

## Thermal Hydrolysis of Orange Peel and its Fermentation with Alginate Beads to Produce Ethanol

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Orange peel (OP) was subjected to thermal hydrolysis at temperature ( $T$ ) and pressure ( $P$ ) values lower than 180 °C and 1000 kPa, respectively, to minimize the energy consumption and obtain a good ethanol yield. The process was conducted in an autoclave, a pressurized reactor for studying the effect of  $T$  (120 to 180 °C),  $P$  (500 to 1000 kPa), gas type ( $N_2$  or  $CO_2$ ), and OP loading ( $2.5 \times 10^{-2}$  and  $5 \times 10^{-2} \text{ gmL}^{-1}$ ) on the reducing sugars yield. The results were compared with those from acid hydrolysis. Hydrolysates were fermented using immobilized yeast on alginate beads, and the highest ethanol yield of 33.14 g/L was obtained from OP treated at 120 °C. Under these conditions, a minimum of 1,407 kWh energy consumption was achieved.

*Keywords:* Ethanol; Thermal hydrolysis; Orange peel; Alginate beads

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### INTRODUCTION

Currently, 84% of energy that is consumed in the world originates from fossil fuels, a non-renewable and rapidly depleting resource. In addition, the use of fuels derived from it results in environmental problems such as emission of greenhouse gases, as well as economic concerns. Therefore, the need to find an alternative energy source has emerged in recent years. Lignocellulosic residues (for example: forestry waste, fruit peel, cereal, or waste paper) as energy sources are a good choice because of their chemical composition, which mostly consists of cellulose, hemicellulose, and lignin. These residues can be processed to obtain ethanol, which can then be used as an alternative combustible fuel (Sun and Cheng 2002; Balat 2011), a biofuel similar to gasoline, but with some advantages over it.

Ethanol is produced mostly from sugar cane or corn, principally in the USA and Brazil. However, in Mexico, as in many other countries, the production of ethanol from sugar cane or corn would not be a sustainable process. Corn and sugar cane production is not sufficient for national consumption in Mexico, as a certain amount is actually imported. Therefore, this process would imply investment in exclusive plantations for biofuel production. On the other hand, Mexico is the fourth worldwide orange producer. Every year, 800 thousand tons of dry waste from the orange industry could be exploited for ethanol production (Rezzadori *et al.* 2012), as this fruit is rich in fermentable sugars, has low lignin levels, is available all year round, and is inexpensive.

Previous studies have reported the successful hydrolysis of OP into sugars and their conversion into ethanol (Grohmann *et al.* 1995; Oberoi *et al.* 2010; Ghorbanpour Khamseh and Miccio 2012). The effects of pH, time, and temperature pretreatment on ethanol production has been studied extensively in the presence of acids (Widmer *et al.* 2010). However, the process with water as a reagent for ethanol production has not been analyzed in terms of energy expenditure and costs. Most of the studies on OP hydrolysis have employed acids. However, these processes are not environmentally friendly because corrosive residues may be produced and acid has to be separated from the hydrolysate. Also, it is necessary to use inoxidizable special reactors with steam injection, which are not easily accessible. Although enzymatic hydrolysis and Simultaneous Saccharification Fermentation (SSF) have several advantages, it is necessary to have an efficient hydrolysis and fermentation from the enzyme mixtures used (Santi *et al.* 2014).

Thermal hydrolysis has the advantage that no chemicals other than water are needed and the cellulose accessibility is enhanced. Thermal hydrolysis of several lignocellulosic materials has been reported at various temperature ( $T$ ) ranges (180 to 250 °C) and pressure ( $P$ ) of approximately 10,000 kPa (Prado *et al.* 2014; Sukhbaatar *et al.* 2014; Zhang and Wu 2014). Nevertheless, it is evident that the glucose yield achieved not only depends on the reaction conditions but also on the biomass source because of the variable chemical compositions (Yoon *et al.* 2014).

Hence, this study investigated the effect of thermal hydrolysis of orange peel (OP) on reducing sugars (RS) and glucose yields under various temperature and pressure conditions. The obtained results were compared with those attained with the widely studied acid hydrolysis method based on proximal analysis of the waste (Lee and Jeffries 2011). Finally, the hydrolysates were fermented with immobilized yeast on alginate beads to improve the ethanol yield (Lee *et al.* 2011), reduce the risk of microbial contamination, improve product tolerance, and to provide the option of relatively easy separation in such a way that the yeast can be reused. Sodium alginate is inexpensive, nontoxic, and easily form beads with yeast (Singh *et al.* 2013).

## EXPERIMENTAL

### Materials

Valencia orange (*Citrus sinensis* cv. *Valencia*) was collected from a local supermarket (Toluca City, Mexico) in bulk in mid-March. The peels were removed (mesocarp and exocarp together), washed, and dried at 60 °C in a Riossa E33D RSU Lab (Monterrey, Mexico) oven for approximately 4 h; they were then milled to 0.25-mm particle size.

Deionized water was used in all experiments. All chemicals were of reagent grade. H<sub>2</sub>SO<sub>4</sub> and CaCl<sub>2</sub> were purchased from Fermont (Monterrey, México); glucose, MgSO<sub>4</sub>, K<sub>2</sub>HPO<sub>4</sub>, and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> were purchased from Baker (Monterrey, México); sodium alginate was purchased from Sigma Aldrich Chemical Co. (India); and *Saccharomyces cerevisiae* yeast was obtained from Safmex S.A. de C.V. (Toluca City, Mexico).

### Methods

#### *Analytical methods*

Ash and moisture content were measured using the Official Methods of Analysis: Association of Official Analytical Chemists (A.O.A.C. 1998). Protein content was

determined by estimating the total nitrogen content using the Kjeldahl method (A.O.A.C. 1998) and multiplying by a conversion factor of 6.25. Pectin was determined by Sudhakar and Maini's method (2000).

The content of hemicellulose and cellulose contents were 144.6 and 119.3 g/kg, respectively, on a dry matter basis, and the lignin content was 21.7 g/kg. A soluble RS content of 211.2 g/kg, protein content of 50.92 g/kg, and pectin content of 189.4 g/kg were determined.

Reducing sugars (RS) were quantified using the Miller method (Miller 1959) with 3,5-dinitrosalicylic acid at 570 nm using a UV-Vis spectrophotometer Hach DR5000 (HACH, Mexico). Glucose, fructose, and xylose contents in hydrolysates were assayed using a HPLC system, equipped with MetaCarb 87C Guard Column (50x) (Varian Inc., Palo Alto, CA, USA). The elution was performed with Milli-Q water at a flow rate of 0.6 mL/min and 65 °C, and monitored by refractive index detector (Prostar 350, Varian Inc., Palo Alto, CA, USA). The presence of 5-hydroxymethyl-furfural (HMF) in the hydrolysates was quantified by HPLC analysis using Eclipse xD8-C18 column, eluted with 50 mM K<sub>2</sub>HPO<sub>4</sub> in methanol at a flow rate of 1 mL/min at 60 °C and monitored by a UV detector.

Ethanol was quantified using a gas chromatography system (CP3800, VARIAN, Mexico) equipped with a wax column (30 m x 0.25 mm, i.d. 0.5 µm). Oven conditions were: temperature 50 °C for 3 min; gradient from 50 to 180 °C at 15 °C/min; split ratio of 50 mL/min; injector and FID temperatures of 180 °C and 250 °C, respectively. The carrier gas used was helium at a flow of 1 mL/min.

### *Hydrolysis*

The OP was hydrolyzed in a Teflon® reactor and in an autoclave at 103.4 kPa and 120 °C for 15, 30, 45, or 60 min with 2.5 g of OP, 100 mL of deionized water, and 0.075 mL of H<sub>2</sub>SO<sub>4</sub>. For thermal hydrolysis, the effect of temperature, pressure, gas type, and OP load on RS yield was studied.

The temperature effect on RS yield was studied by adding 2.5 g of OP to 100 mL of deionized water into a stirred Parr reactor. Pressure was adjusted to 1000 kPa with N<sub>2</sub> gas and at a stirring rate of 350 rpm during 60 min. Samples were taken from the reactor after 15, 30, and 45 min and the temperature was set at 120, 140, 160, or 180 °C.

The pressure effect on RS yield was studied by adding 2.5 g of OP to 100 mL of deionized water into a stirred Parr reactor, and pressure was adjusted to 1000, 800, 600 or 500 kPa with N<sub>2</sub>, stirring rate of 350 rpm, and temperature of 160 °C for 30 min.

The OP load effect on RS yield was analyzed by varying the load from 2.5 to 7.5 g of OP with 100 mL of deionized water at 350 rpm, 160 °C, and 30 min.

Finally, the gas type effect on RS yield was determined by switching from N<sub>2</sub> to CO<sub>2</sub> to adjust the pressure to 1000 and 600 kPa into the reactor, and working with 2.5 g of OP, 100 mL of deionized water, 350 rpm, 160 °C, and 30 min. This change was done to improve the RS yield in the presence of CO<sub>2</sub> and carbonic acid at high temperature and pressure, and decreased pH of the solution, facilitating hydrolysis (Zhang and Wu 2014).

In all cases, reducing sugars, glucose, xylose, and fructose yields were determined. For experiments in which the temperature effect was studied, the solid residues were dried, weighted, and analyzed by infrared spectroscopy with an IR-ATR Bruker Tensor 27 (Bruker, Alemania). Also, glucose, fructose, xylose, furfural, and HMF contents were determined for hydrolysates obtained at different temperatures at 30 min, 600 kPa, and 350 rpm.

### Yeast immobilization and fermentation.

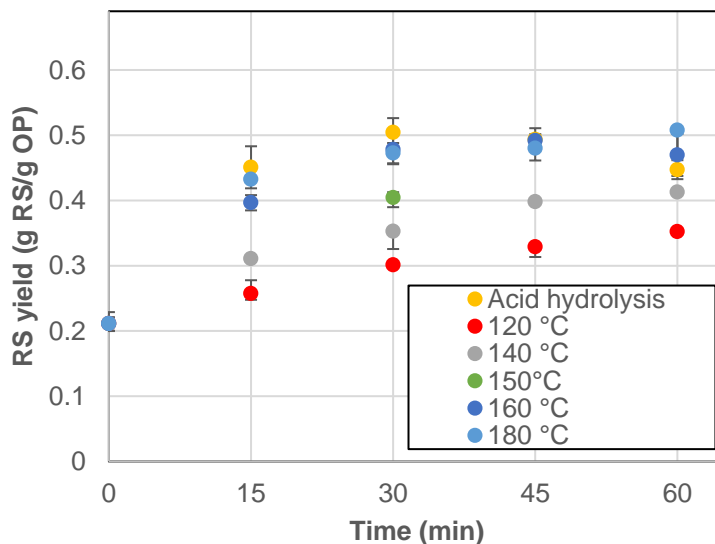
Growth medium was prepared with glucose (100 g/L),  $(\text{NH}_4)_2\text{SO}_4$  (2 g/L),  $\text{MgSO}_4$  (0.4 g/L), and  $\text{K}_2\text{HPO}_4$  (0.4 g/L). The pH 5 was then adjusted with 0.1 M HCl or NaOH solution. It was sterilized and then 0.015 g of yeast was added to 2.5 mL of this sterile solution growth medium. The resulting media was then transferred to an oven at 30 °C for 24 h for incubation. At the end of this time, it was incorporated into 7.5 mL of a solution of alginate prepared with 0.2 g of sodium alginate and water. The mixture was stirred until homogeneous solution was obtained and then was added dropwise into 5 mL of 0.1 M  $\text{CaCl}_2$  solution. This was then decanted to separate the alginate beads of 3 mm diameter size.

The OP hydrolysates were fermented to produce ethanol. For this purpose, the effect of immobilizing the yeast on the ethanol yield was evaluated first. To do so, fermentations with and without immobilized yeast were conducted. For the latter experiment, the hydrolysates obtained as described previously at 160 °C, 600 kPa, 350 rpm, and 30 min were reduced by heating to a concentration of 50 g/L of RS; pH was adjusted to 5 with 0.1 M HCl or NaOH solution, and then was sterilized. Approximately 25 mL of this solution was fermented with yeast immobilized on alginate beads prepared as described in previous section. The fermentation without immobilized yeast was done by mixing 2.5 mL of yeast solution resulting from incubation and 25 mL of sterile hydrolysate solution. Both experiments were performed at 30 °C during 48 h and a stirring rate of 150 rpm in closed flasks.

## RESULTS AND DISCUSSION

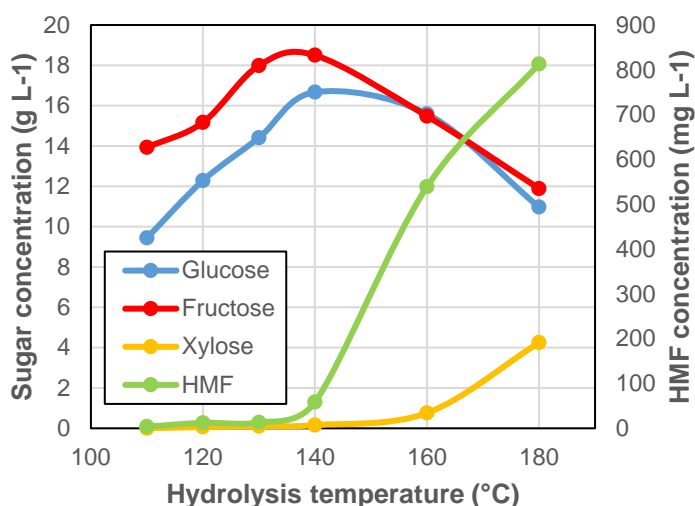
### Hydrolysis

The effect of temperature on RS yield was studied by treating OP at temperature range of 120 to 180 °C at 1000 kPa. In Fig. 1, it can be observed that increasing reaction temperature from 120 to 160 °C leads to higher yields of RS. Nevertheless, this effect is not remarkable above 160 °C and 30 min of reaction.



**Fig. 1.** Temperature effect on RS yield during thermal hydrolysis and comparison with acid hydrolysis

For reference purposes, the obtained results under acidic conditions are also included. It is worth noting that the maximum yield obtained under acidic conditions was practically the same with those at 160 °C and 180 °C. In the acid hydrolysis, the RS yield decreased after 30 min because of the degradation of sugars derived from the reaction into HMF or acids (Oberoi *et al.* 2010; Hu *et al.* 2013). Nevertheless, the increased glucose yield in hydrolysate resulted in a substantial increase in ethanol concentration, as *S. cerevisiae* is a hexose fermenting yeast. Although high temperatures produce higher RS yields than low temperatures, 130 °C was sufficient to produce a high glucose content in the hydrolysate, according to HPLC analysis (Fig. 2). Acid hydrolysis at temperatures higher than 130 °C resulted in production of inhibitory compounds like HMF formed *via* degradation of hexose sugars.



**Fig. 2.** Temperature effect on glucose, fructose, xylose, and HMF concentrations at the end of thermal hydrolysis (30 min, 600 kPa, and 350 rpm)

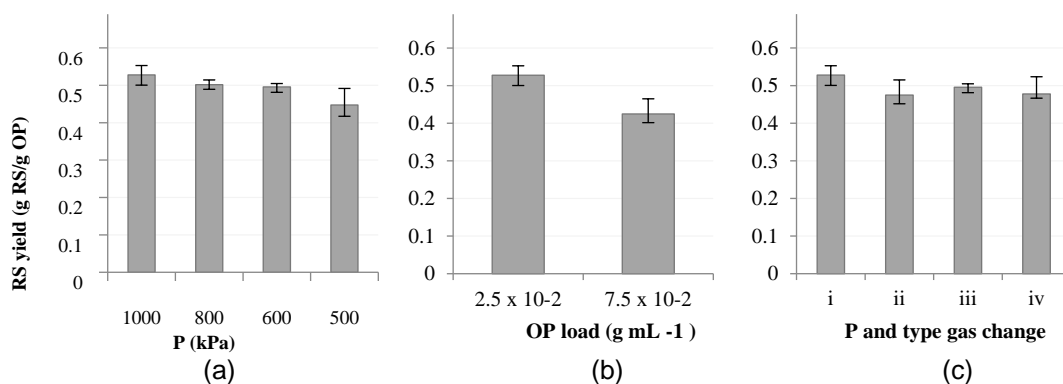
Furthermore, IR-ATR spectra of the solids were obtained before and after hydrolysis (spectra not shown). There was a change in the material's chemical structure with increasing *T*. At 120 and 140 °C, the band intensity at 1200 to 900  $\text{cm}^{-1}$ , attributed to a glycosidic structure, increased because of the breaking of the structure of the polymer to smaller units of oligosaccharides or glycosidic units. The C-O-C stretch and C-H bend peaks at 1105 and 1026  $\text{cm}^{-1}$ , respectively, became more prominent compared with the untreated OP. In contrast, the analysis of the solid obtained after hydrolysis at 160 and 180 °C showed that the intensities of the peaks corresponding to the O-H bond at 3300, 1450, 1344, and 1240  $\text{cm}^{-1}$  as well as the peak from the C-O-C bond at 1050  $\text{cm}^{-1}$ , decreased drastically. Those peaks at 1050, 2880, and 2950  $\text{cm}^{-1}$  that were still present were also observed in the IR-spectrum of the cellulose standard. Hence, at these *P* and *T* conditions, it was assumed that some cellulose, primarily the amorphous structure, was degraded, but not entirely. The results also revealed the interaction of hemicellulose and pectin, which was also solubilized according to the RS yield levels (Zakzeski *et al.* 2012).

Based on the above results, it can be understood that 160 °C and 30 min were the conditions under which cellulose was hydrolyzed. Therefore, the pressure effect was studied at this temperature.

Figure 3a presents the  $P$  effect on RS yield during the thermal hydrolysis at 160 °C for 30 min. The method currently being used to discriminate among the means was ANOVA procedure. According to this method, there were no statistically significant differences between 1000, 800, and 600 kPa with a confidence level of 95%.

When the  $P$  effect on sugar yield was studied for other biomass types, it resulted in higher RS yields when the pressure was increased. This was attributed to the enhanced penetrability of water into the inner part of biomass, crystallinity changes, particle size reduction, or structure damage occurring at high pressure (Castañón-Rodríguez *et al.* 2013; Jin *et al.* 2015).

In this case, the positive effect of pressure when operated at values higher than 500 kPa can be ascribed to the physical state changes and acidity of water. According to the thermodynamic water phase diagram, water changes from vapor (at pressure of 500 kPa and 160 °C) to liquid state (pressure higher than 500 kPa and 160 °C). Therefore, at 500 kPa, the contact between the OP and water was not favored, which negatively affected the hydrolysis reaction yield. In addition, the acidity constant of water is smaller at 500 kPa than when pressure was increased. Because there were no significant differences between 600 and 1000 kPa, a pressure of 600 kPa was chosen to analyze the effect of the other variables on the RS yield during hydrolysis of OP.



**Fig. 3.** Effect of a)  $P$  with  $2.5 \times 10^{-2}$  g/mL of OP, b) OP load at 1000 kPa, and c) Gas type with  $2.5 \times 10^{-2}$  g/mL of OP: i) 1000 kPa-N<sub>2</sub>, ii) 1000 kPa-CO<sub>2</sub>, iii) 600 kPa-N<sub>2</sub>, iv) 600 kPa-CO<sub>2</sub> on RS yield during thermal hydrolysis at 160 °C, 350 rpm, and 30 min

The effect of OP loading on RS yield was studied at two values,  $2.5 \times 10^{-2}$  and  $7.5 \times 10^{-2}$  g/mL. When the OP load was increased, RS yield decreased by 6% (Fig. 3b). Thermal hydrolysis reaction is based on H<sub>3</sub>O<sup>+</sup> ions from water, which react with the β-1,4-glycosidic bond of the polymer. Decreasing the ratio H<sub>2</sub>O/OP would result in smaller quantity of these ions and therefore reduced RS yield. Nevertheless, an ANOVA statistical analysis was applied, and it was concluded that there was no significant difference with changing OP loading, with a confidence level of 95%.

Two gases, CO<sub>2</sub> and N<sub>2</sub>, were used to conduct the hydrolysis reaction. CO<sub>2</sub> gas was considered for use to increase the acidity of solution and consequently to increase the RS yield. Results are shown in Fig. 3c. According to Fisher's least significant difference (LSD) procedure, there were no significant differences observed in this case. It was likely that at 160 °C and 600 or 1000 kPa, the amount of H<sub>3</sub>O<sup>+</sup> and H<sub>2</sub>CO<sub>3</sub> were not sufficient enough to improve the RS yield (Narayanawamy *et al.* 2011), unlike a previous report (Zhang and Wu 2014), where CO<sub>2</sub> positively affected glucose yield when using sugarcane bagasse as the raw material. In this case, the pH of the H<sub>2</sub>O - CO<sub>2</sub> mixture decreased from 4.1 with N<sub>2</sub>

to 3.9 with CO<sub>2</sub>. Hence, as reported by Rogalinski *et al.* (2008), the obtained results of RS yield were not surprising because a decrease in pH was not observed. It is worth pointing out that a pH value of 2.0 or less is caused by the addition of sulfuric acid when acid hydrolysis was applied.

## Fermentation

From the perspective of increasing ethanol yield, it is effective to immobilize yeast in alginate beads, as 80% ethanol yield (EY) (EY- based on theoretical ethanol yield from RS initial concentration) can be attained. This represents a noticeable effect, as only a 35.5% of EY was obtained without immobilized yeast. This can be ascribed to a greater tolerance to ethanol concentration in the medium and a higher concentration of yeast units inside the beads (Shuvashish and Chandra 2010; Lee *et al.* 2011). Therefore, the OP hydrolysates were fermented using yeast immobilized in alginate beads.

The OP hydrolysates were fermented to determine ethanol yield in g per L of hydrolysate and g of ethanol per g of OP. The results are shown in Table 1.

**Table 1.** Results of the Fermentation of OP Hydrolysates: Effect of the Hydrolysis Reaction Temperature ( $T_R$ ) on Ethanol Yield (EY) per Unit Substrate Consumed

Parameters	Pressure reactor conditions					Autoclave	Acid hydrolysis in autoclave
	120	130	140	160	180		
$T_R$ (°C)	120	130	140	160	180	120	120
$t_R$ (h)	0.5	0.5	0.5	0.5	0.5	0.5	0.5
$t_S$ (h)	0.816	0.85	0.883	0.95	1.016	0.75	0.75
Hydrolysis energy (kWh)	1.407	1.466	1.523	1.638	1.752	1.495	1.495
EY g/L	33.14	29.04	13.89	20.20	5.68	28.09	6.31
EY g per g OP	0.166	0.145	0.139	0.101	0.056	0.140	0.063
* $t_R$ reaction time $t_S$ total time in which reactor or autoclave were on							

There was no significant difference at 130, 140, or 120 °C in the autoclave, but at 120 °C in the reactor, there was, with a confidence level of 99% using Duncan's test. In addition, energy consumption at 120 °C in the pressure reactor was less than that in the autoclave. Heating time, fermentation time, and reactor size directly affect energy consumption. However, other factors, such as the cost of equipment, availability, feasibility of large-scale operation, useful life, and maintenance costs are also parameters requiring investigation. The addition of nutrients and reagents to adjust pH prior to fermentation and the type of microorganism also affect the overall process cost. The aim of this study was to analyze the effect of different variables on AR and ethanol yield. It was found that thermal hydrolysis at 130 °C produced more glucose and fructose, and inhibitors were present in a minimum concentration. The results of the fermentation confirm that the presence of inhibitors was an important factor. A temperature of 120 °C was found to be the most convenient because an ethanol production of 33.14 g/L of OP with 1.407 kWh consumed during hydrolysis was achieved. Grohmann *et al.* (1995), using an acid pretreatment and enzymatic hydrolysis with cellulolytic and pectinolytic enzymes, reported

an ethanol production of 38 g/L. Oberoi *et al.* (2010) reported a value of 50.55 g/L using a two-stage acid hydrolysis. Santi *et al.* (2014) achieved an ethanol yield of 15 g/L by acid pretreatment of citrus waste with a novel scaled reactor of steam injection at 180 °C and 150 s, and fermentation with an industrial *S. cerevisiae* strain. Pourbafrani *et al.* (2010) reported an ethanol yield of 0.037 g/L using diluted acid and enzymatic hydrolysis at 150 °C compared with 0.166 g/g reported in this study. Thus, it is important to highlight that the results obtained through the present study are promising because a value of ethanol yield among the above mentioned was obtained. Secondly, it has been shown that a *T* of 120 °C is sufficient to obtain the required reducing sugars to be fermented and no nutrients were added to hydrolysates prior to fermentation. Also, only one hydrolysis step was applied and fermentation was conducted with Baker's yeast immobilized on alginate beads, offering the option to reuse them.

## CONCLUSIONS

1. The RS yield was positively affected by hydrolysis temperature. However, the inhibitor concentration also increased. The glucose and fructose concentration were highest at 130 °C, and the ethanol yield was highest with thermal hydrolysis at 120 °C. The effects of pressure, OP loading, and gas type on RS yield were negligible.
2. Immobilization of yeast onto alginate beads increased the ethanol yield to 44.5%, no extra nutrients were needed, and ethanol production attained the maximum value of 33.14 g/L. Therefore, the proposed process represents a valid and encouraging alternative for the production of ethanol.

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