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Microwaves as a pretreatment for enhancing enzymatic hydrolysis of pineapple industrial waste for bioethanol production

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Abstract

The pineapple industry generates significant amounts of residues which are classified as lignocellulosic residual biomass. In the present paper, microwaves are studied as a pretreatment to improve pineapple waste saccharification. Different microwave (MW) powers (10.625, 8.5, 6.375, 4.25 and 2.125 W/g) and exposure times (1 - 20 min) were applied to the solid part of the waste before enzymatic hydrolysis. Infrared thermography was used to assess temperature evolution and structural modifications were evaluated by Cryo-SEM. Sugar content and fermentation inhibitors (phenols, furfural and hydroxymethylfurfural) were also determined. MW increased sugar yield as long as intermediate powers were used (up to 6.375 W/g). However, high powers and longer treatments resulted in sugar degradation and/or a decrease in the efficiency of the enzymatic hydrolysis process. Temperature records indicated that thermal sugar degradation may occur in those cases. The presence of fermentation inhibitors have been confirmed and related to prolonged MW treatments. Microscopic observations suggested that mild microwave pretreatments may promote microstructural changes that enhance enzyme performance, whereas harsher treatments could increase tissue compactness and reduce the effectiveness of the enzymatic treatment. It is concluded that microwave pretreatments using the appropriate energy supply and exposure time enhances saccharification efficiency, potentially improving further bioethanol yield.

Keywords: Lignocellulose pretreatments; Microwaves; pineapple industrial waste; saccharification, enzymatic hydrolysis; bioethanol.

1. Introduction

The global energy problem and the search for solutions based on sustainable and environmentally friendly renewable energies (Sun & Cheng, 2002) such as biomass and others (EC, 2009) has promoted bioethanol to be a clear alternative to fossil fuels. In this scenario, second-generation bioethanol, i.e. the produced from the fermentation of lignocellulosic biomass (waste and energy crops), deserves especial attention. Unlike first-generation bioethanol, second-generation of this biofuel helps diversify energy supplies without competing in the global food market (Rutz & Janssen, 2008; Bacovsky, 2010). Furthermore, the use of waste as a source for bioethanol production would also add up value to the whole manufacturing process.

The food industry generates significant amounts of residues which are a potential source for bioetanol production. In particular, pineapple production increases annually, and reached 24.79 million tons in 2013 (FAOSTAT, 2016). In addition, the industrialization of these fruit (juice, cannery, minimally processed) generates significant amounts of residues which consist mainly of the peel, core and crown of the pineapple. Pineapple waste usually represents about 50% (w/w) of the total processed fruit (Ketnawa *et al.*, 2012), although some authors have even suggested values up to 80% (Ban-Koffi & Han, 1990). On the one hand, the liquid phase of this residue contains a high content of fermentable sugars (glucose, fructose, sucrose) (Nigam, 1999). On the other hand, the solid phase is a lignocellulosic material which, apart from lignin, consists of cellulose and hemicellulose, polymers which are potentially hydrolyzable into fermentable mono- and disaccharides (Abdullah & Mat, 2008). Consequently, pineapple industrial waste has been investigated as an interesting source for ethanol (Ban-Koffi & Han, 1990; Nigam, 2009; Tanaka *et al.*, 1999; Ruangvirichay *et al*., 2010) and other metabolites production such as citric acid (Imandi *et al*., 2008).

However, bioethanol production from lignocellulosic biomass continues to be a challenge due to the complexity of this material in which cellulose and hemicellulose are densely coated by a hard-to-degrade lignin cover (Taherzadeh & Karimi, 2008). Hydrolysis of cellulose (polymer of D-glucose units linked by β-1,4-glycosidic bonds) and hemicellulose (complex heteropolysaccharide polymer that consists of pentoses, hexoses and uronic acids) could yield fermentable sugars to be used in bioethanol production (Scheller & Ulvskov, 2010). Lignocellulose would need to be disrupted in order to expose cellulose and hemicellulose to further chemical or enzymatic hydrolysis. Therefore, a pretreated lignocellulosic matrix becomes an essential prerequisite to obtain ethanol.

Nowadays, different physical, physicochemical, chemical and biological pretreatments, as well as a combination of all of them, are being assayed for pretreating lignocellulose (Sun & Cheng, 2002). Most of the conventional pretreatments require high temperatures usually reached by convection or conduction heating (Liu & Wyman, 2005). This creates a high energy cost that reduces the efficiency of the process. Therefore, there is a need for alternative methods to conventional pretreatments, among which microwaves have been suggested (Hu & Wen, 2008). The use of microwaves enables a volumetric, targeted and faster heating of the product than conventional heating, since there is direct contact between the product and the electromagnetic field generated by the microwave (De la Hoz *et al*., 2005). Furthermore, Xiong *et al.* (2000) showed that the use of microwaves could change the ultrastructure of cellulose, degrade lignin and hemicellulose and facilitate hydrolytic enzymes to access the lignocellulosic substrate (Kitchaiya *et al.*, 2003; Zhu *et al.*, 2005).

In this context, the aim of the present study was to investigate microwaves as an alternative pretreatment in order to improve the enzymatic hydrolysis yield of pineapple industrial waste.

2. Materials and methods

2.1. Sample preparation

A total of 20 pineapple fruits (*Ananas comosus* [L.] Merr., MD-2 cv.) were obtained from a tropical fruit importer and selected on the basis of their external factors such as the absence of injuries, ripeness and weight. Pineapples were first washed in a sodium hypochlorite solution (0.1%) for 5 min. Next, a pineapple cutter was used to remove the crown and separate the pulp. The resulting waste, consisting of the peel and core, were cut into smaller pieces and pressed in a screw press at 2.5 bar (Vincent Corporation model CP-4), the resulting press ratio being 0.49 (kg pressing cake/kg liquid phase). Liquid phase was removed from the original pineapple waste since it already contains simple sugars which would be directly fermentable (Nigam, 1999). In addition, sugar degradation can take place during microwave heating, for which only the solid part or press cake was subjected to subsequent microwave (MW) pretreatment and hydrolysis. Thus, the resulting press cake (*solid pineapple waste*) was grinded in a blender (Solac Inox Professional 1000W Mixer), introduced in glass flasks (40 g each) and kept frozen (-22 ºC) until the experiments were conducted. The resulting product was named *grinded solid pineapple waste*.

Experiments were performed on 40 g of thawed grinded solid pineapple waste to which distilled water had been added in 1:1 (w/w) proportion, resulting in the final sample identified as *reconstituted pineapple waste*. Distilled water was added to the press cake in order to enhance the microwave pretreatment effect as well as to avoid calcinations, as suggested by some authors (Azuma *et al*., 1984; Ooshima *et al.,* 1984).

2.2. Microwave Pretreatment

Microwave (MW) pretreatment was carried out in a microwave oven provided with a turntable plate (LG MH63340F / MH6340FS) with a frequency of 2.45 GHz. Samples were introduced in microwave intended plastic containers. The samples were treated at the following nominal powers: 170, 340, 510, 680 and 850 W, which resulted in the applied nominal powers: 2.125, 4.25, 6.375, 8.5 and 10.625 W/g; and exposure times from 1 up to 6, 8, 10, 14 and 20 min, respectively. Time exposure limits were defined by the appearance of calcinations or scorching. The power absorbed by the sample at these nominal power levels was estimated by heating 1 kg of distilled water from 10 °C up to 20 °C at 170, 340, 510, 680 and 850 W, according to the international standard IEC 60705 (1999). A thermocouple (HIBOK-14, sensor type K, sensitivity 39 µV °C-1, accuracy ± 0.1 °C) was used for temperature measurements. Experiments were performed in triplicate and results showed an average (and standard deviation) of 129

(3) W for the 170 W, 247.4 (1.2) W for the 340 W, 336 (2) W for the 510 W, 485.5 (1.3) W for the 680 W and 602.0 (0.9) W for the 850 W. Corresponding absorbed powers in W/g were then estimated as 1.61 (0.04), 3.09 (0.02), 4.2 (0.03), 6.07 (0.02) and 7.53 (0.01). Finally, the pH of the samples was adjusted to 5 by adding NaOH 1N (Panreac Química, S.L.U.). Water loss due to microwave processing was determined by weight difference and restored before proceeding with saccharification. Experiments were conducted in triplicate.

2.2.1. Evolution of microwave heating by infrared thermography

In order to estimate the temperatures reached during MW pretreatments, an infrared thermocamera Testo 870-1 (Testo AG) with a spectral infrared range of wavelength from 7.5 to 14 mm, 9 Hz frame rate and detector with 160 x 120 pixels, was used. In order to compare the difference in the heating undergone by the pineapple waste due to the different MW powers and exposure times applied, an image of the bottom surface of the container was taken just after each microwave pretreatment. Testo IRSoft software was used for image analyses, which allowed the study of microwave heating evolution, and the registration of hot spots location as well as maximum, minimum and average temperatures.

2.3. Enzymatic Hydrolysis

Enzymatic hydrolysis was carried out by adding 0.4% (w/v) of cellulase (1.13 U/mg solid) and 0.1% (w/v) of hemicellulase (1.5 U/mg solid) from *Aspergillus niger* (both enzymes purchased to Sigma-Aldrich Química SL (Tres Cantos, Madrid, Spain). In each experiment, the reconstituted pineapple solid waste was introduced in a 100 mL glass beaker and placed in an incubation oven at 50 ºC (PSelecta, model Incudigt) during 24 h. Concentration of the enzymes as well as optimum conditions of the saccharification process were based on previous experiments performed at the same laboratory. Saccharification was undergone on microwave pretreated and non-pretreated samples, which were used as a reference. Experiments were performed in triplicate.

2.4. Physicochemical determinations

The following physicochemical determinations were performed on the reconstituted pineapple waste before pretreatment and saccharification, as well as after saccharification, on samples subjected to MW pretreatments or saccharified without having been pretreated.

2.4.1. *pH, moisture content (x_w) and Total Soluble Solids (TSS).*

pH and moisture content (x_w) were determined as a control measure, using a digital pH-meter and the 20.013 AOAC gravimetric method (AOAC, 1980), respectively. pH can be modified during hydrolysis as a consequence of the different species released to the medium, whereas moisture content is not expected to change significantly. Total soluble solids (TSS) present in the liquid phase were estimated by obtaining the Brix degrees (º Brix) values after saccharification, which were considered a good approximation of the simple sugars released as a consequence of cellulose and hemicellulose hydrolysis.

2.4.2. *Sugar profile*

Identification and quantification of specific sugars present in the liquid phase of the reconstituted pineapple waste was measured by High-Performance Anion-Exchange Cromatography with a Pulsed Amperometric Detector (HPAEC-PAD), using a Metrohm IC chromatograph system equipped with a 716 Compact module and an ICnet 2.0 software program for interpreting the results. A three-step PAD setting was used with the following path intervals (ms) and potentials (V): t_1: 400/E_1 = +0.05 (detection); t_2: 200/E_2 = +0.75 (cleaning); t_3: 400/E_3 = -0.15 (regeneration). The column used was a Metrosep Carb 1 250/4.6 column (250 mml x 4.6 mmID) coupled to a guard column. Analyses were done at 32 ºC, 8.8 MPa, injection volume: 20 µL and using sodium hydroxide 0.1 M as the mobile phase (1 mL/min). Chromatographic measurements required filtration of the liquid (0.45 µm nylon filter) and dilution of the resulting filtered sample (1:2000 v/v in bidistilled water). High-purity standards (Sigma-Aldrich Química SL, Tres Cantos, Madrid, Spain; purity ≥ 99%) of glucose, fructose, sucrose, arabinose and xylose were used to prepare standard calibration curves (2.5, 5, 10, 15, 25 and 50 ppm) and proceed to identification and quantification of the sugars present in the hydrolizates. All the determinations were carried out in triplicate. Sugar content was expressed in g/kg of pineapple waste.

2.5. Determination of fermentation inhibitory compounds

Inhibitory components present on the saccharified samples was evaluated in terms of phenolic content and the presence of furfural (F) and hydroxymethylfurfural (HMF) on the liquid phase of pretreated and non-pretreated samples.

2.5.1. Phenolic content

The Folin-Ciocalteau method was used in order to determine total phenols present in the samples (Singleton *et al*., 1999). Monohydrate gallic acid was used as a standard and results expressed in mg equivalent of gallic acid per mL (mg GAE/mL). All reagents used were of analytical grade (Sigma-Aldrich Química SL, Tres Cantos, Madrid, Spain).

2.5.2. Determination of Furfural (F) and Hydroxymethylfurfural (HMF)

High Performance Liquid Cromatography (HPLC) was used in order to determine the content of furfural and hydroxymethyl furfural, according to the methodology developed by Blanco-Gomis *et al*. (1991). An Alliance® System (Water Co., Mildford, MASS, USA) equipped with a degasser and a 2695 separation module, coupled to a diode array detector (DAD 2996, Waters Co.) was used. A Kromasil ® 100 C-18 column (3 mm x 250 x 4.6 mm I.D.) was used for chromatographic separation. The analyses were performed isocratically at 25 ºC using acetonitrile/water (8:92 v/v) as the mobile phase (1 mL/min) and an injection volume of 10 μL. UV detection was fixed at 280 nm. Quantification was based on external calibration by using standard solutions of F (0 to 5 μg/mL) and HMF (1-100 μg/mL) in 10% methanol-water (v/v). All reagents were purchased from Sigma-Aldrich Química SL (Tres Cantos, Madrid, Spain) and of analytical grade. Purity of standards was $\geq$ 98%.

2.6. Analysis of microstructural changes by Low Temperature Scanning Electron Microscopy - Cryo-SEM.

Low-temperature Scanning Electron Microscopy (Cryo-SEM) was used in order to evaluate the microstructural changes undergone by the reconstituted pineapple waste as a consequence of microwave pretreatments. A Cryostage CT-1500C (Oxford Instruments) coupled to an electronic scanning microscope Jeol JSM-5410 was used for this purpose. First, samples were sublimated in the microscope stage during 20 min at -90 ºC and -5 kV; subsequently, these were moved to another stage and metalized with gold during 1.5 min, at vacuum conditions. Then, sample observation was performed at 15 kV and 15 mm of working distance. Micrographs of the reconstituted pineapple waste before and after microwave pretreatments were taken.

2.7. Statistical Analysis

Statgraphics Centurion XVI® (Manugistics Inc.; Rockville, MD, USA) was used for statistical analyses. Statistically significant differences across the different analyzed results were determined by using one-way or multiple analyses of variance (ANOVA) at 95% confidence level (p-value < 0.05).

3. Results and discussion

3.1. Enzymatic hydrolysis of the reconstituted pineapple waste: sugar profile.

Pineapple waste saccharification resulted in an increase in the amount of sugars present in the samples. Specific changes in the sugar content of the liquid phase were studied by examining the sugar profile before and after saccharification (Table 1). Before saccharification, glucose, fructose and sucrose were the only sugars identified in the samples, whereas the pentoses xylose and arabinose were also present in the enzymatically hydrolyzed ones. The enzymatic treatment produced a statistically significant increase in the glucose and fructose contents, whereas sucrose content slightly decreased after saccharification. The action of the fungal cellulase complex that consists of three groups of enzymes: endoglucanases (EC 3.2.1.4), exoglucanases or cellobiohydrolases (EC 3.2.1.74) and β-glucosidases (EC 3.2.1.21) (Goyal *et al.*, 1991) is most probably the main responsible for glucose increase; On the contrary, fructose increase would not be the result of the enzymatic action but of sucrose inversion (Ban-Koffi & Han, 1990), considering the acid pH (which reduces from 5.0 to 4.28 ± 0.07 during saccharification) of the medium and the fact that selected enzymes are not potentially capable of reversing sucrose. Hemicellulase action led to xylose and arabinose release from hemicellulase. This enzyme complex consists of enzymes that hydrolyze the main chain: xylanases and β-xylosidases (Shallom & Shoham, 2003) to xylan; as well as those responsible for the hydrolysis of hemicellulose branches: α-L-arabinofuranosidases to arabinose (Saha *et al.,* 2005). Nevertheless, xylose and arabinose contents in the saccharified samples were minimal, significantly lower than the content of the other sugars identified (glucose, fructose and sucrose).

Regarding total sugars content, enzymatic saccharification produced a statistically significant sugar increase in the pineapple waste. However, due to the slight increase obtained, pretreatment is recommended in order to disrupt the lignocellulosic structure and facilitate the action of hydrolytic enzymes, thereby increasing the efficiency of enzymatic hydrolysis and the saccharification yield.

3.2. Effect of microwave (MW) pretreatments on pineapple waste saccharification

MW pretreatments significantly modified the result of enzymatic hydrolysis of pineapple waste as it was deduced from the analysis of the total soluble solids of pretreated samples after saccharification (Figure 1). Generally, the application of a MW pretreatment produced an increase in the TSS content when powers up to 6.375 W/g were applied. However, higher powers resulted in a decrease in TSS content, the power being statistically significant. Besides, all the powers applied showed a critical time at which TSS content was reduced indicating that not only high powers but also longer treatments could result in sugar degradation and/or a decrease in the efficiency of the enzymatic hydrolysis process. In fact, both factors and their interaction appeared to be statistically significant.

As for pH and moisture content, significant differences were obtained when comparing the results before and after saccharification; however, none of these parameters were significantly affected by the different MW pretreatments. In particular, pH decreased from the adjusted 5.0 to an average of 4.3 (0.3), as a result of the different species released to the medium during enzymatic hydrolysis, whereas x_w decreased from 92.2 (0.5) to an average of 91.3 (0.3).

In order to go into detail, HPAEC-PAD was used to analyze individual sugars in each pretreated pineapple waste, and compared them to the obtained in the non-pretreated one. All reported values correspond to sugar content after saccharification (Figure 2).

Microwave pretreatments at the lowest power assayed (2.125 W/g) did not lead to any statistically significant modification in glucose content when compared to the non-pretreated waste (Figure 2a). Nevertheless, glucose experienced a significant increase when higher powers were used (4.25 W/g) at exposure times from 10 min. Increasing the applied power to 6.375 W/g led to a statistically significant increase in the glucose yield for any exposure time, as occurred when 8.5 W/g were applied at exposure times up to 4 min. On the contrary, lengthening treatment beyond 4 min at the latter power led to a decrease in the sugar content to that of the reference treatment (NP) or even to lower values in some cases. Finally, the highest MW power applied (10.625 W/g) led to a statistically significant reduction in glucose release in all cases.

Fructose content followed a similar pattern to that of glucose yield but variations in fructose content were more moderate (Figure 2b). Likewise, the greatest fructose yield was observed at intermediate MW powers, specifically at 6.375 W/g. At the lowest microwave power (2.125 W/g) fructose content did not change for any exposure time, whereas a statistically significant reduction in fructose release was observed at the highest microwave power applied (10.625 W/g).

On the contrary, as shown in Figures 2c and 2d, MW pretreatment did not have a significant effect on sucrose and arabinose yields whereas xylose content markedly decreased at almost every power and exposure time applied (Figure 2e). When investigating MW-alkali pretreatments, Hu & Wen (2005) also found out that the yields of xylose dropped significantly when the pretreatment (190 ºC) extended to 40 min. Xylose have been reported to be especially sensitive to hydrothermal treatments as compared to other monosaccharides such as glucose and fructose (Tsubaki *et al.,* 2015). However, xylose was not present in the original biomass, for which a lower concentration of this sugar in the hydrolyzed sample should be a consequence of the effect of MW on hemicelluloses, which contain the xylose monomers. In line with this, depletion of hemicellulose due to the high temperatures reached during MW treatments has also been reported, it completely disappearing at temperatures above 190 ºC (Hendriks & Zeeman, 2009). Therefore, solubilization of hemicellulose and further degradation of xylose, or either direct degradation of hemicelluloses when even higher temperatures are reached during the MW pretreatments, would be responsible for the lower xylose yields.

The effect of MW pretreatment in total sugars content is summarized in figure 2f. As it can be observed, when very low microwave powers are applied (2.125 W/g), insufficient energy is provided in order to disrupt the lignocellulosic structure and therefore promote the subsequent action of the enzymes. On the contrary, increasing microwave power up to 10.625 W/g produces a decrease in the total sugar yield, with respect to the non-pretreated waste, which could be a consequence of sugar degradation due to the high temperatures reached in the microwave (Zhu *et al.*, 2005). This phenomenon has also been observed by other authors such as Binod *et al.* (2012) that reported sample charring at 3 min of treatment when 850 W were applied, whereas at 100 W power charring did not occur even after 30 min. Liu & Wyman (2005) also indicated that temperatures above 190 ºC might trigger sugar degradation. Hydrothermal degradation of sugars and its resulting formation of fermentation inhibitors such as furfural and hydroxymethylfurfural (HMF) have been extensively documented (Palmqvist & Hahn-Hagerdal, 2000; Carvalho *et al.*, 2004). Kim & Pan (2010) noted that organosolvent biomass fractionation have shown that xylose dehydration to furfural mostly occurs at 190 – 205 ºC, and the molar conversion of hexose sugars to HMF is higher at high temperatures (up to 200 ºC), low pH and longer pretreatment times.

Nevertheless, intermediate powers and exposure times did have a positive effect on further enzymatic action. It has been reported that MW pretreatments enhances the saponification of intermolecular ester bonds cross-linking xylan hemicelluloses, as well as other components such as lignin and other hemicelluloses (Jin *et al.*, 1999). Hu & Wen (2008), by using scanning electron microscope images, showed that when switchgrass was treated by MW, many granules appeared on the surface, indicating partial breakdown of the lignin structure. Thus, these structural changes would facilitate the enzymes' access to the potentially hydrolysable components. In our case, intermediate applied powers (6.375 W/g) did always imply a significant improvement in the saccharification yield. In particular, microwave pretreatment at 6.375 W/g – 4 min produced an increase in total sugars of 27% compared to the non-pretreated waste. Similar results were obtained at low-intermediate powers (4.25 W/g) and long exposure times (≥ 10 min) and high-intermediate powers (8.5 W/g) and short exposure times (≤ 4 min); therefore, increasing the microwave power could result in shorter treatments. These results are in line with those obtained by Binod *et al.* (2012).

As for the thermal effect of microwaves, Hu & Wen (2005) reported that when lower temperature levels are reached (70-130 ºC), the increase in the sugar yield obtained is largely due to biomass de-lignification. However, when temperatures exceed 130 ºC, the improvement in sugar yield might be caused by the disruption of the crystalline cellulose instead of by lignin removal. Higher temperatures (> 160 ºC) may also induce the solubilization of lignin (Jackowiak *et al.,* 2011). However, this is a non-desired phenomenon since it results in the release of phenolic compounds which have an inhibitory toxic effect on bacteria and yeast (Hendriks & Zeeman, 2009). High temperatures also promote hemicellulose depletion, which is complete at temperatures above 190 ºC (Hendriks & Zeeman, 2009).

3.3. Analysis of microwave induced heating of pineapple waste by thermography

Some effects of MW on the lignocellulosic biomass may be attributed to the high temperatures reached during the irradiation treatment. Therefore, an infrared camera was used to estimate any temperature changes produced as a consequence of MW treatments, as explained in the Materials and Methods section. Since thermographs were taken immediately after MW pretreatments, temperature losses were considered negligible, as suggested by different authors (Liu *et al.,* 2014; Pitchai *et al.,* 2012; Wang *et al.,* 2001; Zhou *et al.,* 1995).

Temperature distribution during MW heating is summarized in Figure 3, where thermographs corresponding to the different applied powers and exposure times are shown. In each thermography, the cross indicates the point exhibiting the maximum temperature or hot spot (HS); in addition, the temperature scale is given next to the corresponding image.

The analysis of temperature evolution during MW pretreatment reveals that increasing the power and exposure time results in higher temperatures. However, different heating patterns are observed at the different powers applied.

MW irradiation heated from the inside to the outside of the sample surface which implied a non-homogeneous heating process, at least during the first minutes of treatment; this is a consequence of the polar part of the lignocellulosic material being selectively heated by microwaves. During this period, hot spots are created in the heterogeneous material. Some authors have discussed that the recalcitrant lignocellulosic structure may be disrupted by the generated "explosion effect" at the hot spots (Hu & Wen, 2008). Later, homogeneity is reached at different times depending on the power applied; the higher the power the earlier the uniformity is reached. These results are similar to the obtained by Kumar *et al.,* (2014), who showed that lower powers required longer exposure times so as to reach a homogenous heating (150 s for 100 W power in their case). During the non-homogeneous heating of the waste, the registered temperatures are below the boiling point of water, whereas temperature approaches this point when uniformity is reached, as deduced from the scales next to the images. Lengthening the treatment implied reaching temperatures up to 160 ºC, leading again to a loss of temperature homogeneity in the residue.

The location of the hot spot also depended on the heating period; it being centered during the homogeneous temperature period, and displaced at the beginning of the process, as well as once temperature uniformity has been lost. The latter case, as explained by several authors, could be due to the loss of water in the central part of the sample as a consequence of water evaporation; this modifying the maximum energy point which would then locate in the regions where there is still some moisture content (Liu *et al.,* 2014; Pitchai *et al.,* 2012; Wang *et al.,* 2001; Zhou *et al.,* 1995). In fact, scorching of the surface sample was visually confirmed in the samples subjected to high MW powers (6.375 W/g and higher), at long exposure times.

In Figure 4, the registered maximum (T_{max}), minimum (T_{min}) and average (T_{ave}) temperatures are given, as a function of time for the different applied powers. In all cases, as long as power and time increase, there is an increase in all registered temperatures; this being in line with the results reported by Liu *et al*. (2014), Pitchai *et al*. (2012), Wang *et al.* (2001) and Zhou *et al.* (1995). The statistical analysis of the results indicated that both factors, power and time, as well as the interaction between them, were statistically significant.

The registered graphs confirmed the existence of different periods during MW heating, as it had been deduced from the thermographs: an initial or induction period, when temperature is below the boiling point of water; a constant period, in which temperature is around 100 ºC; and a final one, when temperature reaches values above 100 ºC. The exact temperatures reached, as well as the length of each of these periods, depended on the power applied, as discussed below for the maximum and average temperatures.

As for the maximum temperature, which would correspond to the temperature of the hot spot for each power and time, the registered value is slightly below 100 ºC in all cases when MW start to act on the sample: the lower the power applied, the further from 100 ºC the temperature is, approaching the boiling point of water as heating proceeds (2-5 min, depending on the applied power). Then, except for the maximum power applied, temperature remains constant for a specific period, it being coincident with the homogeneous heating period of the samples (Figure 3). Next, when heating stops being homogeneous (Figure 3), there is an increase in the maximum temperature registered, at intermediate and high powers, for which temperatures higher than 100 ºC are reached. Temperatures above the water boiling point would suggest the presence of dry regions in the sample and the concentration of moisture in specific points, which could lead to overheating of some specific regions. This has also been confirmed when analyzing temperature distributions in Figure 3 and by visual inspection of the samples, since scorching of the sample surface was visually observed after some specific treatments. On the other hand, when lower powers were applied (2.125 and 4.25 W/g), all registered values were around 100 ºC until the end of the treatment, which implies a homogenous heating until 20 and 15 min, respectively; the hot spot still being centered in the sample.

Evolution of minimum and average temperature was quite similar in all cases. Therefore, average temperature will be used as a reference, since it can be considered representative of the sample. Average temperature increase is sharper during the first 2 minutes, 5 min in the case of the lowest power. Then, as occurred with the maximum temperature evolution, average temperature remains constant during a specific period, and increases later above 100 ºC, except in the case of the two lowest powers applied, where temperature does not significantly change until the end of the treatment. The phenomena responsible for this evolution are the same as exposed above since, again, average temperature remains constant while the registered heating is homogeneous, and increases when the hot spot displaces as a result of dry regions appearing in the sample.

As explained by Kumar *et al.* (2014), MW power would be more efficient at short times, since more significant temperature changes are then registered. It agrees with the results of the present work (not taking into account the temperatures that produce scorching, since these are a cause for sugar degradation). However, it should be reminded that heating is not the only effect that MW may exert on the residue structure, since the electromagnetic field applied might create non-thermal effects that also accelerate the destruction of the crystalline structures (De la Hoz *et al.*, 2005).

3.4. Presence of inhibitory compounds on the saccharified samples.

3.4.1. Phenolic content

As it can be deduced from the results (Figure 5a), microwave pretreatment causes an increase in the phenolic content of the saccharified samples. This increase would be a consequence of lignin solubilization (Jackowiak *et al.*, 2011) as a result of the microwaves acting on the lignocellulose matrix. Both power and time were statistically significant (p-value < 0.05), treatments longer than 10 min causing a particularly significant increase in the phenolic content. This phenomenon would be related to the high temperatures reached during the treatments; in fact, the evolution of the sample's average temperature (Figure 4c) shows a quite similar pattern to that of total phenols (Figure 5a). Hu and Wen (2008) reported similar results as for the generation of phenolic components due to temperature-induced lignin degradation. Nevertheless, in spite of the significant increase in the phenolic content registered, values are still far from the concentrations that have been reported to be detrimental for ethanologenic microorganisms (Palmquvist *et al.*, 2000; Ando *et al.*, 1986).

It should be pointed out that that reducing sugars may interfere in the Folin-Ciocalteau assay, for which an increased phenolic content could also correspond to an increase in theses sugars' content. Nevertheless, according to Sánchez-Rangel et al. (2013), the presence of reducing sugars is only a problem when they are present and the total phenolic content is low. Therefore, phenolics would be the main components reacting with the Folin-Ciocaltau reagent in samples in which harsher MW treatments have been applied, i.e. when a significant increase has been registered, since sugar depletion is observed in those cases.

3.4.2. Furfural and Hydroxymethylfurfural

Furfural and hydroxymethyfurfural can appear in the saccharified samples due to hydrothermal sugar degradation. Therefore, this would be a consequence of sugar release from the celluloses and hemicelluloses chain during MW treatments and subsequent thermal degradation due to a prolonged treatment. This phenomenon is evidenced on figure 5b,c, were the presence of F and HMF is confirmed when duration of MW treatments is ≥ 10 min. This increase being especially significant when treatments continued up to 15 min. MW power had also a significant effect, since F and HMF contents were almost negligible at the lowest power applied, independently on the exposure time. Several authors confirm that F and HMF do not inhibit *Sacharomyces cerevisiae* when these components are below 2 g/L (Banerjee et al., 1981; Cantarella et al., 2004; Gu et al., 2014; Klinke et al, 2004; Palmqvist et al., 1999; Taherzadeh et al., 1999; Taherzadeh et al., 2000), for which it is deduced that the concentrations obtained in the present study, F ≤ 0.0458 (0.6) g/L and HMF ≤ 1.63 (0.05) g/L, would not negatively affect the fermentation microorganisms. The increase in the F and HMF contents when increasing power and time confirms the hydrothermal degradation of sugars due to the temperatures reached when harsher MW pretreatments are applied, this being in line with temperature records (Figure 4), as well as with sugars content (Figure 2).

3.5. Analysis of microstructural changes by Low Temperature Scanning Electron Microscopy - Cryo-SEM.

Microstructural changes due to MW pretreatments were studied by Cryo-SEM, as explained in the Materials and Methods section. Some representative pretreatments were chosen and compared to a non-pretreated sample in order to identify any changes promoted by MW irradiation. Micrographs of a non-pretreated sample (Figure 6a); a sample pretreated at 6.375 W/g - 5 min, which exhibited high sugar yield (Figure 6b);

and a sample subjected to 8.5 W - 8 min, in which sugar degradation had been identified (Figure 6c), are shown.

In Figure 6a (non-pretreated), rounded packed cells, typical from a parenchymatic tissue, are observed; cell walls and protoplasts are clearly identified using this technique. In addition, certain degree of cell degradation was observed when comparing the pretreated samples with the non-pretreated ones. Less rounded, more irregular cell walls, as well as the separation of the plasmatic membrane from the cell wall were identified (Figure 6b). In spite of not being easily observable using this technique, the different signs of disruption identified suggest that cell degradation is taking place during pretreatments. Therefore, the microstructural changes described evidence that MW pretreatment is affecting the waste microstructure and, therefore, this is going to determine further enzyme action.

In the case of applying intermediate powers and times (Figure 6b,), structure would be affected in a way that enzyme action in the following stage is promoted, as deduced from the results of sugars released to the medium. On the contrary, increasing the power and time applied would cause more significant structural changes, which could result in reduced enzyme efficiency. In fact, in Figures 6c, cell wall roughness is more obvious and suggestive of tissue dehydration. In addition, protoplast plasmolysis is not observed in this case, but rather a significant cell shrinkage which would result in increased tissue compactness. This breakdown of the tissue would be negatively affecting further saccharification, since it would represent a decrease in the available surface area and, therefore, a lower effectiveness of the enzymatic treatment (Arantes & Saddler 2001). Furthermore, it is known that dehydration has a negative effect on enzyme's accessibility to the substrate due to smaller pore sizes and narrowed pore size distribution in cellulose fibers (Laivins & Scallan, 1996).

Results are in line with other published studies in which cell structural modifications, especially cell membrane integrity, as a consequence of MW treatments have been reported (Binod *et al.*, 2012; Hu & Wen, 2008). Sugar yield and structure modifications uphold the beneficial effect of a MW pretreatment in order to improve enzymatic hydrolysis (Hu & Wen 2008), since the exposed surface is increased in a way that cellulose results more accessible to hydrolytic enzymes (Binod *et al.*, 2012).

4. Conclusions

In the context of biofuel production pretreatments of lignocellulosic biomass are being currently studied in order to improve the saccharification step; in particular, microwaves have been suggested as an alternative pretreatment of this residual biomass. On the other hand, pineapple industry produces significant amounts of residues which need to be properly managed. Obtaining added value from these residues would not only reduce environmental impact but would also represent a benefit for the manufacturing industry.

In the present work microwaves have been studied as a pretreatment for improving pineapple waste saccharification. Results of applying different powers and exposure times to the pineapple waste material indicate that microwave pretreatment may increase saccharification performance as long as mild treatments are used. However, low powers and short exposure times do not modify sugar content whereas higher powers and/or exposure times may result in sugar decrease. Infrared thermography and Cryo-SEM microscopy observations indicated that both thermal sugar degradation and increased tissue compactness may be responsible for the lower yield when harsher microwave conditions are used. The presence of phenolic components as a result of lignin solubilization as well as sugar degradation to furfural and hydroxymethylfurfural have also been confirmed when lengthening the treatment, especially when higher powers are used. At milder conditions, however, explosion in hot spots and resulting tissue modifications facilitate enzyme action in the subsequent saccharification step.

The use of infrared thermography for the study of temperature profiles also allowed the identification of different periods during MW heating: a first period, characterized by a heterogeneous heating, the existence of a hot spot in the central region of the sample and temperatures below 100 ºC; a second one, in which a homogeneous heating is reached and temperature remains around 100 ºC; and a final period, when sample overheating have been identified (Temperatures > 100 ºC) and new hot spots appear.

In conclusion, microwave pretreatments using the appropriate energy supply and exposure time allows to enhance the efficiency of lignocellulosics saccharification and, therefore, it may improve bioethanol yield in a subsequent step. This has been proved for industrial pineapple waste, although it could be potentially applicable to other food industry residues.

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Figure Captions

Figure 1. Total Soluble Solids (TSS) content of MW pretreated pineapple waste at different powers and exposure times.

Figure 2. Sugar content of MW pretreated waste at different powers and exposure times: a) Glucose, b) Fructose, c) Sucrose, d) Xylose, e) Arabinose and f) Total Sugars *Indicates statistical differences at the 95% confidence level between MW pretreated and non-pretreated samples (p-value < 0.05).

Figure 3. Thermographs corresponding to the bottom surface of the plastic container containing MW pretreated pineapple waste at different power: 2.125, 4.25, 6.375, 8.5 and 10.625 W/g, and exposure times: 1, 2, 5, 10 y 15 min. HS: Hot Spot.

Figure 4. Temperature evolution graphs: a) Minimum temperature (T_{min}), b) Maximum temperature (T_{max}) and c) Average temperature (T_{ave}) of MW pretreated pineapple waste at different power: 2.125, 4.25, 6.375, 8.5 and 10.625 W/g, and exposure times: 1, 2, 5, 10 y 15 min.

Figure 5. Generation of fermentation inhibitors during MW pretreatments. Total phenols (mg GAE/mL), furfural (µg/mL) and hydroxymethylfurfural (µg/mL) content in the liquid phase of the microwave pretreated pineapple industrial waste. $^{a,b,c\ldots}$ Different superscript letters indicate statistically significant differences at the 95% confidence level (p-value < 0.05).

Figure 7. Scanning electron microscopic images of: a) Grinded solid pineapple waste (without any pretreatment); b) MW pretreated waste at 6.375 W/g – 5 min and c) MW pretreated at 8.5 W/g – 8 min.