Effect of ozonolysis pretreatment on enzymatic digestibility of sugarcane bagasse

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Abstract: Bagasse was pretreated with ozone to increase the enzymatic hydrolysis extent of potentially fermentable sugars. Through a 3×4 factorial design, this research studies the influence of operating parameters (moisture content and retention time) on ozonization pretreatment of bagasse in a fixed bed reactor under room conditions. Enzymatic hydrolysis yields of up to 67% were obtained compared to 20% in non-ozonized bagasse. Moisture content and retention time showed significant effect on ozonolysis. And the most efficient conversion was obtained in 50% w/w humidity and 3.5 h retention time. The study also revealed that smaller particle size in the raw bagasse improved the performance of ozonolysis as well.

Keywords: Sugarcane bagasse, ozonolysis, bioethanol, enzymatic hydrolysis

Citation: Eqra, N., Y. Ajabshirchi, M. Sarshar. 2014. Effect of ozonolysis pretreatment on enzymatic digestibility of sugarcane bagasse. Agric Eng Int: CIGR Journal, 16(1): 151−156.

1 Introduction

Biofuel produced from lignocellulosic materials, so-called second generation bioethanol shows energetic, economic and environmental advantages in comparison to bioethanol from starch or sugar (Alvira et al., 2010). Lignocellulosic material is composed of heterogeneous complex of carbohydrate polymers (cellulose, hemicellulose and lignin). It has evolved to resist degradation and to confer hydrolytic stability and structural robustness, which is attributable to cross linkage between polysaccharides (cellulose and hemicellulose) and lignin via ester and ether linkages. Cellulose and hemicellulose are densely packed by layers of lignin, which protect them against enzymatic hydrolyses. So it is necessary to break lignin seal to expose cellulose and hemicellulose for enzymatic action (Lee et al., 2008).

Received date: 2013-02-02 **Accepted date:** 2014-02-01
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Amongst possible applications, this research focuses on enzymatic hydrolysis of the cellulose fraction, by ozone pretreatment, to glucose and xylose to be fermented in order to obtain fuel-grade ethanol in future researches. The different alternatives tested for lignocellulose pretreatment involve the use of physical, chemical, physicochemical and/or biological methods, e.g. steam explosion, hot water extraction (Rosgaard et al., 2007; Pan et al., 2005), microwave (Keshwani, 2009), ultrasound (Yachmenev et al., 2009), sulfuric acid (Cara et al., 2008), sodium hydroxide, hydrogen peroxide, ammonia fiber explosion, AFEX (Sendich et al., 2008; Kumar et al., 2009), wet oxidation (Sun and Cheng, 2002) and ozonolysis (Silverstein et al., 2007; Zhao et al., 2008).

The main obstacle to producing ethanol economically from lignocellulosic materials is its low digestibility due to the loose link between their components: cellulose, hemicellulose and lignin. It is well known that lignin content significantly impacts enzymatic hydrolysis of lignocellulosic biomass (Mosier et al., 2005).

Ozone is a powerful oxidant that shows high delignification efficiency (Sun and Cheng, 2002). This lignin removal increases the yield in subsequent enzymatic hydrolysis. The pretreatment is usually performed at room temperature and normal pressure and does not lead to the formation of inhibitory compounds that can affect the subsequent hydrolysis and fermentation. Ozone is highly reactive towards compounds incorporating conjugated double bonds and functional groups with high electron densities. Therefore, the moiety most likely to be oxidized in ozonization of lignocellulosic materials is lignin due to its high content of C=C bonds. Ozone attacks lignin releasing soluble compounds of less molecular weight, mainly organic acids such as formic and acetic acid which can result in a drop in pH from 6.5 to 2.

Ozone applications have increased substantially both in number and diversity over the last two decades, and have been used for example in the treatment of ground and industrial wastewaters (Coca et al., 2005). In pulp bleaching in the paper industry, ozone has been widely used and has evidenced high delignification efficiency (Roncero et al., 2003; Shatalov et al., 2008). It has also been applied on several agricultural residues. Silverstein et al. (2007) recently applied ozone pretreatment for conversion of cotton stalks to ethanol by continuously sparing ozone gas through a 10% (w/v) mixture of cotton stalks and water at 4 ºC for 30, 60 and 90 min. Ozone did not cause the expected effect, possibly because of insufficient reaction time, low ozone concentration or contact method. Additional research needs to be conducted to optimize ozonolysis pretreatment operation conditions.

Souza-Corrêa et al. (2013) used mass spectrometry to monitor neutral chemical species from sugarcane bagasse that could volatilize during the bagasse ozonation process. Lignin fragments and some radicals liberated by direct ozone reaction with the biomass structure were detected. The optical results indicated that the ozone interaction with the bagasse material was better for bagasse particle sizes less than or equal to 0.5 mm.

García-Cubero et al. (2009) pretreated wheat and rye straws with ozone and studied the influence of five operating parameters (moisture content, particle size, ozone concentration, type of biomass and air/ozone flow rate) on ozonation pretreatment of straw in a fixed bed reactor under room conditions. The acid insoluble lignin content of the biomass was reduced in all experiments involving hemicellulose degradation. Enzymatic hydrolysis yields of up to 88.6% and 57% were obtained compared to 29% and 16% in non-ozonated wheat and rye straw respectively. Moisture content and type of biomass showed the most significant effects on ozonolysis.

Bes et al. (1989) applied ozone pretreatment to poplar sawdust in a fixed bed reactor results in greatly enhanced susceptibility to cellulose enzyme hydrolysis. Ozone attacks lignin and hemicellulose in preference to cellulose giving water soluble products.

Miura et al. (2012) carried out ozonolysis and subsequent wet-disk milling (DM) on Japanese cedar (*Cryptomeria japonica*) to improve sugar production by enzymatic saccharification. When the moisture content reached more than 40%, ozone consumption decreased, resulting in less delignification. Ozone treatment removed mainly lignin, but also small amounts of polysaccharides.

The aim of this work was to determine the influence of process parameters on the ozonolysis pretreatment of sugarcane bagasse to improve yields at the enzymatic hydrolysis stage. A pilot plant at lab scale with a fixed bed reactor was designed and built for the ozonation experiments. The raw material considered for this study was sugarcane bagasse, the by-product from sugarcane crop. This material was selected as it is widely produced in the region of Khozestan (Iran) and presented a considerably different percentage of lignin. During the trial, the effect of three variables likely to impact the sugarcane bagasse ozonolysis process such as moisture, particle size and retention time was studied.

2 Materials and methods

2.1 Raw material

Sugarcane bagasse was obtained from Khuzestan Haft Tappe Cultivation and Industry sugar production Co. in Iran and ground in a blender, sieved to obtain two different sizes: <1 mm and 3-5 mm and dried in an oven at 45ºC. The composition (%, w/w) of sugarcane bagasse is shown in Table 1.

Table 1 Composition of sugarcane bagasse

Composition	Percentage (%, w/w)
Moisture	9.6
Cellulose	36.1
Hemicellulose	22.3
Lignin	23
Others	9

2.2 Ozonolysis pretreatment

The ozonolysis pretreatment of bagasse performed in a fixed bed reactor (glass chamber 70 cm in height, 50 cm in length and 50 cm in width) under room conditions. At the beginning of each test, ground material was hydrated to the required value, fed into the reactor until total reaction volume was attained and then exposed to an air/ozone gas stream in the fixed bed reactor.

Kinetic experiments (in four retention time were carried out to determine a reaction time long enough to allow total oxidation of the bagasse.

Ozone production was controlled to 10000±10 ppm by varying the air flow rate, and the concentration in the gas phase was measured following the iodometric method (Standard Methods for the Examination of Water and Wastewater, 1995). Reactor outlet gas flow was passed through a 2% KI (potassium iodide) solution to remove any unreacted ozone from the gas stream. The resulting ozone-treated substrate was dried in an oven at 45ºC, stored in a freezer and used for enzymatic hydrolysis.

2.3 Enzymatic hydrolysis

Hydrolysis tests for each sample were performed to determine the improvement in enzymatic saccharification under the different pretreatment conditions. Enzymatic hydrolysis was performed using a mixture of cellulase complex (NS22086) and β-glucosidase (NS22118), and xylanase (NS22083). Enzyme reactions were performed with 0.3 g of freeze stored biomass suspended in 5 mL acetate buffer 0.1 M (pH 4.8); containing 10 FPU/g and 10 CBU/g of substrate (dry basis) at 50ºC for 52 h. Test flasks were shaken in a rotary incubator at 150 r/min. After hydrolysis, 600 μL samples were withdrawn, passed through a 0.22 μm filter and stored for analysis of sugars and other possible compounds (e.g. inhibitors).

2.4 Analytical methods

Glucose, xylose and other possible inhibitors (HMF, furfural, etc.) from enzymatic hydrolysis were also analyzed by HPLC using the VETEX EH-002 column to measure sugar concentration. The mobile phase was 5 mmol/L H_2SO_4 at a flow rate of 0.4 mL/min at 75 ºC. The detection was based on the refraction index measurement.

2.5 Statistical analysis

The experimental work was conducted in three consecutive trials to examine the influence of the ozone pretreatment operation factors [particle size (S), moisture (M), and retention time (T)] on bagasse enzymatic digestibility. Experiments were analyzed through two trails, first determining particle size factorial design in which the variables were evaluated at their levels (Table 2). The impact of the main operating factors and their interactions were calculated and laid out in a normal probability plot to draw the most significant factors of the process. The effect was defined as the change in the response caused by a change in the level of the parameter. The statistic software package SPSS was used for evaluation of the data.

Table 2 Experimental levels of the parameters studied

Factor	Level value			
Particle size, mm	<1		3-5	
Moisture content, % (w/w)	40	50	60	
Retention time, h	1.5	2.5	3.5	4.5

3 Results and discussion

3.1 Determination of particle size

To determine appropriate particle size for the reaction, several ozonization tests were run modifying particle size for sugarcane bagasse. All these experiments were carried out with 10000 ppm ozone, 40%–60% w/w moisture (w.b.) and 1.5 – 4.5 h retention time.

Results obtained showed that ozonization reaction was more effective in smaller particle size, in all cases, and these tests repeated three times in each trail. As it can be seen in Table 3, there is a significant difference in two mentioned sizes. Therefore, from these results bagasse with particle size of 1mm was selected for subsequent trials. In agreement with results obtained by (García-Cubero et al., 2009) when wheat straw and rye straw were pretreated and hydrolyzed, in all cases, smaller particle size in the raw bagasse improves the performance of ozonolysis and by (Souza-Corrêa et al., 2013) that the optical results indicated the ozone interaction with the bagasse material was better for bagasse particle sizes less than or equal to 0.5 mm.

Table 3 Significance level found at particle size of sugarcane bagasse

Particle size of sugarcane bagasse, mm	Paired differences				t	df	Sig.
	Std. deviation	Std. error mean	95% confidence interval of the difference				
			Lower	Upper			
<1 and 3-5 mm	1.62898	0.27150	1.12613	2.22846	6.178**	35	0

Note: ** = Significance level $p \leq 0.01$.

3.2 Effect of ozone pretreatment operation variables on bagasse delignification

After selecting the particle size, a set of experimental runs were performed to analyze the influence of ozone pretreatment on hydrolysis efficiencies by variance analysis of the data related to dissolved sugar percentage (Table 4).

Table 4 Variance analysis of the data related to dissolved sugar percentage

Source	Type III sum of squares	df	Mean square	F	Sig.
Retention time/h	751.987	3	250.662	**223.603	0
Moisture percentage/%	258.433	2	129.216	**115.268	0
Retention time * moisture percent	15.698	6	2.616	**N.S.** 2.334	0.064
Error	26.904	24	1.121		
Total	128166.977	36			
Corrected total	1053.022	35			

Note: Dependent Variable: sugar percentage;
a. R Squared = 0.974 (Adjusted R Squared =0 .963);
N.S. = Not Significant; ** = Significance level $p \leq 0.01$.

As explained above, the influence of moisture and retention time was assessed on selected particle size. This analysis was used to provide a good insight into pretreatment optimization, establishing which parameters have a significant effect on the ozonization of bagasse, either directly or by their interactions. It can be found in Table 4 that there is a significant difference between the levels of retention time and moisture content but there is no significant difference between the interactions. Therefore, the basic factors (retention time and moisture content) had to be analyzed. Possible inhibitors such as furfural or HMF were not detected in ozonated samples, and at hydrolysis stage all experiments led to a reduction in klason lignin and an increase in the fermentable sugar concentrations compared to untreated biomass.

Ozonolysis efficiency is defined as the conversion percentage of bagasse to sugar after hydrolysis. The impact of retention time on conversion percentage of bagasse to sugar in various humidity levels is illustrated in Figure 1. Even though in results by García-Cubero et al. (2009), 2.5 h was considered as retention time at 27,000 ppm concentration, but in this experiment ozone concentration was lower (10,000 ppm) so longer retention time was effective.

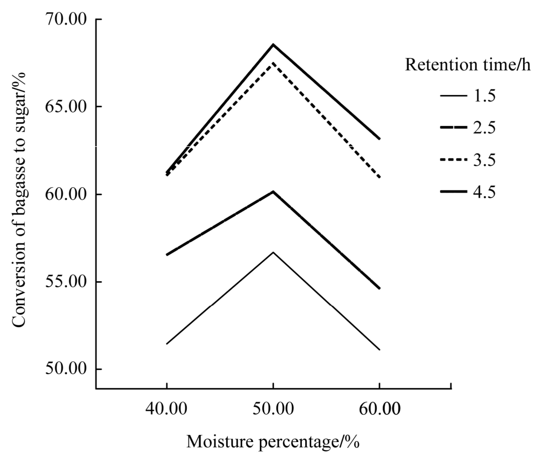

Figure1 Effect of retention time on conversion of bagasse to sugar

Hence ozonation is energy and time consuming, the analysis of retention time factor, using a series of average comparison experiments, was conducted (Table 5), HSD (Honestly Significant Difference) experiment was selected and obtained results are shown in Table 5 that shows that there is no significant difference between 3:30 h and 4:30 h. So in order to save time and energy, 3:30 h retaining time was declared optimized.

Table 5 HSD (Tukey) experiment for four retention time

Retaining time/h		N	Subset		
			1	2	3
Tukey[a] HSD	1.5	9	**A** 53.0835		
	2.5	9		**B** 57.1108	
	3.5	9			**C** 63.1827
	4.5	9			**C** 64.3100

Note: a = 0.05.

The impact of moisture content on conversion percentage of bagasse to sugar in various humidity levels is illustrated in Figure 2.

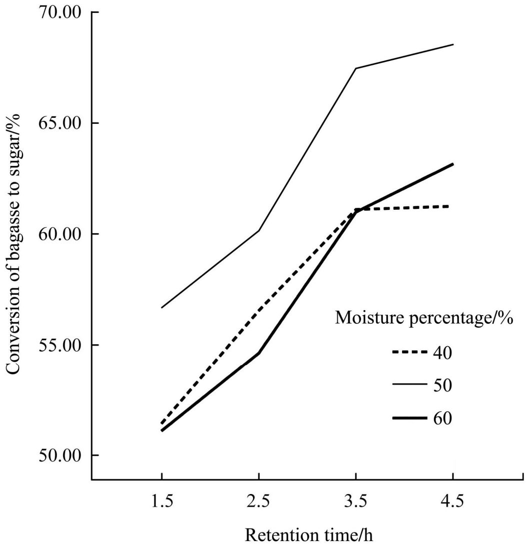

Figure 2 Effect of moisture content of bagasse on conversion to sugar

As it can be seen in Figure 2, 50% w/w moisture (w.b.) content of the bagasse resulted in the highest percentage of conversion, this result contradicts the García-Cubero et al. (2009) research in which 40% moisture content of bagasse was declared optimized.

4 Conclusions

The following conclusions can be drawn from the experiments in ozonated sugarcane bagasse:

- Ozonolysis is an efficient pretreatment for sugarcane bagasse. Ozone degrades lignin and slightly solubilizes the hemicellulose fraction, improving subsequent enzymatic hydrolysis.
- Operating in a fixed bed reactor, size of the bagasse, moisture and retention time proved to be the relevant parameters in sugar conversion.
- The most efficient conversion was obtained at 50% w/w moisture and 3.5 h retention time.
- Ozone pretreatment in smaller particle size results in increase accessibility to the ozone gas, therefore in all the experiment it results in more efficiency.
- Enzymatic hydrolysis yields of up to 67% were obtained compared to 20% in non-ozonated sugarcane bagasse.

Acknowledgements

The authors wish to thank the agricultural research center authorities of Fars province in Iran for providing the necessary equipment for this experiment work and financially supporting this research.

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