissue under high magnification, forming a more porous substrate (Fig. S2 (b), (d) and (f)).

This opening of the cell wall structure increases significantly the fluid and the enzyme accessibility to the substrate and must be a decisive factor in increasing hydrolysis yields. Therefore, lignin removal must be contributing to improving hydrolysis not only by a compositional point of view, as sample wettability increases, unproductive adsorption decreases, and cellulose is more exposed, but also by a morphological contribution, associated to a greater surface area.

Indeed, changes in morphology and chemical composition of CB samples, mainly concerning cellulose enrichment and lignin removal, resulted in improved yields of fermentable sugars released from these samples after 24 h of hydrolysis. Fig. 1 presents the total amount of glucose released by 0.01 g of the dried CB samples after each pretreatment step, together with the hydrolysis yields, and the corresponding composition profile for cellulose, hemicellulose and lignin. Fig. 1 indicates a very clear relationship between the decrease in lignin content, the cellulose enrichment, and the corresponding increase in the yield of glucose released from the samples.

Higher cellulose contents are consistent with improved glucose release, since cellulose is the substrate for hydrolysis. Similarly, the decrease in the lignin amount also contributed to improved hydrolysis yields, as lignin distribution in the cell wall hinders the access of enzymes to cellulose, and because enzymes adsorb unproductively on its surface (Kim and Dale, 2004; Lee et al., 2015; Rezende et al., 2011). Moreover, a lower residual lignin content also represents an improvement in the amount of extractable acid-insoluble lignin that is available in pretreatment liquors to be after converted into lignin nanoparticles.

The highest hydrolysis yields were 86.0% and 78.6%, obtained in the samples sequentially treated with H_2SO_4 1% and NaOH 5% and with H_2SO_4 3% and NaOH 5%, respectively. This result represented a significant increase when compared to the value in CB *in natura* (24.0%). Furthermore, these yields can be considered good results of improved hydrolysis when compared to other pretreatment methodologies reported in the literature (Lee et al., 2015; Li et al., 2012, 2018, 2014; Liu et al., 2016; Pang et al., 2012; Xu et al., 2010; Zhang et al., 2012; Zhu et al., 2016; Zu et al., 2014). Li et al. (2012) obtained a 78.2% hydrolysis yield in corn stover after an alkaline sodium sulphite pretreatment, also using Accellerase 1500 (20 FPU) and Novozyme 188 (10 IU/g) at 50 °C and pH 4.8. These authors also tested four other alkali pretreatments that resulted in lower hydrolysis yields. Lee et al. (2015) applied a two-step acid alkali pretreatment to CB samples and obtained hydrolysis yields from 89.1 to 97.9%, using the same enzymes applied here, but under higher enzyme loads (60 FPU of Accellerase and 30 CBU of Novozyme 188) and lower substrate to liquid ratio (0.4%).

These authors tested different conditions in the acid step, varying H_2SO_4 concentration (0.25 to 1%); reaction temperature (140 to 180 °C) and time (60 to 90 min), but at fixed conditions of the alkali step (2% NaOH concentration, 80 °C for 1 h at a 1:20 solid to liquid ratio). Also, with similar conditions, Li and collaborators (2018) obtained up to 86.5% enzymatic conversion of glucose using corn stover pretreated with dry dilute acid method (2.5% sulfuric acid concentration, 170 °C for 3 min at a 2:1 solid to liquid ratio). It is important to remark that results on hydrolysis yields from different studies are difficult to be compared, since they are prone to the influence of many different variables such as enzyme type and load, substrate to liquid ratio, pH, hydrolysis time, and temperature.

3.2. Experimental design

Considering the remarkable influence of lignin contained in CB solid substrate for glucose release, in addition to the clear effect of the alkali pretreatment on the lignin removal and the intent of recovering the fractionated lignin, the pretreatment conditions in this second step were studied by means of a factorial design approach. The effects of three main factors, named Temperature, Time, and NaOH concentration, were investigated on the total residual amount of lignin on the solid substrate after pretreatments. The conditions of the first acid pretreatment step had already been optimized in previous studies (Rezende et al., 2018), and were thus kept fixed (1% H_2SO_4 (v/v) at 120 °C for 40 min (CB1)). The concentration of 1% H_2SO_4 was also selected because the improvements in the glucose released by hydrolysis did not vary significantly with the acid concentration within the range from 1 to 3% (w/v), as it can be observed in Fig. 1.

Table 2 shows the factor arrangements of the experimental set created by Modde software on the screening and optimization designs. The set of experiments is composed of 16 randomly ordered runs, with two replicates at the central star point. Table 2 also shows the response values obtained for each experiment, which are the average of duplicates of residual lignin concentration (summing up acid-soluble and insoluble lignin) for each sample submitted to the respective alkaline pretreatment.

To improve the non-linear model ability of prediction, the relevant interaction of the quadratic term NaOH x NaOH was included, based on a positive p-value significance expressed for this effect, resulting in a high Q2 value (80.3%). This procedure also improved the model validity and the value of R2. Fig. 2(a) presents the adjusted coefficient plot expressing the effect of the factors independently, the first-grade interactions (Time x NaOH, Time x Temperature and Temperature x NaOH) and the square NaOH x NaOH interaction.

Table 2
Temperature, time and NaOH concentration conditions and total lignin in the solid (response) for the set of alkaline pretreatment experiments used in the screening and optimization designs.

Samples		Design factors			Design response
Number	Name	Temperature (°C)	Time (min)	NaOH (% w/v)	Lignin residual content (%w/w)
1	N1	80	20	1.0	19.95 ± 0.05
2	N2	120	40	0.5	25.10 ± 0.3
3	N3	100	60	0.5	25.00 ± 1.0
4	N4	120	60	0.5	31.70 ± 0.5
5	N5	80	20	2.5	15.00 ± 1.0
6	N6	100	20	2.5	10.30 ± 0.6
7	N7	80	40	2.5	13.27 ± 0.09
8	N8	120	60	1.0	17.12 ± 0.05
9	N9	80	40	1.0	19.00 ± 3.0
10	N10	120	40	1.0	17.00 ± 0.2
11	N11	100	20	1.0	16.00 ± 2.0
12	N12	100	60	1.0	16.33 ± 0.09
13	N13	100	40	0.5	25.00 ± 0.3
14	N14	100	40	2.5	12.00 ± 2.0
15	N15	100	40	1.0	14.80 ± 0.5
16	N16	100	40	1.0	17.60 ± 0.6

According to Fig. 2(a), the most significant terms of the model are NaOH concentration as an independent factor, followed by the quadratic effect of the NaOH x NaOH interaction and the smaller Temperature x Time interaction. NaOH concentration is thus the most relevant parameter to be controlled in order to increase the removal of lignin from the pretreated samples and its transference to the hydrolysate (alkaline liquor). The negative sign of the interaction NaOH x NaOH indicates the curvature of the curve in the response contour (convex in this case). Time and Temperature as independent factors are virtually ineffective for lignin removal, but their association has an indirectly proportional influence in the removal of lignin, with higher temperature and time values resulting in a worse lignin removal.

The worsening in lignin removal as the severity of the pretreatments increases (higher temperature associated to longer reaction times or higher NaOH concentration) may be due to cellulose solubilization in these samples, which would increase the relative lignin percentage. A similar behavior of the interaction effects was observed in sugarcane bagasse pretreated with calcium hydroxide (Rabelo et al., 2013) and also with alkaline hydrogen peroxide (Rabelo et al., 2014). In the first case, although Time and Temperature as independent factors presented positive effects on the enzymatic hydrolysis of the samples (glucose release as the response), the interaction between them was negative. In the case of alkaline hydrogen peroxide, Temperature and Concentration presented positive individual effects, while their interaction was negative for the same response. In both studies, the authors attributed the decrease in glucose release to cellulose solubilization by the alkali media.

Fig. 2(b) shows the observed x predicted responses for each experiment calculated by the model, returning a very good fit. The exception is Experiment 4 (T =120 °C, t =60 min, NaOH = 0.5%), where the deviation can be due to the conjugated negative effects of the association of low NaOH concentration and upper operation time and temperature.

The same set of experiments regarding factor arrangements and responses was submitted to optimization by means of a Central Composite Face (CCF) quadratic design and a Partial Least Square (PLS) projection. By removing the non-significant factor interactions, this optimization presented a very strong and reliable model for the response of residual lignin content (R2 = 94.5%, Q2 = 77.6%, model validity = 92.0% and reproducibility = 87.8%). Fig. S3 indicates how well the set of experiments fitted to the designed model, regarding its capability of prediction.

Fig. 3 is the response of the optimized model of the most adequate working region in order to obtain residual lignin content lower than 10% w/w, based on the most favorable NaOH concentration estimated by the model (1.95% w/v). Lignin content under 10% is reached with any pair (Time, Temperature) whose values sit below the target curve. The almost vertical slope of the curve at temperature around 90 °C indicates that the target cannot be achieved below this temperature. For fixed reaction times below 50 min, lignin content reduces in the solid as temperature increases, and the prediction is that values as low as 6% would be reached at 25 min and 115 °C. It is important to highlight that temperatures around 95–100 °C are very convenient in terms of operation in heating systems. This response contour in Fig. 3 includes the factor arrangement identified as optimal by the screening, differing slightly only by the NaOH concentration (screening optimized conditions: NaOH = 2.0% w/v, Temperature =92.7 °C, Time =28.7 min). Actually, the pretreatment was carried out under this optimal experimental condition, when a lignin residual content of 9.2 ± 0.3% w/w was obtained in the solid. In this sample, the measured glucose released was equal to 36.8 ± 2.6 mg/mL (or 846 ± 60 mg of glucose/g of CS), which corresponds to a hydrolysis yield of 87.4 ± 7.3%. This verification confirms the validity of the model prediction and shows a

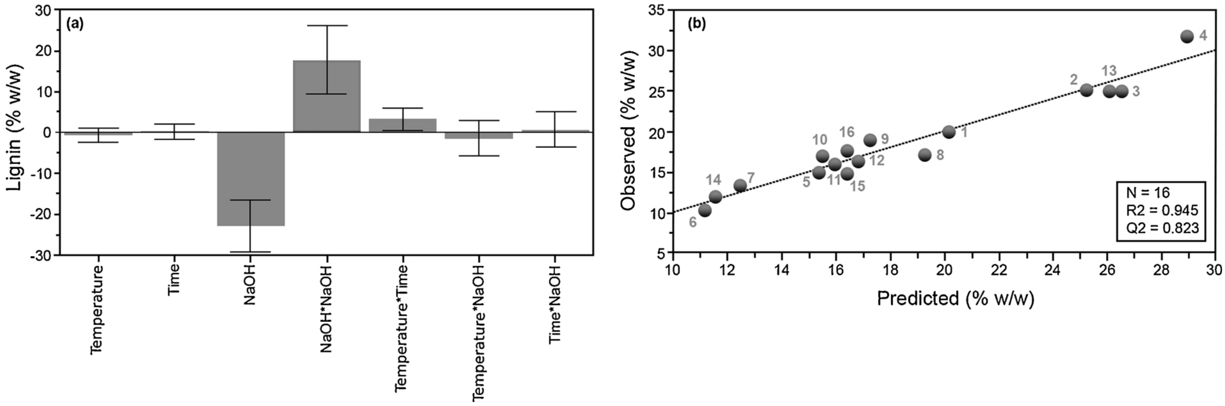

Fig. 2. (a) Coefficient plot after adjustment and inclusion of square NaOH x NaOH interaction, showing a Q2 value = 80.3%; (b) Observed x Predicted Response Plot for the set of experiments after screening adjustment.

value similar to the maximum hydrolysis yield shown in Fig. 1, obtained under more severe conditions (NaOH = 5.0% w/v, Temperature = 120 °C, Time = 40 min).

From the perspective of a potential industrial operation, the response contour indicates that the ideal would be to perform the pretreatment at a given NaOH concentration with a Time x Temperature arrangement to allow the smallest process time associated with the lower working temperature, in order to maximize productivity and reduce energy consumption, thereby minimizing costs.

3.3. Characterization of corn biomass lignin (CBL) recovered from the liquors and of lignin nanoparticles (CBLN)

The amount of corn biomass lignin (CBL) contained in the liquor from the acid pretreatment was equal to 6.2 kg/100 kg biomass (Table 3), which corresponded to 22% of the total lignin in CBIN (28 kg/100 kg biomass, as shown in Table 1). In turn, CBL recovered from the alkali pretreatment in a condition within the optimal region determined by DOE (2% NaOH, at 90 to 95 °C for 20 to 35 min) was 13.5 kg/100 kg biomass (Table 3), which corresponded to 48% (w/w) of the total initial weight of lignin in CBIN. A 1:10 solid to liquid ratio was used in the pretreatments and the concentrations of total lignin in the acid and the alkaline liquors were 9.0 ± 0.6 g/L and 30.0 ± 1.5 g/L, respectively. However, only acid-insoluble lignin can be extracted by means of acidification of the medium. Therefore, considering that 100 kg of CB contained 21 kg of acid-insoluble lignin, only 25% of the extractable lignin was quantified in the acid liquor (5.2 kg) and 27% of this content was isolated as bulk lignin. Alkaline liquor was composed by approximately 53% (11.1 kg) of the starting amount of acid-insoluble lignin and 96% of this content was isolated as bulk lignin.

Table 3
Content of acid-soluble lignin, acid-insoluble lignin and lignin isolated from 100 kg of corn biomass.

Liquor	Total lignin (kg)	Acid-soluble lignin (kg)	Acid-insoluble lignin (kg)	Isolated lignin (kg)
Acid	6.2 ± 0.2	1.0 ± 0.1	5.2 ± 0.2	1.4 ± 0.1
Alkaline	13.5 ± 0.6	2.4 ± 0.6	11.1 ± 0.2	10.7 ± 0.1
Total	19.7 ± 0.6	3.4 ± 0.6	16.3 ± 0.3	12.1 ± 0.1

UV-vis spectra of these two lignin samples (from the acid and from the alkaline liquors) presented absorption bands similar to commercial alkaline lignin at 220 nm, 290 nm, and 310–320 nm (Fig. S4). These characteristic bands are associated with aromatic groups in general (sharp band at 220–230 nm), with non-conjugated phenolic fragments such as those from polylignol, adehydrogenative copolymer of sinapyl alcohol, coniferyl alcohol and a small amount of p-coumaryl alcohol (280–300 nm), and also with the esters of p-coumaric and ferulic acids (shoulder at 310–320 nm) (Klein et al., 2018; Sun et al., 2001).

At various CBL concentrations (5 to 80 mg/L in NaOH 2 mol/L) the characteristic absorptions at 220 and 290 nm linearly increased with an increase in the amount of lignin in the alkaline solution (R2 = 99.9%,

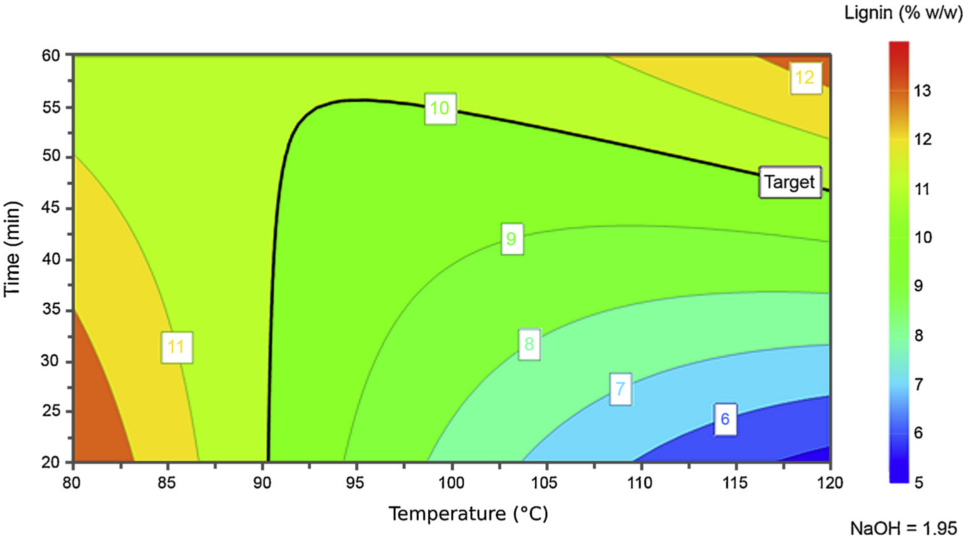

Fig. 3. Optimum region delimited by the model to reach the expected target (residual lignin content < 10%).

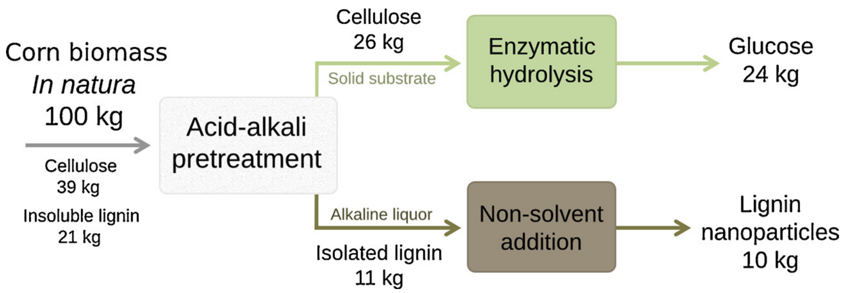

Fig. 4. Block flow diagram for the conversion of corn biomass *in natura* into glucose and lignin nanoparticles.

Fig. S5). This correlation was used to estimate the percentage of impurities in each CBL sample, as aqueous solutions with equal weight concentrations of lignin isolated from acid and alkaline liquors had different intensities of absorption at 220 and 290 nm. Therefore, taking the absorption at 220 nm of commercial alkaline lignin with low sulfonate content as a standard for lignin purity (99%), the estimative is that the CBL of the acid liquor was contaminated with 50% of carbohydrate derivatives, while the CBL of the alkaline liquor presented low percentage of carbohydrate impurities (9.5%). Thus, the two-step acid-alkali pretreatment allowed this lignin-rich liquor to be obtained from the alkali step.

Due to its higher purity and yield, CBL isolated from the alkaline liquor was used to obtain lignin nanoparticles (CBLN). CBLN was prepared by adding water as non-solvent to a solution of CBL in acetone, with a yield of 92.9 ± 6.3%. For an overview of the total use of corn biomass in this work, including the production of lignin nanoparticles, a flow chart diagram with a brief schematic summary is presented in Fig. 4. Considering 100 kg of dried corn biomass *in natura* as starting material, 24 kg of glucose can be obtained by enzymatic hydrolysis at the end of the proposed acid-alkali route, together with 11 kg of isolated lignin. This bulk lignin can be driven to different applications or converted to 10 kg of lignin nanoparticles dispersed in aqueous medium.

Particle size and colloidal stability (ζ-potential) of CBLN were estimated by dynamic light scattering at different pH values. In Fig. 5 it is possible to compare the number average size and the ζ-potential of aqueous dispersions of CBLN in pH range from 2 to 12.

Freshly prepared dispersions of CBLN presented pH 5, when nanoparticle average size was 88 ± 57 nm and ζ-potential was - 39.0 ± 6.1 mV (Fig. 5). In fact, electrostatic stabilization of CBLN dispersions at pH 5–12 is supported by ζ-potential values, which were below - 30 mV for all these samples. Zeta potential values more negative than - 30 mV usually represent sufficient mutual repulsion to ensure stability against aggregation, coalescence, and sedimentation (Hiemenz and Rajagopalan, 1997). Thus, at pH 6–8, CBLN had maximum stability with up to - 54.8 ± 4.8 mV of surface charge and an average size of 60 ± 36 nm. Particle sizes were virtually equal for the samples between pH 6–11 (total average size of 58 ± 35 nm). In turn, at pH 12 most of CBLN was solubilized and the detected number average size for this sample was 26 ± 18 nm.

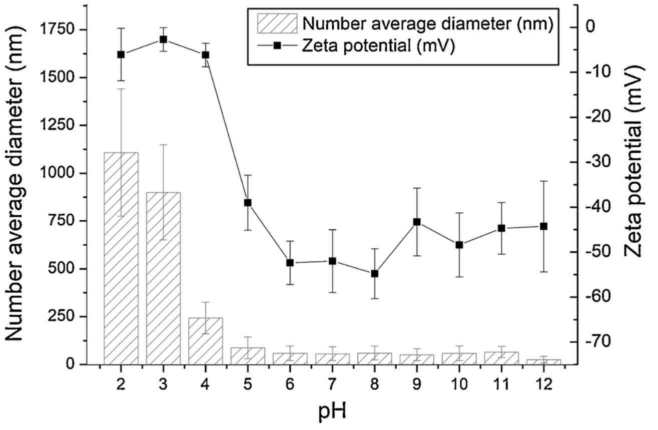

Fig. 5. Number average diameter (left axis) of corn biomass lignin nanoparticles (CBLN) in aqueous dispersion at different pH values, associated to the corresponding zeta potential for each sample (right axis).

Fig. 6 shows TEM images of CBLN at different pH values. Fig. 6(c–f) shows that sphere-like shaped CBLN at pH 5 and 7 forms a fractal-like network when dried. These results were comparable to a study reported by Tian and collaborators (2017b), who produced nanoparticles with *ca.* 200 nm from cellulolytic enzyme lignin of corn stover through the dialysis method. These nanoparticles were stable at pH values between 4 and 10 and also presented a strong tendency of aggregation when freeze-dried. The authors concluded that this phenomenon was probably associated to the presence of a high content of hydrophilic groups due to carbohydrate impurities (Tian et al., 2017b).

On the other hand, bellow pH 4 the zeta potential values were not negative enough to lead to electrostatic stabilization of the nanoparticles (Fig. 5). CBL extracted from the alkaline liquor is highly acid-insoluble; thus, at pH 2 to 4, CBLN are prone to preferentially coalesce with the neighboring particles, creating large clusters, as shown in Fig. 6(a, b). This result also agreed with the large values obtained for number average size of these particles (Fig. 4).

Therefore, the results presented herewith indicated that the addition of water as non-solvent was an effective method for manufacturing lignin nanoparticles from corn biomass lignin extracted after a sequential diluted acid-alkali pretreatment. The method was simple and CBNL presented colloidal stability in a wide range of pH values (pH 5 to 11). These nanoparticles can be readily applied to the production of nanocomposites, among other potential utilization.

4. Conclusions

The two-step pretreatment applied here allowed the selective acid extraction of hemicellulose and the alkali extraction of lignin from corn biomass. Optimization of lignin removal was achieved by experimental design, which showed that NaOH concentration was more influential than temperature and time in the alkali step. A 2% NaOH concentration was proven to be enough to obtain substrates with less than 10% of residual lignin, using temperatures from 90 to 95 °C and relatively short times (20–35 min). Experimentally designed pretreatment was effective in improving hydrolysis yields as well as the extraction of lignin, which was successfully converted into lignin nanoparticles. Therefore, the methodologies presented here confirm the suitability of combining lignin valorization to the enhancement of fermentable sugar release for bioethanol production through a more integrated approach.

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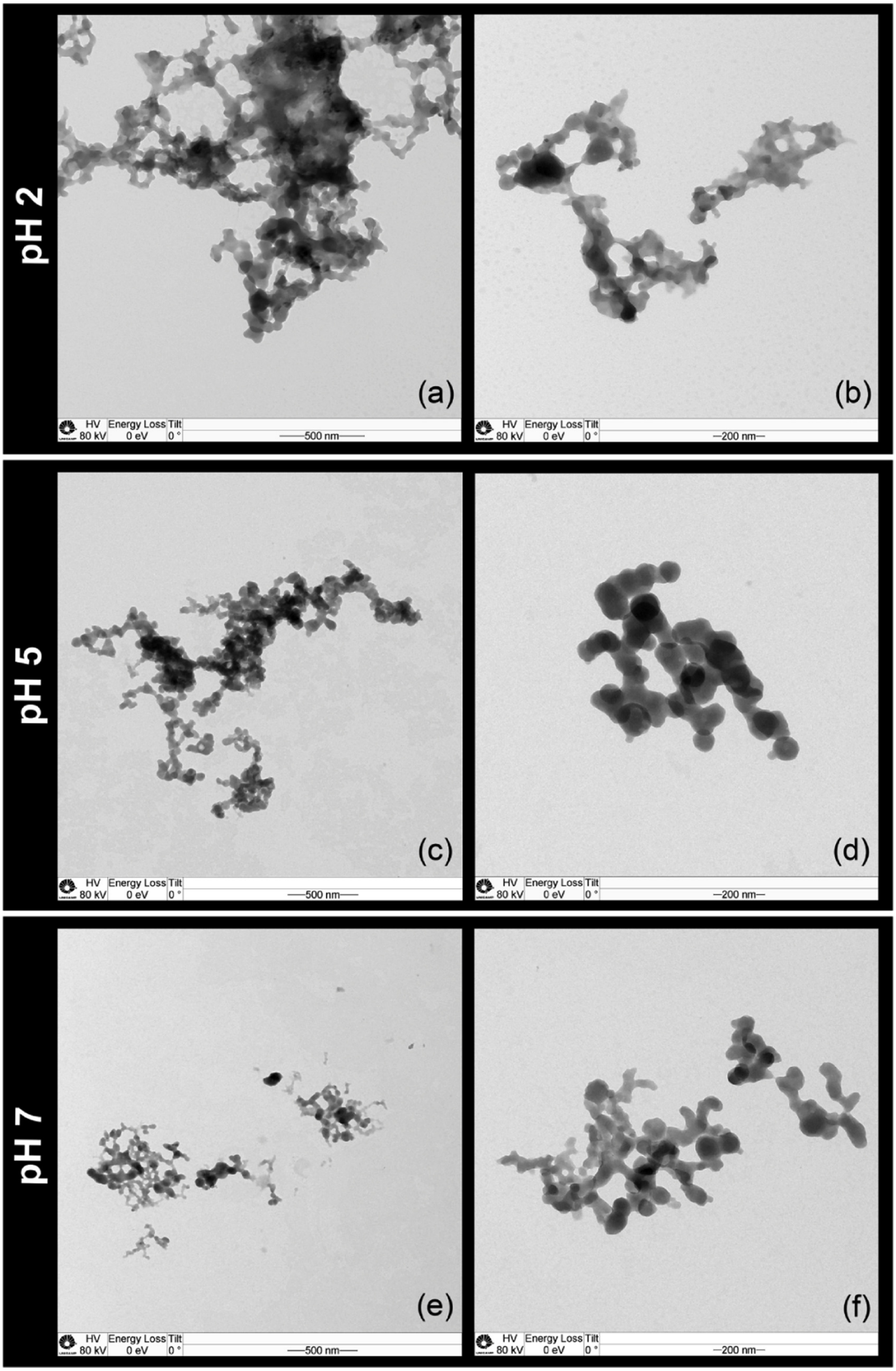

Fig. 6. TEM micrographs of CBLN at: (a, b) pH 2; (c, d) pH 5; and (e, f) pH 7. The concentration was 20 μg/mL for all aqueous dispersions. Scale bars: 500 nm (a, c, e) and 200 nm (b, d, f).

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Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.indcrop.2019.111649.

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