Microwave-Assisted Alkali Pretreatment for Enhancing Pineapple Waste Saccharification

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The effectiveness of microwave-assisted sodium hydroxide pretreatments to enhance the saccharification performance of pineapple waste was evaluated. Microwave alkali pretreatments for short exposure times (up to 60 s) significantly improved the yield of the enzymatic hydrolysis compared with non-pretreated waste. The greatest increase of fermentable (35.7%) and total sugars (33.5%) was obtained at 6.375 W/g for 5 s. However, longer exposure times resulted in sugar degradation and released fermentation inhibitors, such as phenols or hydroxymethylfurfural (HMF), as a consequence of thermal degradation. Nevertheless, the obtained phenols values were not sufficient to inhibit subsequent fermentation. Scanning electron microscope (SEM) images confirmed that applying microwaves for short exposure times promoted structural changes that improved enzymatic hydrolysis. By contrast, an increase in the severity of the treatment led to a compacted structure, which hindered access to enzymes and consequently reduced the release of sugars into the medium.

Keywords: Microwave-assisted alkali pretreatment; Enzymatic saccharification; Pineapple waste

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INTRODUCTION

Pineapple (*Ananas comosus*) is one of the most important and appreciated tropical and subtropical fruit crops for the processing industry; it is used for juice, canned, and minimally processed fruit (Bartholomew *et al.* 2003; Reinhardt and Rodriguez 2009). Up to 75% of the whole processed fruit is industrial waste, consisting of peeled skin, core, and crown (Buckle 1989; Abdullah 2007). Pineapple industrial waste has been investigated as an interesting source for bromelain enzyme extraction, phenolic antioxidants, organic acids, fiber, vinegar, and biogas (Larrauri *et al.* 1997; Roda *et al.* 2016). Moreover, this waste is a potential raw material for bioethanol production, as it contains a high amount of fermentable sugars and potentially hydrolyzable cellulose and hemicellulose (Nigam 1999; Abdullah and Hanafi 2008; Ruangviriyachai *et al.* 2010).

The use of lignocellulosic biomass for biorefining continues to be a challenge due to the cellulose crystallinity and the complexity of the structure. Cellulose and hemicellulose are densely covered by layers of lignin, which protects them against enzymatic saccharification (Dalgaard *et al.* 2006). For these reasons, pretreatment of lignocellulose is necessary to disrupt their recalcitrant structure and successfully hydrolyze the biomass. Pretreatments are one of the least technologically mature and most expensive steps in the bioethanol production process (Laser *et al.* 2002). Of the different pretreatment

methods, alkali pretreatment is known for its ability to alter the lignin composition and, therefore, increase the digestibility of the biomass (Durot *et al.* 2003; Kristensen *et al.* 2008; Pedersen and Meyer 2010). However, this chemical pretreatment usually requires a high temperature, generally reached by conduction or convection heating (Pedersen and Meyer 2010). The high energy cost reduces the efficiency of the process. In addition, sugars and lignin may be degraded to furfural, hydroxymethylfurfural, and phenolic compounds, which strongly inhibit microbial fermentation (Taherzadeh and Karimi 2007).

Microwave irradiation is a clear alternative to conventional heating in many areas because of its high energy-efficiency, rapid heating, and easy operation (Binod *et al.* 2012). Xiong *et al.* (2000) found that microwaves change the ultrastructure of cellulose and break down the hemicellulose and lignin layers. Thus, microwave heating enhances enzymatic hydrolysis (Azuma *et al.* 1984). Moreover, microwaves can be easily combined with chemical pretreatments to increase efficiency and reaction rate (Zhu *et al.* 2006; Hu and Wen 2008; Binod *et al.* 2012).

The present study examined the efficiency of microwave-assisted sodium hydroxide pretreatment in pineapple waste. The effect of microwave power and exposure time on enzymatic saccharification and the release of fermentation inhibitors was evaluated. Low temperature scanning electron microscopy (cryo-SEM) and infrared thermography were applied to assess microstructural changes and microwave heating during saccharification, respectively.

EXPERIMENTAL

Raw Material and Sample Preparation

Pineapples (*Ananas comosus* [L.] Merr., "MD-2" cv.) were washed in 0.1% sodium hypochlorite for 5 min. The crown and the pulp were removed, and the peel and core waste were pressed in a laboratory screw press at 2.5 bar (CP-4, Vincent Corporation, Tampa, FL, USA) to separate the liquid phase from the original pineapple waste, as it already contains fermentable sugars. Finally, the resulting press cake (solid phase) was ground in a blender (Avance Collection Blender HR2097/00 800 W, Philips, Amsterdam, The Netherlands) and stored at -22 °C.

Microwave-assisted Alkali Pretreatment (MAP)

A combined alkali and microwave pretreatment was carried out by mixing 40 g of thawed, ground solid pineapple waste and 40 mL of NaOH 0.5 N (Panreac Química S.L.U., Barcelona, Spain) at room temperature (20 °C) for 1 h in the line of previous experiences by Zhu *et al.* (2006), Hu and Wen (2008), and Binod *et al.* (2012). The samples were vacuum filtered in a filter flask attached to a Büchner funnel and a vacuum pump (N86KN.18 model Laboport®, KNF Neuberger GmbH, Freiburg, Germany). The retentate (solid phase) was treated in a microwave oven with a turntable plate (LG MH63340F / MH6340FS), with a frequency of 2.45 GHz at 170 W, 340 W, or 510 W. The applied powers were 2.125 W/g, 4.25 W/g, and 6.375 W/g, with exposure times of 5 s, 10 s, 20 s, 40 s, 60 s, 120 s, and 180 s. The appearance of calcination phenomena defined the time exposure limits. Samples were reconstituted by mixing the solid waste and the liquid phase (permeate). Water loss during microwave processing was determined by the difference in weight and restored. Finally, the samples were adjusted to pH 5 with 37% HCl (Panreac

Química, S.L.U., Barcelona, Spain) for subsequent enzymatic hydrolysis. Experiments were carried out in triplicate.

Study of Microwave Heating by Infrared Thermography

A Testo 870-1 thermal imaging camera (Testo AG, Lenzkirch, Germany) with a spectral wavelength range from 7.5 µm to 14 µm, 9 Hz image refresh rate, and an infrared resolution of 160 x 120 pixels was used to estimate the temperatures reached during MAP. An image of the bottom surface of the container was taken at the end of each microwave pretreatment. The infrared images were analyzed by Testo AG IRSoft software.

Enzymatic Hydrolysis

Enzymatic saccharification was conducted by mixing 0.4% (w/w) of cellulase (1.13 U/mg solid, Sigma-Aldrich Química SL, Madrid, Spain) and 0.1% (w/w) of hemicellulase (1.5 U/mg solid, Sigma-Aldrich Química SL) from *Aspergillus niger* (L.) with the pretreated wastes in a 100-mL glass beaker, which was placed in an incubation oven (Incudigit, JP Selecta S.A., Barcelona, Spain) at 50 °C for 24 h.

Non-pretreated samples were used as a reference for assessing the effectiveness of MAP. To do this, 40 g of thawed, ground solid pineapple waste was diluted in distilled water in 1:1 (w/w) as described above for MAP before saccharification.

Sugars Determination

Sugars in the liquid phase for both the pretreated and non-pretreated wastes were measured by high-performance anion-exchange chromatography with a pulsed amperometric detector (HPAEC-PAD) on a Metrohm chromatograph system (Herisau, Switzerland) consisting of a 716 IC Compact module and Metrohm ICnet 2.0 software for data analysis. For separation, a Metrosep Carb 1 250/4.6 column (250 mm x 4.6 mm I.D.) was coupled to a guard column at 32 °C, 8.8 MPa, injection volume of 20 µL, and 0.1 M sodium hydroxide as the mobile phase (1 mL/min). The samples were diluted (1:2000 v/v in bidistilled water) and then filtered (0.45 µm nylon filter) before injection. High-purity standards (Sigma-Aldrich Química SL; purity ≥ 99%) of glucose, fructose, sucrose, arabinose, and xylose were used to prepare standard calibration curves (2.5 ppm, 5 ppm, 10 ppm, 15 ppm, 25 ppm, and 50 ppm). All measurements were conducted in triplicate. The applied potentials and time periods were as follows: t_1, 400 ms / E_1 = +0.05 V (detection); t_2, 200 ms / $\underline{E}_2$ = +0.75 V (cleaning); $\underline{t}_3$, 400 ms /E_3 = -0.15 V (regeneration).

In this study, the term fermentable sugars means glucose, fructose, and sucrose, as these sugars are naturally fermented by *Saccharomyces cerevisiae* (Hahn-Hagerdal *et al.* 2007; Matsushika *et al.* 2009). Likewise, the term total sugars refers to fermentable sugars plus pentoses (arabinose and xylose) released from saccharified pineapple wastes.

Determination of Fermentation Inhibitors

The following inhibitory compounds were determined in triplicate in the liquid phase of the non-pretreated and MAP samples followed by enzymatic saccharification.

Determination of total phenolic content

The total phenolic content was measured using the method developed by Waterhouse *et al.* (2003), with Folin-Ciocalteu reagent (Sigma-Aldrich Química SL) and monohydrate gallic acid (Sigma-Aldrich Química SL) as the standard. Results were expressed as mg of gallic acid equivalents per mL of pineapple waste (mg GAE/mL).

Determination of furfural and hydroxymethylfurfural

The furfural (F) and hydroxymethylfurfural (HMF) contents were determined by high-performance liquid chromatography (HPLC) as described by Blanco-Gomis *et al.* (1991). An Alliance® HPLC system (Waters, Milford, MA, USA) equipped with a degasser, a 2695 separation module, and coupled to a diode array detector (DAD 2996, Waters). The chromatographic separation was performed on a Kromasil® 100 C-18 column (3 µm × 250 mm × 4.6 mm inside diameter) (Sigma-Aldrich). Analyses were done isocratically at 25 °C, an injection volume of 10 µL, and using acetonitrile/water (8:92 v/v) as the mobile phase (1 mL/min). Final ultraviolet (UV) detection was conducted at 280 nm. Standard solutions of F (0 to 5 µg/mL) and HMF (1 to 100 µg/mL) were prepared by dissolving analytical grade reagents (Sigma-Aldrich; purity ≥ 98%) in water with 10% (v/v) methanol.

Low Temperature Scanning Electron Microscopy (Cryo-SEM)

Microstructural changes in pretreated and unpretreated wastes were analyzed by cryo-SEM on a Cryostage CT-1500C unit (Oxford Instruments, Witney, UK), coupled to a Jeol JSM-5410 scanning electron microscope (Jeol, Tokyo, Japan). Samples were sublimated in the microscope stage for 20 min at -90 °C and -5 kV and then moved to another stage and coated with gold for 3 min at vacuum conditions (0.2 kPa). Samples were observed at 15 kV, 15 mm working distance, and a temperature ≤ -130 °C.

Statistical Analysis

Statgraphics Centurion XVI® (Manugistics Inc., Rockville, MD, USA) was used for statistical analyses, including one-way and multifactor analyses of variance (ANOVA) across the different results. Multiple regression analysis using the Pearson product moment correlations between each pair of variables were conducted. A 95% confidence level was used in all cases; a p-value lower than 0.05 indicated a statistically significant difference.

RESULTS AND DISCUSSION

Effect of Microwave-alkali Pretreatment on Sugar Yield

To assess the suitability of the microwave pretreatment, the total and fermentable sugars in each pretreated sample were determined and compared with the non-pretreated controls. All reported values correspond to sugar content after enzymatic hydrolysis (Fig. 1). Fermentable and total sugars followed similar patterns for all treatments because fermentable sugars (glucose, fructose, and sucrose) comprise most of the total sugars.

However, the applied microwave power, the exposure time, and the combination of both factors caused a statistically significant increase in sugar content. For all applied powers, microwave irradiation at short exposure times (up to 60 s) generated an increase in total and fermentable sugars yields. At a low microwave power (2.125 W/g), the highest increases in fermentable (32.6%) and total sugars (33.6%) were obtained at t = 10 s, but there were no statistically significant differences with the results at t = 5 s and t = 15 s. At an intermediate power (4.25 W/g), the largest increases in fermentable (27.9%) and total sugars (26.7%) were achieved at t = 5 s, although no statistically significant differences were observed with the results at t = 10 s and 20 s. The highest increases of fermentable (35.7%) and total sugars (33.5%) were obtained at t = 5 s when high microwave power was applied. However, these values showed no statistically significant differences with any

pretreatment up to 20 s. Therefore, as suggested by Binod *et al.* (2012), for every microwave irradiation power, there was an optimal treatment time. Longer exposure times resulted in decreased sugar content. Higher applied microwave power accelerated decreases in sugar content. Binod *et al.* (2012) observed the same phenomenon in MAP for sugar cane bagasse, and charring was found for any applied microwave power at different exposure times. However, Zhu *et al.* (2005) and Singh and Trivedi (2013) did not report sugar reduction in wheat and rice straw at long exposure times. Notably, there was no further reduction of sugar yield with high microwave power (6.375 W/g) and 120 s exposure time, suggesting that sugars may have been entirely degraded at this point. Some useful components and sugars might be decomposed by pretreatments at high temperatures with high microwave power and long exposure times (Zhu *et al.* 2005).

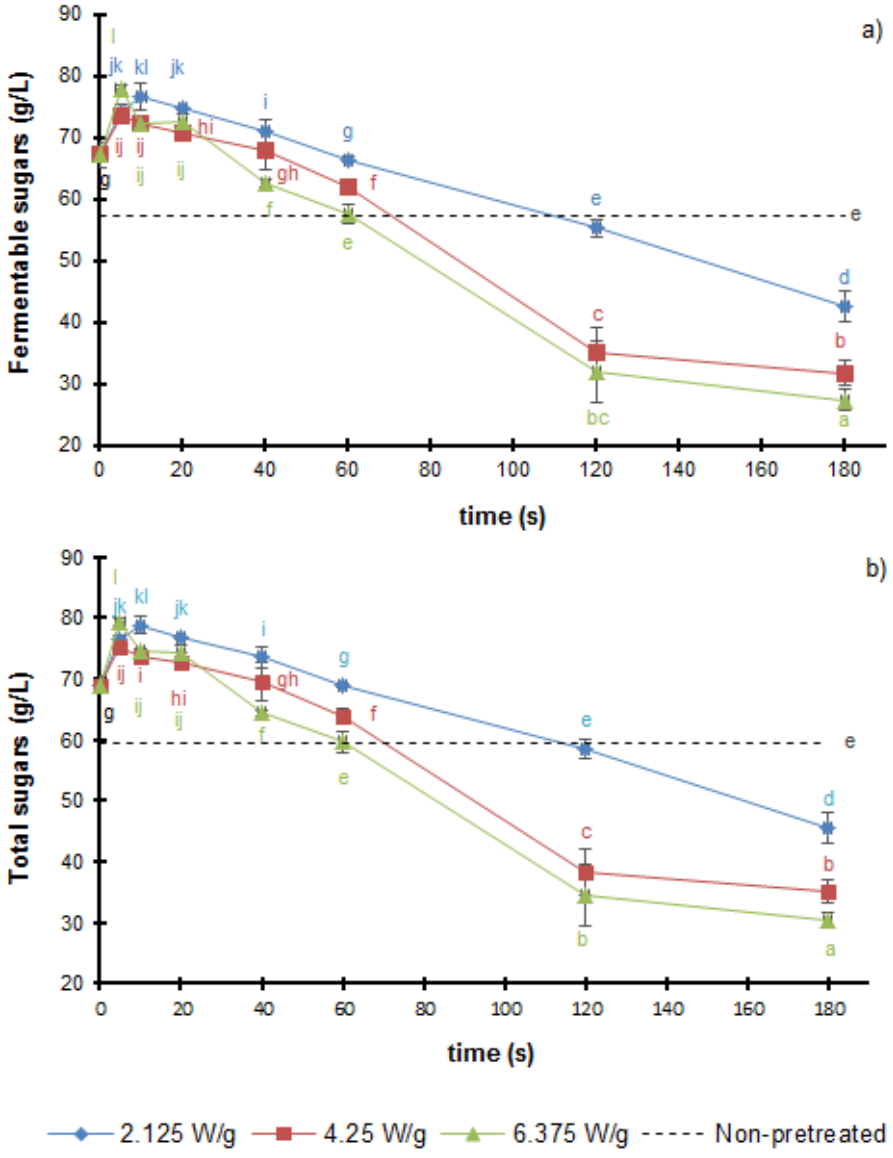

Fig. 1. a) Fermentable sugars (g/L) and b) total sugars (g/L) of MAP samples at different power and exposure times followed by enzymatic saccharification. Data followed by different lowercase letters are statistically different according to the multiple range test (95% confidence level).

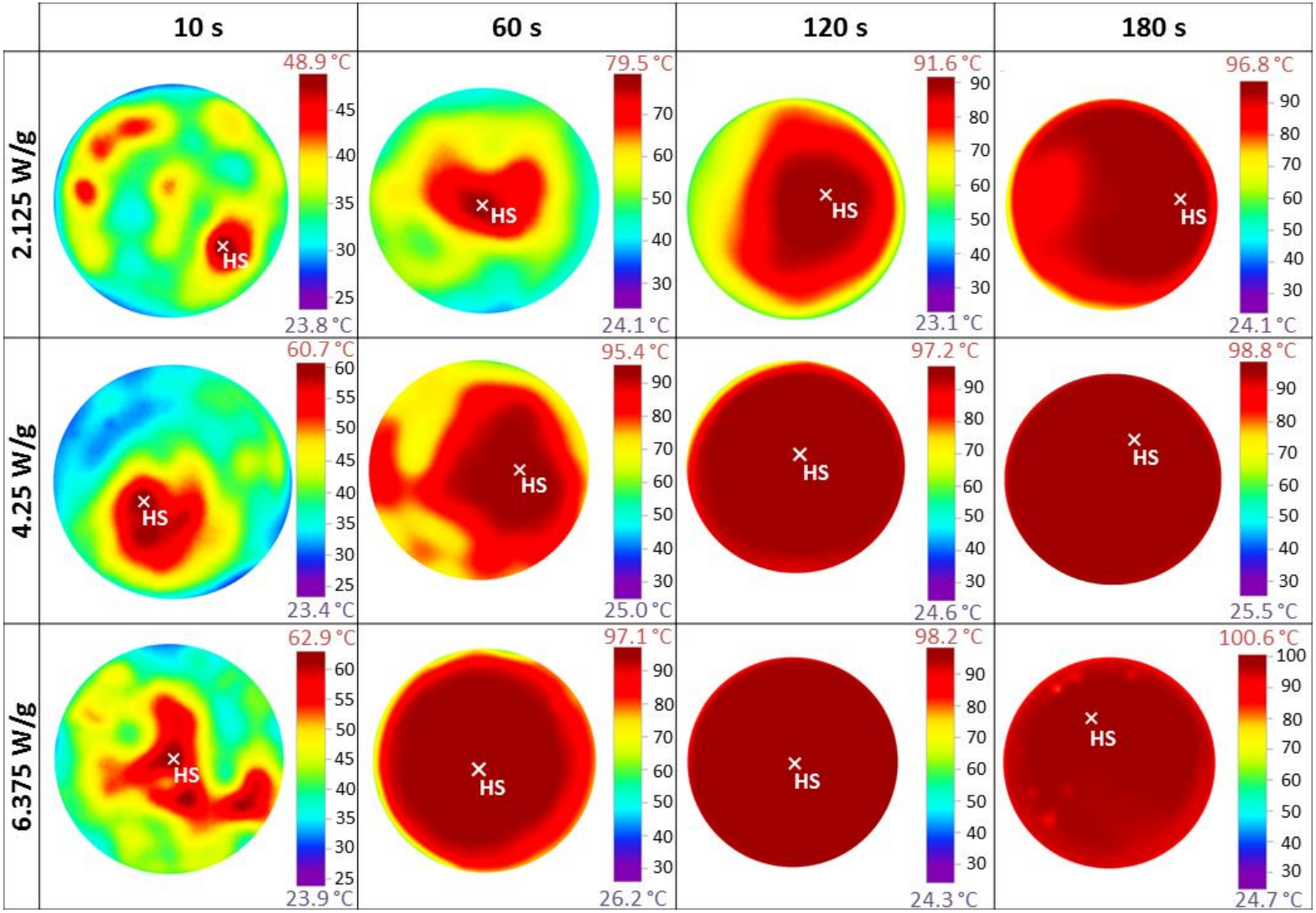

Fig. 2. Thermal images and temperature scales corresponding to the bottom surface of the recipient containing MAP pineapple waste at different powers: 2.125 W/g, 4.25 W/g, and 6.375 W/g, and exposure times: 10 s, 60 s, 120 s, and 180 s; white cross, hot spot (HS)

Analysis of Microwave Heating of Pineapple Waste by Thermography

Some of the effects produced by the application of microwaves may be due to the temperatures reached during pretreatment. Consequently, the temperatures of the samples during microwave pretreatment in an alkaline medium were evaluated *via* thermal images of the base of the waste containers. Figure 2 shows the thermography data and the maximum temperature or hot spot (HS) in each image. With short exposures ($t < 60$ s), the appearance of the HS was followed by inhomogeneous heating of the sample by the microwaves, leading to conductive heating from these points. This evolution is characteristic for microwave heating (Lidström *et al.* 2001; Hu and Wen 2008) and is due to polar regions of the lignocellulosic material being selectively heated. The recalcitrant lignocellulosic structure may be disrupted by an "explosion effect" at the hot spots (Hu and Wen 2008).

Despite the initially heterogeneous heating, homogeneity of the entire sample was reached within a few minutes, although this effect took longer at lower power levels. In particular, more than 180 s was needed at the lowest power (2.125 W/g), more than 120 s was needed at intermediate power (4.25 W/g), and over 60 s was needed at the highest power (6.375 W/g). The results were similar to another report where longer exposure times were required at lower power levels; an applied power of 100 W required 150 s to reach a uniform temperature in the waste (Kumar *et al.* 2014).

Figure 3 shows the maximum and average temperatures throughout the treatment for all power levels. Exposure time, applied power, and the interaction between these two factors each had a statistically significant effect on the maximum and average temperatures. The evolution of the average and maximum temperature was fairly similar for all power levels. When 4.25 W/g and 6.375 W/g were applied, there was a more pronounced increase in temperatures up to 40 s of treatment; from then on, the temperature changed more gradually until the end of treatment. With the lowest applied power (2.125 W/g), the greatest temperature increase was between 20 and 40 s, and its evolution was considerably slower than in the previous cases. This result suggested that microwave power was less efficient at long times rather than at short times, where the greatest temperature changes were recorded (Kumar *et al.* 2014). This initially faster warming was due to the emergence of the HS, while the subsequent slow evolution overlapped with conductive heating. The maximum temperature did not change greatly after 20 s for the highest power, after 20 s to 40 s for the intermediate power, or after 40 s for the lowest power. Although the final temperatures were significantly different depending on the power applied, the greatest differences between the various power levels occurred between 10 s and 120 s of treatment. Finally, heating was not the only effect that microwaves exerted on the residue structure. The electromagnetic field might have created non-thermal effects that also could have accelerated the destruction of the crystalline structures (De la Hoz *et al.* 2005).

Effect of the Pretreatments on the Release of Fermentation Inhibitors

Figure 4 shows the total phenol content in the liquid phase of the MAP and subsequently saccharified waste. Microwave pretreatment in an alkaline medium produced a significant increase in the total phenol content compared to non-pretreated waste (0.145 mg GAE/mL ± 0.004). In general, the phenol content was gradually raised as the time of exposure and the power applied were increased. Statistical analysis indicated that the time of exposure, the power applied, and the interaction between these two factors had a significant effect on the total phenols.

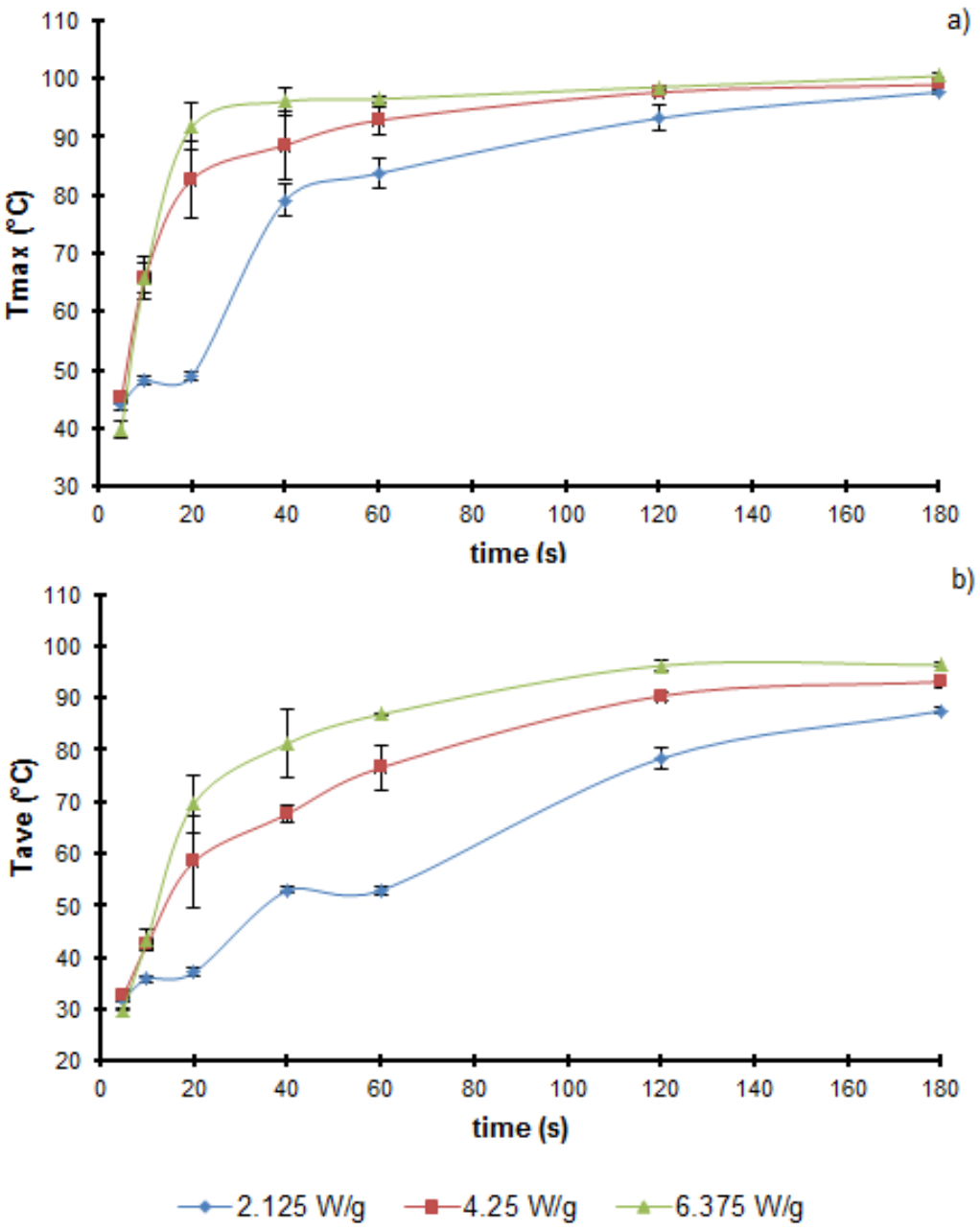

Fig. 3. a) Maximum temperature (T_{max}) and b) average temperature (T_{ave}) in pretreated pineapple waste at different powers (2.125 W/g, 4.25 W/g, and 6.375 W/g) and exposures (5 s to 180 s)

Hu and Wen (2008) suggested that the increase in temperature *via* microwave pretreatment promotes lignin degradation and, thus, a rise in the phenol content. Vázquez-Gutiérrez *et al.* (2013) applied high pressures to onion waste and showed that increasing the severity of the treatment enhances the total phenol content.

Ando *et al.* (1986) showed a 30% reduction in the yield of phenolic compounds from *S. cerevisiae* yeast when 1 g/L of 4-hydroxybenzoic acid was added, while Palmqvist and Hahn-Hägerdal (2000) reported no significant effects when adding 2 g/L of this phenolic compound. In this case, the obtained values were not sufficient to inhibit subsequent fermentation.

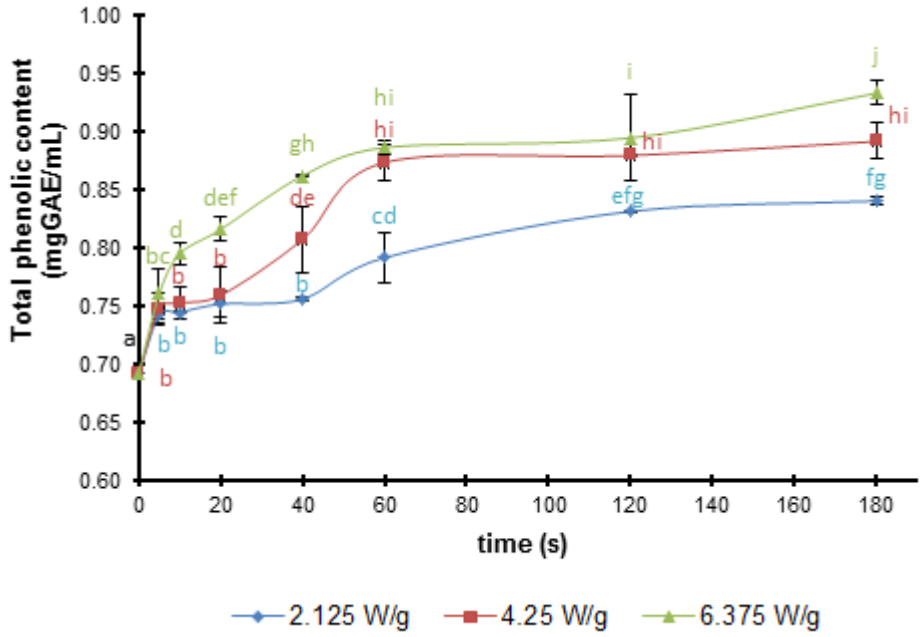

Fig. 4. Total phenolic content (mg GAE/mL) in microwave-assisted alkali pretreated samples followed by enzymatic saccharification. Different lowercase letters indicate a statistical difference according to the multiple range test (95% confidence level).

Figure 5 shows the HMF content of MAP and subsequently saccharified waste. Furfural was not detected in the liquid phase of the analyzed waste, which could be due to the low pentose content in the initial waste that can be considered negligible (Conesa *et al.* 2016). In addition, the microwave pretreatment in an alkaline medium caused a marked increase in HMF content, such that both nominal power applied and exposure time were significant. The interaction between these two factors was also significant because the differences due to applying different power levels declined as exposure time increased. All values were significantly higher than those obtained when microwaves were not applied to the waste. These values were consistent with those reported in other studies. Specifically, Gabhane *et al.* (2013) found that increasing the application time and power of the microwave pretreatment of banana waste increases the temperature, resulting in greater degradation of hexoses to HMF. In this case, all of the values obtained ranged from 1.282 ± 0.015 g/L to 2.148 ± 0.018 g/L, with the lowest produced at a power of 2.125 W/g and a time of 5 s. The negative effects on the growth and fermentation rate of *S. cerevisiae* were reported for HMF concentrations above 1.0 g/L (Banerjee *et al.* 1981; Taherzadeh *et al.* 2000). Sanchez and Bautista (1988) hypothesized that the main effect of HMF (2 g/L) was the prolongation of the lag phase on fermentation.

Consequently, the use of MAP at short exposure times (t < 60 s) significantly improved the yield of the enzymatic hydrolysis and kept the concentration of the generated inhibitors below the level to inhibit the subsequent fermentation. As the purpose of the study is to ferment the pretreated and saccharified waste without any extraction, these inhibitors will not be concentrated in the samples avoiding problems in the fermentation step.

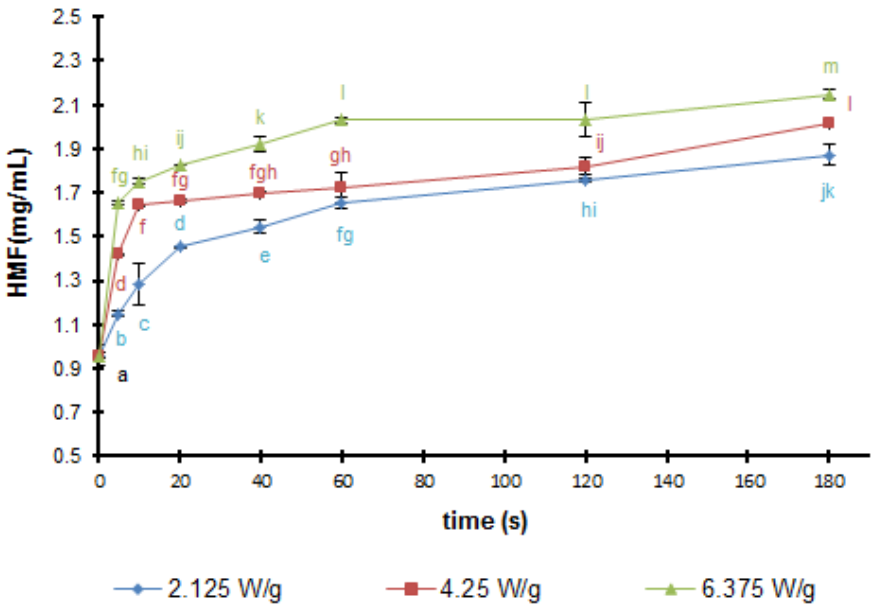

Fig. 5. HMF content (mg /mL) of microwave-assisted alkali pretreated samples followed by enzymatic saccharification; data followed by different lowercase letters are statistically different according to the multiple range test (95% confidence level).

Analysis of the Relationships between the Study Variables

Table 1 shows the Pearson correlation coefficients among each pair of analyzed variables. Each pair of variables had a significant linear relationship ($p < 0.05$). Fermentable and total sugars were strongly and negatively correlated with all the studied variables, meaning that these variables tended to decrease as the HMF, total phenolic content, and average and maximum temperatures increased. These results were in line with those previously obtained because HMF is generated from hexose degradation as temperature increases.

The remaining variables were strongly and positively correlated with the maximum and average temperatures. The total phenolic content showed a positive correlation with the HMF content and the measured temperatures. This effect was due to further degradation of the lignin structure and the subsequent production of HMF as the temperatures rose. All temperatures were interrelated.

Table 1. Pearson Product Moment Correlations between Each Pair of Analyzed Variables

	FS	TS	TPC	HMF	T_{max}	T_{ave}
FS	1.000	0.9997*	-0.8251*	-0.6717*	-0.6747*	-0.8460*
TS	0.9997*	1.000	-0.8287*	-0.6761*	-0.6759*	-0.8483*
TPC	-0.8251*	-0.8287*	1.000	0.8740*	0.8473*	0.9233*
HMF	-0.6717*	-0.6761	0.8740*	1.000	0.8749*	0.8661*
Tmax	-0.6747*	-0.6759*	0.8473*	0.8749*	1.000	0.9443*
Tave	-0.8460	-0.8483*	0.9233*	0.8661*	0.9443*	1.000

* Indicates statistically significant correlations ($p < 0.05$)
FS, fermentable sugars; TS, total sugars; TPC, total phenolic content; HMF, hydroxymethylfurfural; T_{max}, maximum temperature; T_{ave}, average temperature.

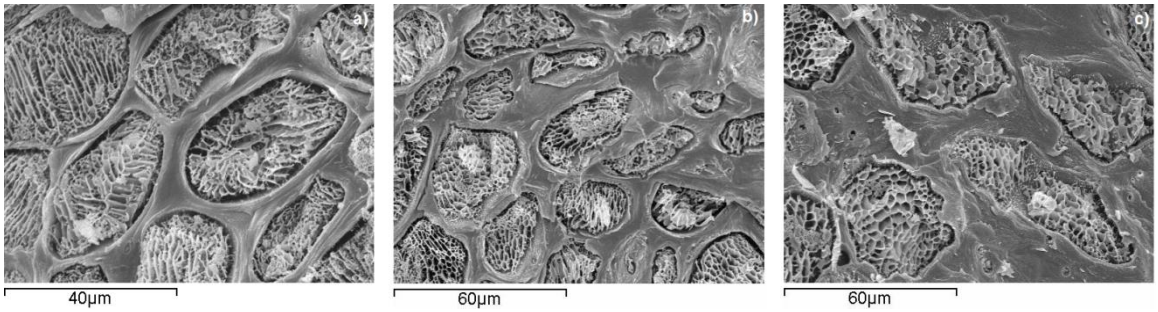

Fig. 6. Cryo-SEM images of a) unpretreated sample, b) MAP waste treated at 6.375 W/g for 10 s, and c) MAP pretreated at 6.375 W/g for 180 s

Analysis of Physical Structure Changes by Cryo-SEM

Cryo-SEM observations of non-pretreated and MAP pineapple waste at 6.25 W/g and 10 s or 180 s showed that pretreatments induced structural changes in the lignocellulosic biomass (Fig. 6). The non-pretreated sample (Fig. 6a) possessed rounded packed cells with continuous surface areas. Moreover, protoplasts and cell walls were clearly identified. In contrast, microwave pretreated samples at a short exposure time (10 s) had a rougher surface area, and the separation of the plasmatic membrane from the cell wall was clearly identified. As suggested by Binod *et al.* (2012), microwave-assisted alkali pretreatments remove external fibers and increase the surface area, which makes cellulose more accessible to enzymes. These effects were directly related to the sugar increase for MAP at short exposure times (< 60 s). Similar structural changes have been reported by Binod *et al.* (2012) for sugarcane bagasse microwave-alkali pretreated at short exposure times. With increasing microwave exposure time (Fig. 6c), cell wall roughness was greater, which suggested tissue dehydration. Laivins and Scallan (1996) indicated that dehydration had a negative effect on enzyme accessibility to the substrate due to small pore sizes and narrowed pore size distributions in cellulose fibers. These results were in line with those obtained for high exposure times, in which MAP reduced the saccharification performance compared with non-pretreated samples.

CONCLUSIONS

1. The use of microwave alkaline pretreatments for short exposure times (up to 60 s) improved the yield of enzymatic hydrolysis compared with non-pretreated waste. The highest increase in fermentable (35.7%) and total sugars (33.5%) was obtained at t = 5 s when high microwave power was applied (6.375 W/g). However, longer exposure times resulted in sugar degradation.
2. The content of fermentable and total sugars showed a statistically significant negative correlation with the maximum and average temperatures in the samples pretreated by microwave in an alkaline medium.
3. The content of fermentation inhibitor compounds, such as total phenols or HMF, increased as microwave power and exposure time rose. This increase was correlated with the rise in temperatures. However, the total amount of phenol values was not sufficient to inhibit subsequent fermentation. Nevertheless, the effect of HMF content on pineapple waste fermentation should be studied.

4. Applying microwaves during short exposure times promoted structural changes that improved enzymatic hydrolysis. In contrast, an increase in the severity of the treatment compacted the structure and thus hindered access by the enzymes, which reduced the sugars released into the medium.

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