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OPTIMIZATION OF HYDROTHERMAL PRETREATMENT FOR ENZYMATIC HYDROLYSIS OF BANANA PSEUDO STEM USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT

In this study different conditions of hydrothermal pretreatment were evaluated for conversion of banana pseudo stem to fermentable sugars.
A Central Composite Design (CCD) was used to obtain regression equations in function of the following variables: solid/liquid ratio (1/10; 1/12.5; 1/15), temperature (170 °C, 190 °C, 210 °C) and reaction time (10 min, 15 min, 20 min).
The cellulose digestibility improved in all conditions tested, with maximum digestibility achieved at 210 °C for 10 minutes and 1/15 of solid/liquid ratio. The Glucose yield at optimal conditions was 58.4 g/kg with an excellent recovery of cellulose of 98%.
Thus, the hydrothermal pretreatment demonstrated to be an effective process in increasing cellulose concentration and producing fermentable sugar from recalcitrant lignocellulosic biomass. Furthermore, based on the design response surface methodology, an optimum condition of each pretreatment could be obtained from the statistical models built. All the variables studied influenced the enzymatic sugar release.

Keywords: Lignocellulosic residues; thermal process, glucose release, central composite design, enzymatic digestibility

INTRODUCTION

There has been an increasing worldwide interest in alternative sources of energy. The stepwise increase in oil prices, in addition to global warming caused by the buildup of greenhouse gases, drives the development of industrial processes based on renewable energy. Renewable energy accounted for nearly half of all new power generation capacity in 2014, led by growth in China, United States, Japan and Germany, with investment remaining strong at around $ 270 billion and costs continuing to fall (**IEA, 2015**).
The availability of renewable cellulosic feedstock is almost unlimited around the globe. Large-scale production of bioenergy requires utilization of lignocellulosic biomass feedstock's that do not compete with food sources. Using biomass like cellulosic agricultural residue is a potential promising natural renewable, inexpensive, cost effective and sustainable source, which can be used considerably and commercially for production of bioenergy as bioethanol (**Gupta and Verma, 2015**). Potentially, bioethanol can be produced by fermentation of sugars derived from hydrolysis of lignocellulosic biomass. Given the wide availability of agro-industrial residue in Brazil, it becomes a very attractive option to use this material for biofuel production.
Annually about 1×10^{10} tons of lignocellulosic biomass are produced worldwide (**Sánchez and Cardona, 2008**). Lignocellulose consists mainly of carbohydrates (up to 75%) that can become an essential source of fermentable carbohydrates for the production of liquid biofuels as well as a large variety of commodity chemicals and biodegradable materials (**Zhao and Zhang, 2012**). The arrangement of these components inside the biomass makes it an extremely complex structure. Cellulose and hemicellulose are tightly linked to a lignin component through covalent and hydrogenic bonds that make the structure highly robust and resistant (**Limayem and Ricke, 2015**). Due to this recalcitrant nature, it is necessary to subject the biomass to physical and / or chemical pretreatments before its hydrolysis into fermentable monosaccharides. Pretreatment is needed to provide accessibility to enzymes for the hydrolysis of cellulose and hemicellulose. Thus, the pretreatment may to decrease content of cellulose and hemicellulose of agroindustrial residues, and it is also cost effective, and environmentally sustainable. Many review papers have reported pretreatment as one of the most expensive units in the cellulosic ethanol production process and, indeed, the development of cost effective pretreatment technologies has become the most important challenge of biorefinement (**Njoku and Uellendahl, 2012; Zheng and Zhang, 2009**).
In the field of biomass utilization, the hydrothermal process is the most used method for obtaining monosaccharides from agricultural residues (**Takata *et al,* 2013**). In this method, the thermal degradation of biomass takes place in water causing a break of glycosidic linkages of the carbohydrates, especially hemicelluloses. Due to its complex nature, hemicellulose does not have the same crystal structure and resistance as cellulose and therefore degrades more easily when subjected to heat treatment (**Tekin *et al*., 2015**). A part of the hemicellulose is degraded into acetic acid, formic acid, 2-furfural and other degradation products. During pretreatment, small amounts of cellulose are also solubilized as oligomers, free glucose and some are degraded into 5-hydroxy-methylfurfural (**Gan *et al*., 2015**). Hydrothermal pretreatment improves enzymatic hydrolysis of cellulose through an increase in pore size to enhance enzyme penetration, and an increase in accessible cellulose by decreasing its crystallinity and its association with lignin (**Zhang *et al*., 2014**). Nevertheless, this process does not require addition and recovery of any chemicals but water, and it only causes limited equipment corrosion problems (**Ruiz *et al*., 2013**).
The biomass chosen for this purpose is banana pseudo stem. Brazil is one of the world's largest food producers and ranks fifth in world production of bananas with an average production of 7 million tons per year. For each ton of bananas harvested, around 4 tons of lignocellulosic wastes are generated; among which 75% consists of banana plant pseudo stem (**Souza *et al*., 2014**).
Several studies were performed using this method of pretreatment in different biomasses such as wheat straw (**Jensen-Ambye *et al*., 2013**), sugarcane bagasse (**Zhuan *et al*., 2014**), and sugarcane straw (**Silva *et al*., 2010**). Authors also reported pretreatment parameters such as temperature, reaction time and solid/liquid ratio that may be critical for maintaining the performance of the system. Therefore, in this paper, an experimental design—response surface methodology (RSM) was employed with a orthogonal central composite design (CCD) to investigate the influence of independent variables of pretreatments (temperature, time and solid/liquid ratio) on the response variable (total glucose yield). Unlike classical optimization, response surface methodology (RSM) involves the integration of mathematical and statistical techniques to analyse the effects of several independent response variables. Also, this method leads to a reduced number of experiments which results in time saving and the use of less material and reagent (**Tye and Lee, 2015; Tekin *et al*., 2014**).

MATERIALS AND METHODS

Banana pseudo stem ("Prata Anã" variety) were manually harvested in March 2012 from plantations in Janaúba, Minas Gerais, Brazil. The raw materials were dried at room temperature for seven days, milled in hammer mill into particles with a size of 20 mesh for hydrothermal pretreatment. The milled materials were stored in plastic bags at room temperature until further use. The standard reagents of furfural, 5-hydroxymethylfurfural (HMF), D-glucose, D-xylose and the chemical reagents methanol and acetic acid were purchased from Sigma–Aldrich. Chemical reagents including sulfuric acid, citric acid, sodium phosphate, acetone, and formic acid were obtained from Vetec Fine Chemical (Duque de Caxias, RJ, Brazil). Enzyme solutions, cellulases, β-glucosidase (Cellic CTec2) and hemicellulases (Cellic HTec2) were kindly supplied by Novozymes A/S (Bagsvaerd, Denmark). All other reagents used in this study were of analytical grade.

Hydrothermal pretreatment of banana pseudo stem

Hydrothermal pretreatment was carried out in a Parr reactor (model 4848 M) equipped with a mechanical stirrer and cooling coils inside for a better temperature control. Fifteen experiments have been performed with continuous pretreatment of banana pseudo stem under different pretreatment conditions. CCD was performed with four central points and three levels for each analyzed variable: temperature (170 °C, 190 °C and 210 ºC), pseudo stem/water concentration (1/10, 1/12.5; 1/15) and reaction times (5, 10 and 15 min, the time excluded heating and cooling periods). The pretreated materials were separated into solid and liquid fractions. The solid fraction was washed thoroughly with water, sealed in a hermetic vessel to retain moisture. The liquid fraction had the pH measured and then stored at -20 °C as well as the solid fraction for further analysis.

Biomass pretreatment and composition analysis

The chemical composition of the untreated and treated banana pseudo stem were milled in a Wiley mill into particles with a size of 40–60 mesh, oven-dried at 45 °C to constant weight, and then selected to determine chemical composition. Ash, carbohydrate (glucan, xylan, galactans, mannans and arabinans) and lignin (soluble, insoluble and total) contents were determined. Firstly, biomass samples were submitted to Soxhlet extraction with acetone for five hours. Extractive-free biomass (0.3 g) was treated with 72% (wt %) H_2SO_4 (3 mL) at 30 °C for 1 h with occasional mixing. The resulted hydrolysates were diluted with 84 mL of deionized water (to 4 wt % H_2SO_4) and autoclaved at 121 °C for 1 h. After autoclaving, the hydrolysates were filtered for gravimetric determination of acid-insoluble lignin. The resulting filtrates were used for soluble lignin and sugar content. Acid-soluble lignins were determined by ultraviolet spectroscopy using an equation, according to SCAN-CM 71:09:

C (g/L) = 4.53 (*Al* – *Ac*) / 300, Al and Ac correspond to the absorbance at a wavelength of 215 nm and 280 nm, respectively.

Concentrations of biomass sugars (arabinose, galactose, glucose, xylose, and mannose) in the filtrate were determined using the high-performance liquid chromatography (HPLC) system Dionex ICS3000 (Dionex Co. – Sunnyvale, CA, USA), equipped with a pulsed amperometric detector (PAD) with a gold electrode and a CarboPac PA1 column (ThermoScientific, USA). Prior to injection, samples were filtered through 0.45-mm HV filters and a volume of 25 µL was injected into the chromatography system at room temperature and a flow rate of 1 mL/min. Calibration curves were prepared for each sugar with results expressed in absolute values. Cellulose and hemicellulose contents were calculated from the individual sugars using mathematical procedures as described in the same reference above. Ash content was analyzed by gravimetric method. Samples (0.5 g) were incinerated at 575 °C for 3 h. After calcination, the samples were cooled in a desiccator to room temperature and weighed. The incineration was repeated until constant mass.

Analysis of organic acids and furfurals

The liquid fraction was also characterized by the determination of degradation products: organic acids (formic and acetic acid) and furfurals (2-furaldehyde and 5-hydroxymethylfurfural - HMF). A HPLC system equipped with refractive index detector (RID) and an Aminex HPX-87H column at 45 °C was used to analyze organic acids. Millipore water was used as the mobile phase at flow rate of 0.6 mL/min. Another HPLC system equipped with a Phenomenex C18 (2) 100A (250 mm x 4.6 mm, 5.0 µm) column at 25 °C and UV detector in 279 nm was used for furfurals analysis with methanol 11.2% (vol.%) and acetic acid (1%) 88.8% (vol.%) as the mobile phase at flow rate of 0.8 mL/min.

Biomass saccharification

Commercial enzyme solutions, cellulases, β-glucosidase (Cellic CTec2) and hemicellulases (Cellic HTec2) were applied in a biomass saccharification experiment.

An Enzymatic digestibility test was performed in 250 mL Erlenmeyer flasks containing 4 g (dry weight) of banana pseudo stem with 50 mL of sodium citrate buffer, 50 mol m^{-3}. Enzyme loading was specified for cellulase 5.4% (wt %) respectively, in pH 4.8 with addition of 0.3% (w/v) sodium azide to prevent microbial growth. Hydrolysis reactions were allowed to proceed for 72 h at 50 °C with continuous agitation on a Thermoshaker at 150 rpm. Incubations were finished by heating to 100 °C for 5 min after which samples were centrifuged at 13,000 *g* for 5 minutes. The supernatants were recovered by aspiration and frozen prior to analysis. All determinations were performed in duplicate.

Released sugar monomer (e.g., glucose) were quantified using a HPLC with a refractive index detector (Shimadzu Corporation, Kyoto, Japan) and an Aminex-HPX-87P column (Shimadzu) at 80 °C and a flow rate of 0.6 mL/min with 5 mol m^{-3} H_2SO_4 as the eluent. Structural carbohydrates were calculated by comparing the area of standard sugar through a calibration curve of each compound.

Cellulose hydrolysis rates (mainly components of pre-treated biomass) were calculated using the following equation:

Glucan saccharification yield (%) = *Amount of glucose* (g) x 0.9 x 100 / *Amount of cellulose in pretreated pseudo stem* (g).

Statistical analysis

Hydrothermal pretreatment was optimized by adjusting the independent variables solid/liquid ratio (S/L), temperature and reaction time by means of a Central Composite Design (CCD) composed of 18 runs, 3 variables with 3 levels and 4 center points. The dependent variable was glucose concentration.

Quality of the statistical model equation was expressed by coefficient of determination (R^2) and its significance was evaluated by the F-test at 95% confidence. Effects of the variables were measured by analyzing Pareto charts and surface response graphics, considering correlation and curves. CCD and statistical analysis were performed using *Statistica*® 13.0 software.

RESULTS AND DISCUSSION

Raw material composition

Similar to levels observed in other lignocellulosic biomass (**Berchem *et al*., 2017**; **Boussarsar *et al*., 2009**; **Petersen *et al*., 2009**), the cellulose is the major constituent of banana pseudo stem, comprising approximately 40% of its dry mass (Table 1). However, hemicellulose content appears at relatively lower levels than other lignocellulosic biomass.

Banana pseudo stem also contains a relatively low amount of lignin (Table 1). This can be advantageous for ethanol production as this compound can limit enzymatic hydrolysis of lignocellulosic biomass by lignin-carbohydrate complex formation.

The highest ethanol yield was observed in sugarcane bagasse followed by banana pseudo stem, due to their high cellulose content (Table 1). These values were based on theoretical fermentation yield calculations, however they suggest banana pseudo stem might be promising lignocellulosic material for ethanol production. Furthermore, the carbohydrate fraction of the biomass represents their potential for biochemical conversion of sugars into lignocellulosic ethanol (***Dowe and Mcmillan, 2008)***.

Ash percentage in banana pseudo stem is quite high when compared to results found for other materials (Table 1). This difference can be due to inorganic fraction of banana pseudo stem that is manly composed by potassium and calcium, whereas lignocellulosic materials contain mainly silica (**Hwang *et al*., 2013; Mohapatra *et al*., 2010**).

Pretreatment process conditions

Separation of individual components present in biomass is important to biorefinery, since the composition of different biomass affects efficiency of processing and the choice of pretreatments required to maximize the recovery of sugars. This pretreatment should be cost-effective, minimize the production of toxic waste and result in the recovery of most of the lignocellulosic components in a useable form in separate fractions (**Agbor *et al*., 2011**).

A similar effect was observed over different pretreatment conditions, removing mainly hemicellulose and retaining cellulose and lignin fractions in the solid portions (Table 2). In hydrothermal pretreatment, most of the hemicellulose fraction is dissolved in the liquid phase, while cellulose and insoluble lignin are

retained in the solid portion fractions (**Lei *et al*., 2013**). However, the proportion of soluble compounds depends on the operation conditions (**He *et al*., 2015**). In addition to separating a large proportion of hemicellulose fraction, hydrothermal pretreatment causes the breakage of the cellulosic structure favoring subsequent enzymatic hydrolysis procedure (**Sun *et al*., 2014**).

In temperature over 200 °C there was a reduction of around 90% of hemicellulose with the largest removal at 218 °C for 15 min (96.7%) indicating that high temperature was positive for this compound hydrolysis (Table 2). However, a small amount of xylose, as the predominant neutral monosaccharide of the hemicellulose structure, was recovered in the liquid fraction (Table 2). This might be explained by the fact that the xylose, under these severe conditions, can be further dehydrated to form organic acid and furan compounds (Table 3) (**Ibbett *et al*., 2014**).

Cellulose is mainly contained in the solid fraction after pretreatment, a direct consequence of the removal of the hemicellulose portion (Table 2). Cellulose content in the solid fraction reached levels of 48.5% at 170 °C (1/15; 10 min), increased to 59.0% at 190 °C (1/12.5; 15 min), remaining close to the latter at 210 °C and then decreased to 53.8% at 218 °C (1/12.5; 15 min). These results suggest increasing pretreatment temperature to a certain extent (up to 210 °C) improves the glucose concentration in the solid fraction. However, cellulose fraction is more resistant, small amounts of glucose that could be found in liquid fractions may also undergo dehydration reactions under the most severe pretreatment conditions. Note that cellulose quantities in liquid fractions decreased with the increase of pretreatment severity. It is consistent with an early study (Sun et al., 2014) that the glycosidic bonds in the chain segments within the amorphous portion of cellulose started to be ruptured at temperatures above 200 °C.

Lignin also presented increasing contents in the solid portion when temperature increased up to 210 °C (Table 2). During hydrothermal pretreatment, lignin cycles between solid and liquid phases are caused by simultaneous depolymerisation and repolymerisation reactions (**Weingarten *et al*., 2011**). The content of lignin in liquid fraction increased with increasing pretreatment severity (Table 2). It may be inferred that covalent bonds between lignin and hemicellulose improve lignin solubility during lignin deconstruction. This result could also correspond to the decrease of the total sugar content at harsh conditions.

Analysis of furfurals and organic acids

During the hydrothermal pretreatment process, especially at higher pretreatment temperatures, acetic acid, furfural and HMF were generated (Table 3). These compounds are the main concern in pretreated biomass. Under acidic conditions, glucose and xylose can be dehydrated into HMF and furfural (**Sun *et al*., 2014**). Organic acids are believed to be generated from the rehydration of these furans. These products are known as inhibitors to anaerobic digestion and can form insoluble lignin-like structures that deposit on the pretreated fibers and therefore decrease accessibility for the cellulases.

The runs 2, 9 and 4 showed no furan generated during pretreatment, which also presented the highest hemicellulose yields in solid fraction (Table 2). These conditions might be very mild for sugar dissolution in the liquid fraction, which would generate less degradation products.

The levels of organic acids and furan compounds released rise as the temperature increases towards 210 °C (Table 3). Their maximal concentrations were observed at run 7 due to the higher temperature and longer pretreatment time favouring furfural and HMF formation, as well as their degradation in acetic acid or formic acid (Table 3). However, at 218 °C the conversion of the inhibitors decreased due to the reactions between xylose and furfural which are highly polymerized insoluble carbonaceous species (**Kristensen *et al*., 2009**).

Table 1 Chemical composition and maximum theoretical ethanol yield of banana pseudo stem and other lignocellulosic biomass

Compounds/yield	Unit	Lignocellulosic biomass			
		Banana pseudo stem[a]	Sugarcane Bagasse[b]	Corn Stover[b]	Wheat traw[b]
Cellulose	(dry w %)	40.7 ± 1.0	45.0	37.2	35.0
Hemicellulose		14.1 ± 0.3	26.0	28.2	22.3
Total lignin		13.2 ± 0.1	20.0	15.3	15.6
Ash		10.2 ± 0.0	2.1	8.4	6.5
Maximum theoretical ethanol yield	(L/dry ton)[b]	291.4	322.9	266.6	251.1

a - Present study, b- Calculated considering the total cellulose conversion in the sample. (**Boussarsar *et al*., 2009**; **Petersen *et al*., 2009**; **Dowe and Mcmilan, 2008**).

Table 2 Chemical composition of banana pseudo stem after hydrothermal pretreatment

Run	Conditions			Solid fractions (dry w %)			Liquid fractions (dry w %)		
	Temperature (°C)	Time (min)	Solid/Liquid (g/mL)	Cellulose	Hemicellulose	Lignin	Cellulose	Hemicellulose	Lignin
1	170	10	1/10	54.0 ± 0.7	9.7 ± 0.0	16.7±0.2	31.9 ± 1.6	6.0 ± 0.2	15.5±0.9
2		10	1/15	48.5 ± 0.7	15.3 ± 0.4	14.1±0.1	36.3 ± 0.2	5.4 ± 0.0	16.6±0.1
3		20	1/10	53.9 ±0.5	8.7 ± 0.2	17.2±0.1	33.3 ± 1.9	6.3 ± 0.4	19.7±0.6
4		20	1/15	53.4 ± 0.5	10.2 ± 0.1	16.9±0.1	35.5 ± 1.9	6.9 ± 0.2	16.9±0.0
5	210	10	1/10	58.3 ± 0.1	0.9 ± 0.0	24.4±0.0	0	0.1 ± 0.1	32.5±0.5
6		10	1/15	60.1 ± 0.2	0.8 ± 0.0	20.9±0.2	0	0.1 ± 0.0	38.4±0.5
7		20	1/10	55.7 ± 0.1	0.6 ± 0.0	24.2±0.5	0	0.1 ± 0.0	45.8±0.2
8		20	1/15	60.9 ± 0.7	1.2 ± 0.1	20.5±0.2	0	0.2 ± 0.0	37.1±0.2
9	162	15	1/12.5	50.4 ± 0.3	14.0 ± 0.2	14.0±0.0	38.8 ± 0.4	4.7 ± 0.1	14.1±0.1
10	218	15	1/12.5	53.8 ± 1.4	0.5 ± 0.0	25.4±0.0	0	0.2 ± 0.0	39.9±0.0
11	190	8	1/12.5	61.1 ± 0.1	4.2 ± 0.0	17.0±0.0	27.2 ± 1.7	4.3 ± 0.4	21.8±0.9
12		22	1/12.5	62.3 ± 2.2	4.9 ± 0.2	17.5±0.1	23.4 ± 1.7	2.8 ± 0.2	22.1±0.7
13		15	1/9	62.9 ± 0.2	4.2 ± 0.1	19.1±0.2	23.4 ± 0.0	2.1 ± 0.0	28.3±0.3
14		15	1/16	58.8 ± 0.3	3.0 ± 0.1	19.3±0.1	18.8 ± 0.1	1.6 ± 0.0	32.2±0.5
15		15	1/12.5	59.0 ± 0.9	3.3 ± 0.3	17.5±0.1	24.4 ± 1.3	2.7 ± 0.1	26.7±0.0
16		15	1/12.5	59.3 ± 1.2	3.0 ± 0.1	18.1±0.3	20.5 ± 0.4	1.7 ± 0.0	31.2±0.1
17		15	1/12.5	59.0 ± 0.7	3.1 ± 0.0	18.5±0.5	23.4 ± 0.2	2.0 ± 0.1	30.7±0.1
18		15	1/12.5	59.8 ± 0.3	3.1 ± 0.1	18.3±0.3	22.8 ± 0.2	1.9 ± 0.0	31.2±0.0

Table 3 Furfurals and organic acids of banana pseudo stem after hydrothermal pretreatment

Run	Temperature (°C)	Time (min)	Solid/liquid (g/mL)	Acetic Acid	Formic Acid	Furfural	5-hydroxymethylfurfural
				----------(g/L)----------			
1	170	10	1/10	1.11	0	0.08	0.0046
2		10	1/15	0.94	2.06	0	0
3		20	1/10	0.89	1.33	0.11	0.011
4		20	1/15	0.93	2.36	0	0
5	210	10	1/10	2.60	1.54	0.54	1.48
6		10	1/15	2.89	5.09	0.62	1.38
7		20	1/10	8.55	10.2	0.68	1.62
8		20	1/15	2.53	0	0.32	0.72
9	162	15	1/12.5	1.94	1.87	0	0
10	218	15	1/12.5	4.49	5.43	0.43	1.17
11	190	8	1/12.5	0.88	1.63	0.32	0.19
12		22	1/12.5	3.57	5.85	0.26	0.16
13		15	1/9	3.79	4.56	0.58	0.49
14		15	1/16	0.77	0	0.38	0.42
15		15	1/12.5	1.43	2.23	0.42	0.33
16		15	1/12.5	1.42	2.31	0.46	0.42
17		15	1/12.5	1.40	2.28	0.43	0.40
18		15	1/12.5	1.42	2.33	0.42	0.44

Enzymatic hydrolysis

The differences in the glucose yields can be are related to the conditions of each test of the experimental design (Figure 1). It was observed there was a high glucan-to-glucose convertibility in pretreated biomass (45-98%) when compared to the untreated one (8%). Furthermore, an enhancement in enzymatic hydrolysis of lignocellulosic biomass is common in pretreatment (**He *et al*., 2015**).

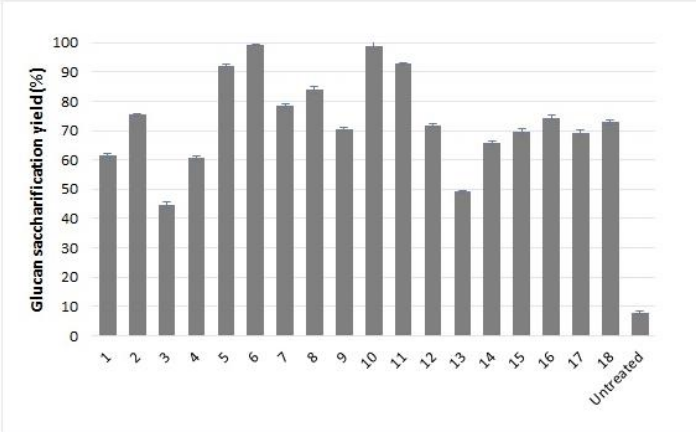

Figure 1 Glucose sacharification yield after thermic pretreatment (run 1 at 18) or not (untreated) of banana pseudo stem

It was also observed that the increase in glucose yields is directly proportional to the increase of hydrothermal temperatures. The highest glucose yield was above 95% in runs 6 (210 °C) and 10 (218 °C). However runs 3 (170 °C) and 13 (190 °C) were less effective, yielding less than 50% of glucan, this may be due to the low hemicellulose removal in these pretreatment steps when compared to runs 6 and 10 (Table 2). The hemicellulose fraction could physically block the access to cellulose by cellulases (**Sun *et al*., 2014**).

The runs 5, 6 and 10, pretreated at the highest temperatures (above 200 °C) presented the best enzymatic digestibility. A more severe pretreatment might produce pre-hydrolyzed cellulose, which could enhance the enzymatic hydrolysis of cellulose. However, compared to the other pretreatments at a higher temperature, run 7 (210 °C) presented lower glucose release. This might have been caused by the higher concentrations of inhibitory compounds (Table 3). Kristensen et al. (2009) suggested end-product inhibition is the main factor affecting sugar yields at high substrate concentrations, but the exact extent and mechanism of the inhibition is still unknown.

The highest solid/liquid ratio (S/L) contributed to higher cellulose-to-glucose conversions, for example, in S/L ratio of 1/15 the sugar yields were greater than in 1/10 ratio (Figure 1). In addition, in run 13 with 1/9 ratio and run 14 with 1/16 ratio showed around 45% and 60% saccharification yield respectively. Highly concentrated solids during pretreatment and enzymatic hydrolysis offers several industrial advantages, including smaller reactor size, increased rates of hydrolysis and lower water consumption levels.

The saccharification was also affected by reaction time. At higher reaction time glucose yields were low. Runs 5, 6, 10 and 11, which were performed in a shorter reaction time (10 min), presented the highest glucose yields. Moreover, biomass pretreatment in shorter times may contribute to reduce process costs in second-generation ethanol production.

Statistical analysis and optimization

ANOVA was used through Statistica software to estimate the influences of three factors on glucose release: temperature, time and S/L ratio.

By applying multiple regression analysis on the experimental data, the following second-order polynomial equation was formed. This describes the adjusted models for glucose yields in function of the terms coded for each independent variable, after removal of terms relating to non-significant variables ($p > 0.05$).

Glucose release = 41.5 + 17.7 *temperature* – 0.7 $temperature^2$ – 8.1 *time* + 6.4 $time^2$ + 5.8 *S/L* – 8.4 S/L^2 – 2.4 (*temperature x time)* + 0.02 (*temperature x S/L)* + 2.02 (*time x S/L)*

When values from temperature, time and S/L were substituted in the above equation, the predicted responses were obtained as shown below (Figure 2).

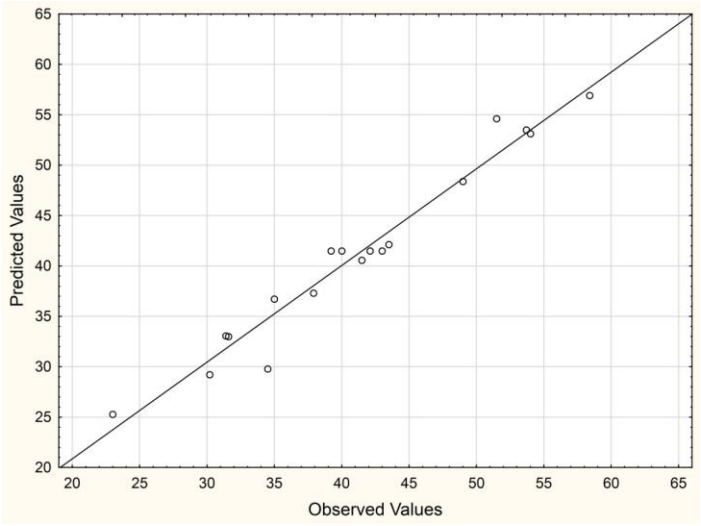

Figure 2 Experimental and predicted values of glucose release (g/kg) of the central composite design

Statistical analysis of the different combinations of variables and their responses are shown in table 4. All the linear and quadratic effects of solid/liquid ratio are significant at $p < 0.007$ level.

ANOVA is required to test the significance and adequacy of the models. In general, the smaller the p-value and larger the F-value, the more significant the corresponding coefficient terms are. A statistically insignificant lack of fit, as evidenced from the p-value being greater than 0.05, the model further indicates no reasons to suspect the goodness of the fit.

Fisher's variance ratio (F-value) is the ratio of the mean square owing to the regression of the mean square of the error. It measures the variation in the data regarding the mean. ANOVA of the multiple regression revealed linear and quadratic models of solid/liquid ratio from the factorial design which can be used to predict the responses as evidenced from the high F-values. The quadratic (except solid / liquid ratio) and combined effects of the variables were not statistically significant on the response.

Table 4 ANOVA table of the adjusted models for glucose released from enzymatic hydrolysis

Factor	Sum of squares	Degrees of freedom	Mean sum of squares	F-statistic	p-value
Temperature (Linear)	937.3	1	937.3	120.3	0.000004
Temperature (Quadratic)	1.043	1	1.043	0.1	0.7
Time (Linear)	196.4	1	196.4	25.2	0.001
Time (Quadratic)	80.9	1	80.9	10.4	0.01
Solid/Liquid (Linear)	99.7	1	99.7	12.8	0.007
Solid/Liquid (Quadratic)	137.4	1	137.4	17.6	0.003
Temperature by Time	11.7	1	11.7	1.5	0.3
Temperature by Solid/Liquid	0.001	1	0.001	0.0002	1.0
Time by Solid/Liquid	8.2	1	8.2	1.1	0.3
Error	62.4	8	7.8		
Total sum of squares	1532.1	17			
R^2	0.95930				
Adjust R^2	0.91352				

The multiple correlation coefficient (R^2) of the regression equations obtained from ANOVA was 0.95930 (R^2 adjusted = 0.91352), showing a satisfactory adjustment of the model indicating the model could explain 95% variability in the responses ($p < 0.05$). It indicates that only 5% of the total variation is not explained by the model. Therefore, the response glucose release was successfully modeled based upon the reaction conditions using linear regression.

Effects for temperature, time and solid/liquid ratio variables at the significance level of 5% ($p < 0.05$) were represented in Pareto charts (Figure 3).

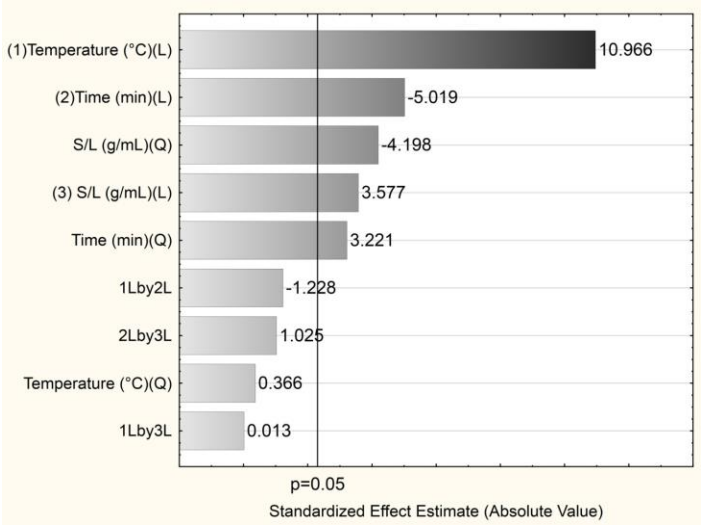

Figure 3 Pareto charts of the effect for temperature (ºC), time (min) and solid/liquid (g/mL) ratio after thermic pretreatment of banana pseudo stem

The analysis of the significant parameters suggests linear effects of temperature and solid/liquid ratio have positive signs (Figure 3). This indicates that an increase in these variables within the ranges studied, increases glucose yield in enzymatic hydrolysis. On the other hand, the linear effect of time has a negative effect, indicating that increase in time leads to decrease of glucose yield. The solid/liquid ratio presented a positive effect, on the other hand, the quadratic effect was negative. This indicates a region of optimum value of S/L to glucose yield.

A response surface plot is the projection of response surface in 3D. These graphical representations visually highlight the regression equations. Each plot represents the number of combinations of two independent variables tested while the other two independent variables are maintained at their center point levels. The response of glucose yields to varying pretreatment conditions show the interactions between temperature and time (Figure 4a), solid/liquid ratio and time (Figure 4b), and temperature and solid/liquid ratio (Figure 4c).

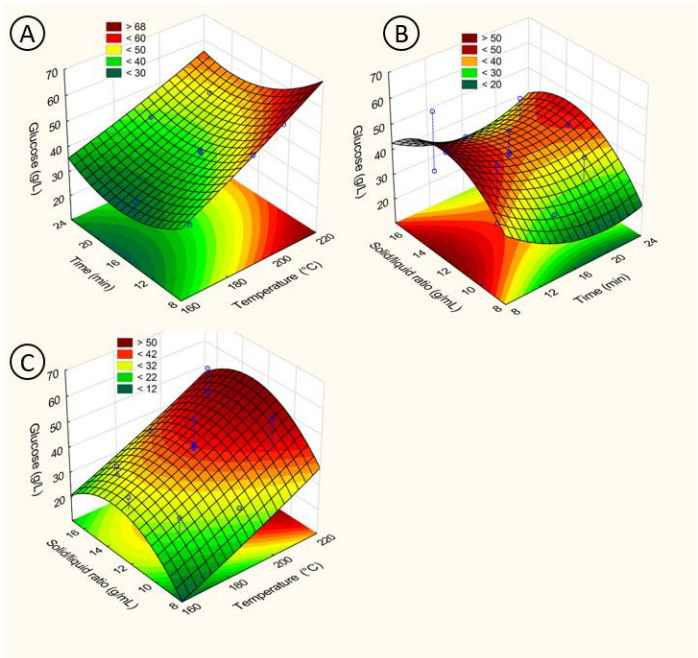

Figure 4 Response surface plot of glucose release in function of the temperature and time (A), solid and liquid ratio and time (B) and temperature and S/L ratio (C).

The results in figure 4 clearly indicate that a combination of higher temperature levels, the lowest reaction time and solid/liquid ration between 1/12.5-1/15 generate the maximal glucose concentration. Run 6 (210 °C, 1/15, 10 min) gave the highest glucose yield of 58.4 g/kg while run 3 (170 °C, 1/10, 20 min) gave the lowest glucose yield of 23.0 g/kg. Similar results were obtained for saccharification of different biomass sources such as wheat straw (Petrik ***et al.*, 2013**) and sugarcane bagasse (**Costa *et al.*, 2014**).

In general, the increase of pretreatment temperature increases glucose yield from cellulose under enzymatic hydrolysis. A relative small part of the glucan was already converted to monomer glucose during the pretreatment. Higher temperature may improve this conversion. However, severe conditions could inhibit a glucose-to-ethanol fermentation process. Some studies agree that longer reaction times coupled with higher pretreatment temperatures can disrupt plant cell walls and improve enzymatic access to cellulose. However, they can also result in the production of by-products as acetic acid, formic acid or furfural. These compounds can inhibit the activity of several enzymes and the fermentation process (**Cybulska *et al.*, 2013**; **Maache-Rezzoug *et al.*, 2011**).

CONCLUSIONS

The hydrothermal pretreatment demonstrated is an effective process in increasing cellulose concentration and producing fermentable sugar of the banana pseudo stem. Through of the RSM, an optimum condition of each pretreatment may be obtained from the statistical models built. All the variables studied influenced the enzymatic sugar release. Thus, optimised conditions were a temperature of 210°C, solid/liquid ratio of 1/10 (g/mL) and 10 minutes of reaction time resulting in a saccharification yield of 98%.

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