Triticum spelta straw hydrothermal pretreatment for the production of sugar syrups via enzymatic hydrolysis

Paolo Becarelli[a], Rodrigo da Rocha Olivieri de Barros[b], Raul Alves de Oliveira[b], Leonardo Tognotti[c], Elba Pinto da Silva Bon[b*]

[a]Department of Engineering of Energy, Systems, Territory and Construction (DESTeC) - University of Pisa - Largo Lucio Lazzarino 1 – 56122 Pisa, Italy.

[b]Department of Biochemistry - Laboratório Bioetanol, Federal University of Rio de Janeiro, CEP 21941-909, Rio de Janeiro, RJ, Brazil.

[c]Department of Chemical Engineering, University of Pisa, 56100, Pisa, Italy.

*Corresponding author: Elba Pinto da Silva Bon (elba1996@iq.ufrj.br)

Avenida Pedro Calmon s/n, Bloco P, P4. Cidade Universitária, CEP 21941-596, Ilha do Fundão, Rio de Janeiro, RJ, Brazil. Tel: + 55 21 3209-6590

Abstract

The oil price volatility and the serious environmental problems related to its use such as climate chage have been feeding the interest for alternative feedstock with lower cost as well as wider geographic distribution such as the plant biomass mostly represented by the residues of the agro-industry. These materials can be used to develop a new integrated chain of processes in a similar way to that developed for crude oil in biorefineries where the complete utilization of the main biomass components, e.i., the cellulose, the hemicellulose and the lignin will be used to produce fuels and feedstock for the chemical and the biochemical industry. Within this challenging scenario this study evaluated the production of sugar syrups from the spelt straw (*Triticum spelta)* to contribute to this complex chain of processes and to aggregate value to this biomass residue. This work reports a statistical experimental design for hydrothermal pretreatment of spelt straw aiming the conversion of the straw into glucose sugar syrups with high efficiency and yield. The best pretreatment conditions were obtained at 180 °C for 10 minutes that allowed the production, via enzymatic hydrolysis, of a glucose syrup of 29.58 g L^{-1} in a reaction mixture presenting 100 g L^{-1} of pretreated straw. The cellulose content of the pretreated straw and the corresponding glucose concentration in the hydrolysate was mathematically modelled with relative deviation smaller than 5% as a function of pretreatment parameters temperature and residence time.

1. Introduction

In the last 50 years the increase in world population alongside the progressive industrialization of developing countries, has more than quadrupled the world's energy needs. The high volatility in the price of crude oil and the environmental issues such as climate change related to its use have been feeding, over the years, the interest to seek alternative renewable feedstock with wider geographic distribution and lower cost, such as the biomass[1],[2].

As an example, Brazil initiated in 1970 a program to replace gasoline with ethanol to reduce its dependence during politically and economically unstable periods; in this program, the sucrose juice from the sugarcane was chosen as feedstock to industrial production of ethanol, presently known as 1G sugarcane ethanol. As a consequence, agricultural and technological studies were greatly intensified, leading Brazil to a favorable position in terms of renewable energy supply [3]. The sucrose syrup that is directly fermented to ethanol is obtained by the cane crushing that generates large amounts of sugarcane bagasse, a residue majorly burned in the mills for cogeneration [24]. Presently both the surplus bagasse as well the sugarcane straw have been studied and used for the production of biomass ethanol nevertheless the necessary technological and economical advances are still high in the agenda of the industry sector [24]. Production of biomass ethanol is advantageous as the sucrose syrup from the cane crushing is also used for sugar production generating competition between the food market and energy market [4]. This competition is likewise generated in the production of fuel ethanol from corn and cereal in the US and in Europe [25], [26], [27]. In this scenario the production of biomass ethanol would avoid imbalance in the food market nevertheless this technology still calls for significant industrial improvements to convert the biomass structural polysaccharides biomass components into fermentable sugars [5].

The cell wall of plants are the source of the lignocellulosic biomass, a rich and chemically complex material, whose structure is represented mainly by the physical-chemical interaction between cellulose, a linear polymer of glucose, hemicellulose, a highly branched mostly C5 sugars polysaccharide of variable composition and lignin, a very complex macromolecule rich in aromatic subunits [6], [7], [8]. The conversion of lignocellulosic biomass into fermentable monosaccharides can be made via acid or enzymatic hydrolysis. For the use of enzymatic hydrolysis, a preferable route, the lignocellulosic materials must undergo a pretreatment step to alter a structured that has evolved to resist the action of physical, chemical and biological external agents [6], [12]. The pretreatment is therefore intended to expose as much as possible the chain of the cellulose to the multi-enzymatic process to convert this polysaccharide into glucose molecules [9].

The biomass pretreatment is of crucial importance and is still at the very center of scientific and industrial research as the enzymatic production of sugar syrups with high concentrations and high yields requires an cost attractive and technically effective pre-treatment to render the material more accessible to the enzymes action. The hydrothermal treatments, mostly known in the scientific literature as liquid hot water (LHW) or hot compressed water (HCW), are based on the use of sub-cooled water in order to maintain the liquid state at high temperatures (160 to 240 °C). The process alters the native structure of the biomass through the partial removal of the hemicellulose fraction, combined to alterations of the lignin structure, rendering the cellulose more accessible to the subsequent enzymatic hydrolysis step [12]. Differently from the steam explosion pretreatment the LHW does not present a rapid decompression at the end of the process and does not use catalysts or chemicals. Pending on the process conditions in terms of residence time and temperature the hemicellulose is solubilized in different proportions of oligosaccharides and mostly C5 sugars

monomers. The process conditions also dose the extension of the C5 sugars dehydration to form furfural, the C6 sugars dehydration to form 5-hydroxi-methyl-furfural (HMF) as well as the extension of the removal of acetic acid from the hemicellulose chain. The process also solubilizes phenolic compounds from the lignin. Collectively the furfural, HMF, acetic acid and phenols released to the pretreatment liquid stream are microbial inhibitors that have been hindering the biotechnological use of this C5 sugars liquid stream.

This study evaluated the production of glucose sugar syrups from the spelt straw (*Triticum spelta*) using statistical experimental design to evaluate the hydrothermal pretreatment of spelt biomass followed by enzymatic hydrolysis. Spelt, also known as spelt wheat or hulled wheat originated from Asia and is one of the oldest crops of mankind. Indeed, in Europe spelt was an important staple cereal during the Bronze and the Middle Ages. Presently, after the grains are processed for food the spelt biomass (the chaff, glumes and stems) lags as a by-product or waste that can be used as raw material for biomass combustion for sustainable heat production [10]. The spelt straw is widely available in Italy, particularly in the region of Garfagnana where in spite of its use as animal feed the vast majority is discarded as waste.

2. Materials and Methodology

2.1. Materials

A total amount of 6 Kg of *Triticum spelta* was collected in the region of Garfagnana, Northern Tuscany, Italy and sent by mail to the Laboratório Bioetanol of the Federal University of Rio de Janeiro (www.bioetanol-ufrj.com.br) where all the experiments were conducted. The material was firstly subjected to moisture determination using a moisture analyzer Gehaka (São Paulo, Brazil) model IV-3000 that records the decrease in weight and estimates the instantaneous moisture until the humidity value stabilizes with a variation less than 0.1%.min^{-1}. The equipment was settled at 105 ºC in auto-dry mode.

2.2. Hydrothermal pretreatment

Before the hydrothermal pretreatment the spelt straw was processed in a cutting mill using a Retsch (Haan, Germany) machine model SM-300 equipped with a sieve with a cut-off of 2 mm to select a size standardized sample avoiding a wide variability in the particle size distribution that hinders statistically valid and reproducible results [11] as well as to homogenize as much as possible the variability in composition of this plant material. The hydrothermal pretreatment experiments were carried out in a reactor Parr Instruments (Moline, Illinois, U.S.A) model 4520 with the capacity of 1 liter. A total of 30 g (dry weight) of biomass was placed in the reactor followed by the addition of 300 mL (around 30% of the reactor capacity) of distilled water. After a careful mixing the pH of the biomass-water suspension was measured to verify a pH in the range 5 to 6 that minimize the formation of biomass derived microbial inhibitors. The reactor was afterwards sealed by a safety lock, the electric heater put into position, and the control thermocouple connected. The reactor

was flushed with nitrogen up to a pressure of 5 bar and left to drain slowly from the exhaust valve. This operation was repeated 5 times in succession to ensure an inert atmosphere inside the reactor avoiding oxidation of the material at high temperatures. Finally, the reactor was pressurized with nitrogen at a working pressure of 20 bar, the temperature was set to a specific setpoint and the stirrer was settled to 100 RPM and heater start-up. In the measurement of the residence time, it was assumed for the onset of the hydrothermal process the time when temperature reached its set-point. After the predetermined treatment time, the heater was turned off and the reactor was cooled down with ice and water. Upon reaching a temperature of 40 °C the valve was opened to drain the nitrogen until the atmospheric pressure, and the vessel was open to retrieve the pretreated material, which was composed by the solid cellulignin and the liquid hemicellulose derived stream that were separated by filtration using a vacuum pump. A sample of the liquid fraction was stored and frozen for the analysis of the inhibitors concentration as these data were inserted in the statistical model. The solid fraction was washed with 600 mL of distilled water to remove inhibitors due to its impregnation by the liquid fraction.

2.3. Use of experimental design to optimize the hydrothermal pretreatment of spelt straw biomass

In this work it was used an experimental design known as Central Composite Design (CCD) that allows the analysis of the interactions and the optimization of two factors previously shown to be the most significant. It consists of a central point, which will be executed in multiple replicas giving an internal estimate of pure error, and axial points that will determine the quadratic terms [13], [14]. It was chosen to cautiously perform 3 repetitions of central point considering the possibility of experimental errors. The coded matrix of experiments for temperature and residence time considering 2 input variables are shown in Table 1.

Table 1 – Coded matrix for complete CCD, 2^2

Experiment	Temperature (X_1)	Residence time (X_2)
1	-1	-1
2	1	-1
3	-1	1
4	1	1
5	-1.41	0
6	1.41	0
7	0	-1.41
8	0	1.41
9	0	0
10	0	0
11	0	0

To proceed with the pretreatment experiments it was necessary to associate the coded values with the real ones. The choice of the extremes of the interval was

based on experiments already done for sugar cane straw that is a similar material and consistent with the operation of the equipment. The temperature range was of 120 ºC to 240 ºC and the residence time of 10 minutes to 120 minutes. By placing the ends of the two intervals with the coded values of ± 1.41, with a linear interpolation it was possible to obtain the real value matrix of the CCD, presented in Table 2.

Table 2 – Real values matrix for complete CCD, 2^2

Experiment	Temperature (ºC)	Residence time (min.)
1	137	26
2	223	26
3	137	104
4	223	104
5	120	65
6	240	65
7	180	10
8	180	120
9	180	65
10	180	65
11	180	65

Each pretreatment condition was repeated several times to obtain 30 grams of pretreated spelt straw allowing performing triplicates of the enzymatic experiments. The end of this phase allowed the evaluation of the response variables Y to validate the model.

As outputs, six different responses were evaluated: (i) the cellulose content of the HCW treated straw; (ii) the glucose concentration of the sugar syrup and (iii) the glucose yield after 48 hours enzymatic hydrolysis; (iv) the acetic acid and (v) the furfural and (vi) HMF concentration in the liquid fraction from the pretreatment. The software *Statistica* 7.0 (Statsoft, USA) was used to gather all data of the complete CCD experimental design and allowed to evaluate the effect of each variable, the analysis of variance (ANOVA), the response surfaces and a possibility of mathematical model to be validated for each response.

2.4. Chemical characterization of spelt straw and of the solid fraction from the HCW pretreatment

Lignocellulosic materials are majorly formed by the structural components cellulose, hemicellulose and lignin besides nitrogen compounds, proteins, chlorophyll, waxes and minerals [15] that are usually quantified in both the raw and pretreated materials according to well established procedures ([15], [16], [17].). For the spelt straw material characterization, the materials soluble in water or alcohol, known as biomass extractives, were extracted in two sequential steps in a Soxhlet that used water and alcohol. For that 6 g of the straw were added

to cellulose cartridges and 190 mL of distilled water added in the collecting flask. The electric heater was adjusted for about 4-5 extraction cycles per hour during 24 consecutive hours. After the extraction, the water with dissolved extractives was removed and replaced with 190 mL of alcohol, for the second extraction step. At the end of this process the cellulose cartridge containing the extracted straw was left to dry in a vacuum oven at 60 °C. The extractives content of the straw sample corresponded to the weight difference in the filter weight before and after the extraction procedure, taking into account the moisture content.

The extracted spelt straw was submitted to acid hydrolysis, in triplicate, for the total hydrolysis of the cellulose and the hemicellulose. Approximately 0.3 g of dry material were placed in the hydrolysis flasks and added of 3 mL of sulfuric acid at 72%. The mixture was agitated with a magnetic stirrer bar that was immersed in a water bath at 30 °C for one hour and after this time interval added of 84 mL of distilled water to reduce the acid concentration to 4%. The flasks were transferred to an autoclave for one hour in an environment saturated with water vapor at 121 °C. After cooling, the mixture was filtered with a glass fiber filter previously dried and weighed and the solid lignin-rich fraction (IL) was dried and weighted. The liquid fraction was used for the spectrophotometric measurement of soluble lignin (SL), such that the percentage of total lignin corresponded to the sum of IL and SL. The liquid fraction was analyzed by HPLC for the determination of the concentrations of the biomass derived monosaccharides.

For the ash content determination 1 g of spelt straw was placed in a ceramic crucible, previously dried and weighted, and transferred to a muffle furnace (Thermo Scientific (Whaltan, Massachussets, U.S.A) model Lindberg/Blue M) at a temperature of 575 °C for 6 hours. After this period the crucibles were cooled down and weighted to quantify the ash content based on initial mass considering the moisture content. All experiments were performed in triplicate. The same procedure was used for the residue obtained after the acid hydrolysis to quantify the content of ash insoluble in acid and by difference estimate the insoluble lignin content.

The final normalized chemical composition of the spelt straw was calculated considering the concentration of each monomeric sugar, the total lignin (IL+SL) content, the total extractives and aches, based on the initial spelt straw mass considering the moisture content.

The characterization of the microbial inhibitors, furfural, HMF and acetic acid in the liquid fraction from HCW pretreatment were done by HPLC.

The microbial inhibitors furfural and HMF are formed by dehydration of C5 and C6 sugars while acetic acid a natural component of the hemicellulose and a potent inhibitor of the yeast *Saccharomyces cerevisiae* (REF), is also released during the hydrothermal process. As such, the quantification of the concentration of acetic acid, furfural and HMF in the liquid stream were carried out. High levels of lactic acid and phenols also can be found in liquid fraction

depending likewise on the process conditions and the severity degree of HCW pretreatment.

To quantify these sugars apparatus of HPLC became mandatory and the concentrations in the various streams, all the 11 samples were analyzed by HPLC directly on the liquid of pretreatment without pass thru acid hydrolysis of HCW liquid fraction.

2.5. Enzymatic hydrolysis

The enzymatic hydrolysis is a crucial step for the conversion of the cellulose content of the biomass in glucose syrups. The enzymatic hydrolysis of cellulose is catalyzed by the cellulase enzymes *endoglucanase* EC 3.2.1.4 and *exoglucanase* EC 3.2.1.91 that acting synergistically with the enzyme *β-glicosidase* EC 3.2.1.21, perform the complete hydrolysis of cellulose to glucose. Total cellulase activity is usually expressed as filter paper unit (FPU) defined by Ghose (1987) [18] and measured by the procedure described by the National Renewable Energy Laboratory[19]. All materials from the different pretreatment conditions were submitted to enzymatic hydrolysis to comparatively evaluate the efficiency of each pretreatment condition. For comparison the enzymatic hydrolysis of the untreated material was also done.

The enzymatic hydrolysis experiments were done with the commercial powder cellulase preparation Power Cell (Prozyn, Brasil) that presents a FPase activity of 412.98 FPU g^{-1}. In the hydrolysis experiments it was used an enzyme load of 10 FPU g^{-1} of dry pretreated or raw biomass that corresponded to 0.2421 g of the enzyme power per hydrolysis experiment. The glucose concentration prior to the enzymatic hydrolysis was measured and discounted from the final results.

The 100 mL reaction mixtures presented 10 g of dry pretreated biomass added of an enzyme mass corresponding to an enzyme load of 10 FPU per gram of dry biomass. The reaction flasks were added of 50 mL of 100 mM sodium citrate buffer solution (pH 4.8) and the necessary amount of distilled water, taking into account the biomass moisture content, to have a final biomass concentration of 100 g L^{-1}. The flasks were placed in a rotatory incubator shaker New Brunswick Scientific (Edison, New Jersey, USA) model Innova 4230, at 200 rpm that allowed the solid to remain in suspension and 50 °C. Samples of 1.5 mL were taken at 24, 48, and 72 hours and boiled for 10 minutes for enzyme inactivation before they were centrifuged at 7000 rpm for 10 minutes, to separate the liquid glucose syrup from the solid lignin rich fraction. Glucose concentration was measured in a YSI 2730 Biochemistry Analyzer.

3. Results and discussion

3.1. Chemical composition of raw spelt straw and of the HCW pretreated straw in different conditions

The spelt straw biomass with 14.9% moisture presented 38.25 ± 0.67 % of cellulose, 24.28 ± 0.42 % of hemicellulose, 14.77 ± 1.17 % of lignin, 5.71 ± 0.08

% of ash and 7.14 ± 1.30 % of extractives. These values represent the mean value and the standard deviation for 3 independent experiments.
Table 3 presents the results of the chemical composition, in terms of cellulose, hemicellulose, lignin and ash for the spelt straw differently pretreated by HCW.

Table 3 – Chemical composition of raw spelt straw and that of the pretreated materials in different HCW pretreatment conditions.

Condition	Cellulose (%)		Hemicellulose (%)		Lignin (%)		Ash (%)		Extractives (%)	
	Mean.	Std dev.	Mean.	Std dev.	Mean.	Std dev.	Mean.	Std dev.	Mean.	Std dev.
Raw biomass	38.25	0.67	24.28	0.42	14.77	1.17	5.71	0.08	7.14	1.30
1 (137 ºC / 26 min.)	45.34	0.26	27.11	1.13	14.77	0.36	2.79	0.25	-	-
2 (223 ºC / 26 min.)	47.82	0.56	0.00	0.00	50.04	0.78	3.74	0.12	-	-
3 (137 ºC / 104 min.)	41.47	1.05	25.52	0.21	24.21	0.46	3.56	0.08	-	-
4 (223 ºC / 104 min.)	35.53	0.43	0.00	0.00	62.53	1.20	3.07	0.19	-	-
5 (120 ºC / 65 min.)	40.23	0.14	25.58	0.07	26.25	0.82	3.64	0.32	-	-
6 (240 ºC / 65 min.)	24.47	0.51	0.00	0.00	80.95	0.10	4.16	0.32	-	-
7 (180 ºC / 10 min.)	57.74	1.07	8.02	0.18	35.83	0.95	3.76	0.56	-	-
8 (180 ºC / 120 min.)	50.27	0.42	1.85	0.03	49.95	0.70	2.89	0.22	-	-
9 (180 ºC / 65 min.)	52.66	0.81	2.87	0.04	40.94	1.03	2.97	0.12	-	-
10 (180 ºC / 65 min.)	54.03	1.06	3.09	0.23	37.39	0.23	4.08	0.30	-	-
11 (180 ºC / 65 min.)	55.47	1.36	2.85	0.09	41.19	0.59	4.49	0.41	-	-

The results show that for the treatment at 120 ºC for 65 min. and at 137 ºC, regardless of the treatment time of 26 or 104 min. the hemicellulose content of the pretreated material was comparable to that of the untreated material suggesting ineffectiveness. In accordance to that the percentages in the cellulose content were quite comparable, considering the variability of this plant derived material and that inherent to a multi-step experimental procedures. The increase of the temperature and treatment time to 180 ºC from 10 to 120 min. increased significantly hemicellulose removal such that the pretreated material presented from 8.02 to 1.85 % residual hemicellulose and the corresponding increase in the normalized cellulose, in the range of 57.74 to 50,27%, and lignin in the range of 35.83 to 49.95% indicating the preservation of the cellulose in the treated material. The further temperature increase to 223 °C for 26 and 104 min. resulted in the complete removal of the hemicellulose as well as the decrease of cellulose to 47.82%, for the 26 min. treatment and of 35.53% for the 104 min. treatment. At 240 °C for 65 min. it was observed, besides a complete hemicellulose removal, as expected, an even higher cellulose degradation whose normalized amount decreased to 24.47% alongside the normalized increase in the lignin content that jumped to 80.95%. As the preservation of the cellulose content is of paramount importance the temperature condition around the central points, i.e., 180 ºC from 10 to 120 min (conditions 7, 8, 9, 10 and 11) favored a higher recovery of cellulose alongside and efficient removal of hemicellulose. These results also indicated a higher influence of the pretreatment temperature on the chemical composition in comparison to the residence time. Nevertheless these results were quite encouraging the full picture of the treatment effectiveness would depend on the glucose concentrations and yields upon the subsequent enzymatic hydrolysis step. Moreover the level of inhibitors in the pretreatment liquid stream from the different conditions would be of importance as inhibitory levels can limit the usefulness of the C5 syrups via biotechnological routes besides. As such an

optimized hydrothermal pretreatment condition must balance the highest possible glucose concentration from the enzymatic hydrolysis of the lignocellulose to an acceptable concentration of inhibitors in the liquid stream.

3.2. Enzymatic hydrolysis

All eleven samples of treated straw as well the raw spelt straw were subjected to enzymatic hydrolysis. The determination of glucose concentration in the 24, 48 and 72 hours samples showed that the 48 hours sample already presented the highest glucose concentration that was equivalent to that of the 72 hours sample. Table 4 presents the mean value and the standard deviation for glucose concentration and glucose yield after 48 hours of enzymatic hydrolysis. The glucose yield was calculated based on the following equations.

$$\text{Glucose yield } (\%) = \frac{\text{mass of glucose } (g)}{\text{mass of cellulose in pretreated biomass } (g)} * 100$$

$$\text{Glucose yield } (\%) = \frac{[\text{glucose concentration}] * V}{\text{cellulose content} * (\frac{180}{162}) * [\text{biomass}] * V} * 100$$

Where, cellulose content corresponds to that on each pretreatment condition (Table 3), biomass concentration ([Biomass]) was of 100 g L^{-1} and the volume of enzymatic hydrolysis (V) was of 0,1 L.

Table 4 – Glucose concentration (g L^{-1}) and glucose yield (%) of raw and pretreated spelt straw biomass after 48h of enzymatic hydrolysis.

Condition	Glucose 48h (g/L)		Glucose yield (%)
	Mean.	Std dev.	Mean.
Raw biomass	8.01	0.05	18.85
1 (137 ºC / 26 min.)	13.61	0.22	27.01
2 (223 ºC / 26 min.)	24.20	0.72	45.54
3 (137 ºC / 104 min.)	15.05	1.54	32.67
4 (223 ºC / 104 min.)	21.67	1.26	54.89
5 (120 ºC / 65 min.)	10.24	0.32	22.91
6 (240 ºC / 65 min.)	16.33	0.22	60.07
7 (180 ºC / 10 min.)	29.58	3.10	46.11
8 (180 ºC / 120 min.)	28.10	0.76	50.31
9 (180 ºC / 65 min.)	28.53	0.34	48.76
10 (180 ºC / 65 min.)	28.89	1.24	48.12
11 (180 ºC / 65 min.)	30.32	0.38	49.19

From the results presented in table 4, the highest glucose concentrations corresponded to pretreatment conditions carried out at 180 ºC from 10 to 120

min. (conditions 7, 8, 9 10 and 11), reaching concentrations in the range of 28 and 30 g L^{-1}, in accordance to the biomass cellulose contents of the corresponding materials.

The highest glucose yield of 60% was observed for the highest studied temperature of 240 ºC for 65 min. However, in this condition the cellulose content of the lignocellulose dropped to 24.47% resulting in glucose syrup of 16.33% that was 55% lower than the average concentration observed for the treatments carried out at 180 ºC.

Higher glucose concentrations and yields could be obtained by the increase, in the reaction mixture of the biomass load from 100g/L to 150g/L and the use of a more effective cellulases preparation.

3.3. Microbial inhibitors concentration in HCW liquid fractions

The concentration of furfural, resulting from the dehydration of C5 sugars, of HMF resulting from the dehydration of C6 sugars and of acetic acid, released from the hemicellulose chain in the liquid stream of spelt straw differently treated by HCW are shown in Table 5. It can be noticed that the concentration of the acetic acid varied around 10 fold (from 0,44 to 4,27 g L^{-1}) according to the increase of the temperature from 137 ºC to 240 ºC. Nevertheless residence time at the same temperature increased the acetic acid release from the hemicellulose molecule the effect of the temperature was more prominent. In all cases the acetic acid concentration did not reach the threshold inhibitory concentration of 6.00 g L^{-1} for yeast *Saccharomyces cerevisiae*, widely used for ethanol fermentation[20]. This is a quite interesting finding as it would benefit 2G ethanol production from both the C6 and the C5 sugars using a GMO yeast. The acid acetic concentration for the conditions that associated hemicellulose removal to cellulose preservation, i.e. condition 7 (180 ºC, 10 min.) of 1.63g/L and conditions 8 to 11 (180 ºC, 65 and 120 min.) in the range of 2.62 to 3.38 g/L, were even lower and as such not inhibitory.

Table 5 – Acetic acid concentration (g L^{-1}) furfural concentration (g L^{-1}) and HMF concentration (g L^{-1}) in liquid fraction from spelt straw biomass pretreated by HCW in different conditions.

Condition	Acetic acid (g/L)	Furfural (g/L)	HMF (g/L)
Raw biomass	-	-	-
1 (137 ºC / 26 min.)	0.44	0.01	0.01
2 (223 ºC / 26 min.)	3.81	1.90	0.96
3 (137 ºC / 104 min.)	0.69	0.03	0.01
4 (223 ºC / 104 min.)	3.98	0.50	0.89
5 (120 ºC / 65 min.)	0.25	0.00	0.00
6 (240 ºC / 65 min.)	4.27	0.20	0.85
7 (180 ºC / 10 min.)	1.63	0.67	0.06
8 (180 ºC / 120 min.)	3.38	3.96	0.58
9 (180 ºC / 65 min.)	2.62	3.38	0.26
10 (180 ºC / 65 min.)	3.01	3.98	0.33
11 (180 ºC / 65 min.)	2.76	2.38	0.30

For the acetic acid concentration, the highest level was obtained in the condition number 6 reaching 4.27 g L^{-1}. For concentration of acetic acid up to 100 mM (6 g L^{-1}) results in an actual increase of the ethanol yield, while higher concentrations provoke its decrease. From other works, 6.00 g L^{-1} of acetic acid is the minimum inhibitory concentration for *Saccharomyces cerevisiae* yeast. Other studies show that acetic acid at 167 mM (10.02 g L^{-1}) and lactic acid 548 mM (49.36 g L^{-1}) completely inhibited growth of *Saccharomyces cerevisiae* both in minimal medium and in media which contained supplements[21].

Considering furfural concentration, the highest level corresponds to conditions of DOE central point reaching values between 2.38 and 3.98 g L^{-1} that are inhibitory for the yeast *Saccharomyces cerevisiae* that is severely affected in the presence of furfural at 2 g L^{-1} or higher[22]. However, the furfural concentration in the condition 7 (180 °C,10 min.), was of 0.67 g L^{-1} and not inhibitory. The inhibition by HMF is not as severe as that for furfural as HMF significantly inhibits the yeast growth and decreases ethanol fermentation above 5g L^{-1} reaching full inhibition at 15 g L^{-1}[23]. As in the present study the maximum HMF concentration observed corresponded to 0.96 g L^{-1} (condition 2), HMF, individually would be harmful to a C5 fermentation process.

3.4. Statistical analysis

All the mathematical models of DOE pretreatment experiments are a second order models since it's composed of two independent input variables, temperature (X_1) and residence time (X_2).

The response variables (Y_k) are:

Y_1 = Cellulose content in pretreated solid fraction (%);
Y_2 = Glucose concentration after 48h of enzymatic hydrolysis (g L^{-1});
Y_3 = Glucose yield after 48h of enzymatic hydrolysis (%);
Y_4 = Acetic acid concentration in pretreated liquid fraction (g L^{-1});
Y_5 = Furfural concentration in pretreated liquid fraction (g L^{-1});
Y_6= HMF concentration in pretreated liquid fraction (g L^{-1}).

In this way it is possible to have a model composed of 6 terms for each output variable:

$$\square_{\square} = \square_{\square} * \square_1^2 + \square_{\square} * \square_2^2 + \square_{\square} * \square_1 + \square_{\square} * \square_2 + \square_{\square} * \square_1 * \square_2 + \square_{\square}$$

The *f* term is fixed, and comes from the center point, where both X_1 and X_2 assume 0 as value; the first step will be to verify if all of the other 5 terms significantly influence the solution or can be ignored through p-value test using Pareto's Diagram from *Statistica* 7.0.
Being the pretreatment an experiment that has not an optimal reproducibility (mainly due to the little instability of the temperature control of the reactor), we adopt a confidence interval of 95%. A confidence interval estimates how the

variation of variables (in this case, parameters *a*, *b*, *c*, *d*, *e* and *f* is really due to statistical significance instead of experimental error or random variability; so, in adopting a too high confidence interval, while the results extracted can be more accurate, there is the risk to cut out variables that have a minor but not zero significance on our experiment.
In particular, to decide which of the above terms will be present in the mathematical model, the software *Statistica 7.0* runs the p-value test; this test gives an estimate between 0 and 1 of how much is probable that the variability between the 11 samples is random or due to significance. So, if the p-value is lower than 0.05, we assume the term to be in mathematical equation.

After discarding the non-significant terms of each mathematical model, it is possible to obtain the following equations:

$$\square_1 = -11.0133 * \square_1^2 - 3.2200 * \square_1 - 3.3473 * \square_2 + 53.8980$$

$$\square_2 = -8.39669 * \square_1^2 + 3.23423 * \square_1 + 28.49943$$

$$\square_3 = -4.83835 * \square_1^2 - 1.45855 * \square_2^2 + 11.67891 * \square_1 + 2.62276 * \square_2 + 48.70956$$

$$\square_4 = 1.544996 * \square_1 + 0.363982 * \square_2 + 2.439455$$

$$\square_5 = -1.55012 * \square_1^2 + 2.66855$$

$$\square_6 = 0.079528 * \square_1^2 + 0.380798 * \square_1 + 0.084298 * \square_2 + 0.328607$$

In these equations presented above, the values used for X_1 and X_2 should be used in coded form, that is, values between -1.41 and +1.41.

In addition to the construction of the mathematical models for each of the output variables was verified the validity of each of the models based on analysis of variance (ANOVA) through the F-test. The null hypothesis is rejected if the F calculated (F-calc.) from the data is greater than the critical value of the F-distribution (F-dist.) for some desired false-rejection probability (e.g. 0.05). Table 6 below shows the validity of models according to 5% of significance level.

Table 6 – Validity of mathematical models according to ANOVA and F-test.

Output variable	Significance (%)	F-calc.	F-dist.	R^2 (%)	Validity
Cellulose (%)	5	19.86	4.347	92.81	OK
Glucose concentration (g L^{-1})	5	44.4	4.459	94.29	OK
Glucose yield (%)	5	16.26	4.534	94.25	OK
Acetic acid concentration (g L^{-1})	5	6.78	5.117	55.22	OK
Furfural concentration (g L^{-1})	5	13.14	4.347	88.97	OK
HMF concentration (g L^{-1})	5	39.82	4.459	93.68	OK

According to the data presented in Table 6, all 6 mathematical models can be counted as valid since the value of F calculated was higher than the F value of the distribution for all output variables at a significance level of 5%. In addition, except for acetic acid concentration, all mathematical models presented a coefficient of determination (R^2) greater than 88%.

Since all models can be considered valid, by previous discussed information such as the microbial inhibitors concentrations, glucose concentration in the enzymatic hydrolysis and hydrolysis yields, some good options can be estimated for the pretreatment conditions in terms of temperature and residence time due to the relevance of these parameters and the exclusion of unfavorable conditions.

3.5. Evaluation of HCW pretreatment best conditions

Among all the output variables evaluated by the DOE, special emphasis can be given to the glucose concentration from the enzymatic hydrolysis since this parameter strongly depends on the cellulose content of the pretreated material as a rich cellulose pretreated material will increase the possibility of a high glucose concentrations released during the enzymatic hydrolysis step.

On the other hand, considering the microbial inhibitors concentrations, it can be said that they favor the definition of restriction conditions. For, if a certain pretreatment condition releases a high concentration of an inhibitory compound the operating region in this condition is not recommended.

As a irrelevant parameter for the definition of an optimum pretreatment condition it is possible to mention the glucose yield, since even in conditions where both the cellulose content and the glucose is low can result in a higher glucose yield. Figure 1 below shows the glucose concentration after 48 hours of enzymatic hydrolysis as a function of temperature and residence time.

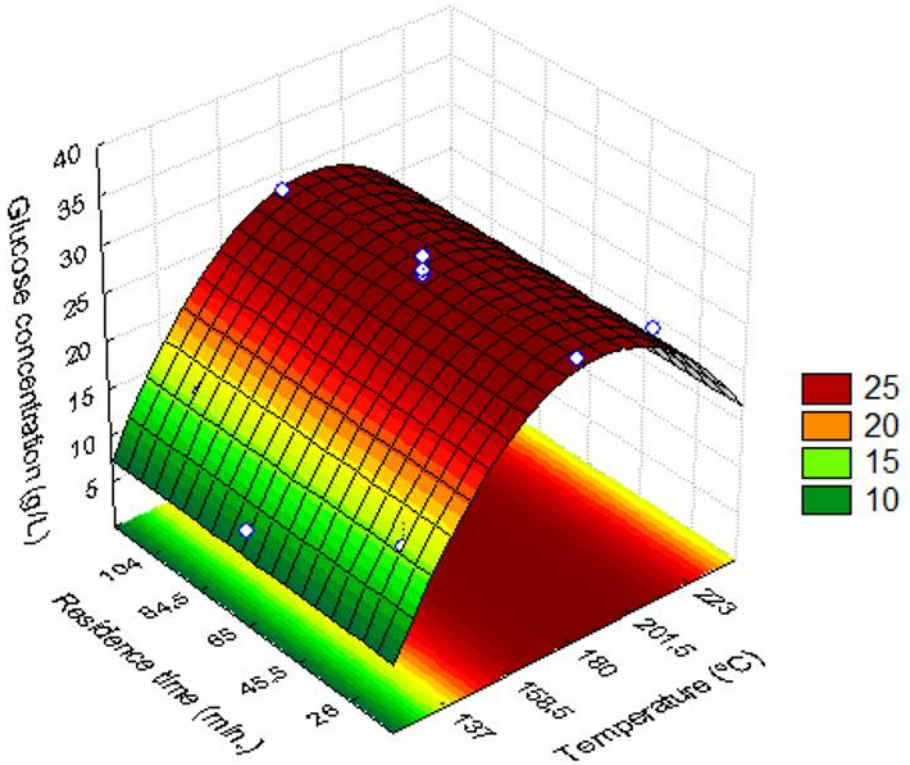

Figure 1 – 3D Fitted surface of glucose concentration after 48h of enzymatic hydrolysis as a function of temperature (ºC) and residence time (min.).

According to the observed in figure 1, the highest glucose concentration occurs at temperatures close to 180 ° C and the residence time does not exert a great influence on this output variable and can be used in its minimum DOE value that is equivalent to 10 min.

The temperature value of 180 °C refers exactly to the value at the central point of this input variable in DOE, however for the residence time the central value was 65 minutes. As has already been observed by the triplicate of the central point (experiments numbers 9, 10 and 11) that for 65 minutes of residence time at 180 ºC, are generated high concentrations of inhibitory compounds mainly furfural, which begins to exert inhibitory activity in the production of CO_2 in values higher than 1.5 g L^{-1} and inhibitory activity in the yeast strain at concentrations greater than 2.0 g L^{-1} [22].

In view of these observations it can be considered that one of the possible best pretreatment conditions are located at a temperature of 180 ° C and 10 minutes of residence time. This condition corresponds exactly to the DOE experimental condition number 7 and respect the restriction conditions related to the maximum concentration of microbial inhibitors and also shows a high concentration of glucose released in the enzymatic hydrolysis step as a result of a high cellulose content in the pretreated solid fraction.

3.6. Mathematical model performance on best condition

In order to evaluate the functionality of the mathematical models obtained by the DOE in this session, the observed behavior and the predicted behavior of the 6 models were evaluated in relation to the one of most favorable pretreatment conditions, which occurred at 180 ºC for 10 minutes of residence time. Table 7 presents the behavior of the models for 6 output variables in one of the best pre-treatment conditions defined in this work.

Table 7 – Evaluation of output variables tested at 180 ºC and 10 minutes of residence time.

Output variable	Model predicted value	Observed experimental value	Relative deviation
Cellulose content (%)	57.25	57.74	0.9%
Glucose concentration (g L^{-1})	28.50	29.58	3.8%
Glucose yield (%)	49.87	46.11	-7.5%
Acetic acid concentration (g L^{-1})	2.08	1.63	-21.5%
Furfural concentration (g L^{-1})	2.67	0.67	-74.9%
HMF concentration (g L^{-1})	0.24	0.06	-75.4%

Based on the results observed in Table 7, it can be concluded that only two of the six mathematical models presented deviations lower than 5% in relation to the value observed experimentally, the cellulose content and glucose concentration after 48 h of enzymatic hydrolysis showed deviations of 0.9% and 3.8%, respectively. The mathematical models related to the concentration of furfural and HMF presented deviations equivalent to 75% of the value observed experimentally.

4. Conclusions

The straw of the switchgrass Spelt (Triticum aestivum var. spelta) was used for the production of biomass sugar syrups via hydrothermal pretreatment and enzymatic hydrolysis. Under the best pretreatment conditions it was possible to obtain glucose syrup with 29.58 g L^{-1} in a reaction mixture with 100 g L^{-1} pretreated biomass load that corresponded to XX % glucan hydrolysis yield. The concentration of acetic acid, furfural and HMF in the glucose syrup were of 1.63 g L^{-1}, 0.67 g L^{-1} and 0.06 g L^{-1}, respectively, which were not inhibitory for the use of the biomass glucose syrup for ethanol production.
The best hydrothermal pretreatment conditions corresponded to 180 °C for 10 minutes. Using a statistical design of experiments for different pretreatment conditions it was possible to model mathematically 2 of 6 output variables with relative deviation smaller than 5% such as the cellulose content in the solid pretreated straw as well as the glucose concentration after 48 h of enzymatic hydrolysis.

5. References

[1] Perera F. Pollution from Fossil-Fuel Combustion is the Leading Environmental Threat to Global Pediatric Health and Equity: Solutions Exist. Int J Environ Res Public Health 2017;15(1).

[2] Wagner L, Ross I, Foster J, Hankamer B. Trading Off Global Fuel Supply, CO2 Emissions and Sustainable Development. PLoS ONE 2016;11(3):e0149406.

[3] Soccol CR, Vandenberghe LPdS, Medeiros ABP, Karp SG, Buckeridge M, Ramos LP et al. Bioethanol from lignocelluloses: Status and perspectives in Brazil. Bioresour Technol 2010;101(13):4820–5.

[4] Lee RA, Lavoie J-M. From first- to third-generation biofuels: Challenges of producing a commodity from a biomass of increasing complexity. Animal Frontiers 2013;3(2):6–11.

[5] Harmsen PFH. Literature review of physical and chemical pretreatment processes for lignocellulosic biomass. Wageningen: Wageningen UR, Food & Biobased Research; 2010.

[6] Marriott PE, Gómez LD, McQueen-Mason SJ. Unlocking the potential of lignocellulosic biomass through plant science. New Phytol 2016;209(4):1366–81.

[7] Kumar AK, Sharma S. Recent updates on different methods of pretreatment of lignocellulosic feedstocks: a review. Bioresour Bioprocess 2017;4(1).

[8] Lee HV, Hamid SBA, Zain SK. Conversion of lignocellulosic biomass to nanocellulose: structure and chemical process. ScientificWorldJournal 2014;2014:631013.

[9] Mosier N, Wyman C, Dale B, Elander R, Lee YY, Holtzapple M et al. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour Technol 2005;96(6):673–86.

[10] Kiš D, Jovičić N, Matin A, Kalambura S, Vila S, Guberac S. Energy value of agricultural spelt residue (*Triticum spelta L.*) – forgotten cultures. Teh. vjesn. 2017;24(Supplement 2).

[11] B. Hames, R. Ruiz, C. Scarlata, A. Sluiter, J. Sluiter, and D. Templeton. Preparation of Samples for Compositional Analysis: Laboratory Analytical Procedure (LAP) - Technical Report NREL/TP-510-42620; Available from: https://www.nrel.gov/docs/gen/fy08/42620.pdf.

[12] da Silva AS, Sobral Teixeira RS, Oliveira Rd, Santana V, Barros RdRO de, Antonieta M et al. Sugarcane and Woody Biomass Pretreatments for Ethanol Production. In: Chandel AK, Silva SSd, editors. Sustainable

degradation of lignocellulosic biomass: Techniques, applications and commercialization. Rijeka, Cr: InTech; 2013.

[13] Box GEP, Hunter WG, Hunter JS. Statistics for experimenters: An introduction to design, data analysis, and model building. New York: Wiley; 1978.

[14] Rodrigues MI, Iemma AF (eds.). Planejamento de experimentos e otimização de processos. 3rd ed. Campinas: Casa do Espírito Amigo Fraternidade Fé e Amor; 2014.

[15] A. Sluiter, B. Hames, R. Ruiz, C. Scarlata J. Sluiter, D. Templeton and D. Crocker. Determination of Structural Carbohydrates and Lignin in Biomass: Laboratory Analytical Procedure (LAP) - Technical Report NREL/TP-510-42618; Available from: https://www.nrel.gov/docs/gen/fy13/42618.pdf.

[16] A. Sluiter, R. Ruiz, C. Scarlata, J. Sluiter and D. Templeton. Determination of Extractives in Biomass - Technical Report NREL/TP-510-42619: Laboratory Analytical Procedure (LAP); Available from: https://www.nrel.gov/docs/gen/fy08/42619.pdf.

[17] A. Sluiter, B. Hames, R. Ruiz, C. Scarlata J. Sluiter, and D. Templeton. Determination of Ash in Biomass: Laboratory Analytical Procedure (LAP) - Technical Report NREL/TP-510-42622; Available from: https://www.nrel.gov/docs/gen/fy08/42622.pdf.

[18] Ghose TK. Measurement of cellulase activities. Pure and Applied Chemistry 1987;59(2):257–68.

[19] Adney B, Baker J. Measurement of Cellulase Activities: Laboratory Analytical Procedure (LAP);Available from: https://www.nrel.gov/docs/gen/fy08/42628.pdf.

[20] Larsson S, Palmqvist E, Hahn-Hägerdal B, Tengborg C, Stenberg K, Zacchi G et al. The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. Enzyme and Microbial Technology 1999;24(3-4):151–9.

[21] Narendranath NV, Thomas KC, Ingledew WM. Effects of acetic acid and lactic acid on the growth of Saccharomyces cerevisiae in a minimal medium. J Ind Microbiol Biotechnol 2001;26(3):171–7.

[22] Sanchez B, Bautista J. Effects of furfural and 5-hydroxymethylfurfural on the fermentation of Saccharomyces cerevisiae and biomass production from Candida guilliermondii. Enzyme and Microbial Technology 1988;10(5):315–8.

[23] Baptista C V R. Efeitos inibitórios do hidroximetilfurfural na fermentação alcoólica: avaliação por Citometria de Fluxo: Dissertação para obtenção do grau de mestre em Engenharia Alimentar; Available from: https://www.repository.utl.pt/bitstream/10400.5/6697/1/Tese%20Versao%20Definitiva.pdf.

[24] Pereira, Sandra Cerqueira; Maehara, Larissa; Machado, Cristina Maria Monteiro; Farinas, Cristiane Sanchez (2015): 2G ethanol from the whole sugarcane lignocellulosic biomass. Em: *Biotechnology for Biofuels* 8 (1), pág. 44. DOI: 10.1186/s13068-015-0224-0.

[25] Ben Fradj, N.; Jayet, P. A.; Aghajanzadeh-Darzi, P. (2016): Competition between food, feed, and (bio)fuel: A supply-side model based assessment at the European scale. Em: *Land Use Policy* 52, pág. 195–205. DOI: 10.1016/j.landusepol.2015.12.027.

[26] Kristoufek, Ladislav; Janda, Karel; Zilberman, David (2016): Comovements of ethanol-related prices: evidence from Brazil and the USA. Em: *GCB Bioenergy* 8 (2), pág. 346–356. DOI: 10.1111/gcbb.12260.

[27] Popp, J.; Lakner, Z.; Harangi-Rákos, M.; Fári, M. (2014): The effect of bioenergy expansion: Food, energy, and environment. Em: *Renewable and Sustainable Energy Reviews* 32, pág. 559–578. DOI: 10.1016/j.rser.2014.01.056.