# Simultaneous Saccharification and Fermentation of Pine Sawdust (*Pinus pseudostrobus* L.) Pretreated with Nitric Acid and Sodium Hydroxide for Bioethanol Production

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With the purpose of taking advantage of pine sawdust residue coming from a sawmill located in Michoacán, México, a pretreatment with nitric acid and sodium hydroxide was performed. Also, the production of bioethanol by enzymatic hydrolysis was investigated. Using a response surface method, the intermediate points for the optimal $HNO_3$ concentration were determined. Results showed that using $HNO_3$ as a pretreatment leads to higher ethanol yields at an optimal concentration of 10.90% $HNO_3$. After a 30-min pretreatment with 10.90% $HNO_3$ at 114.32 °C, followed by 1% NaOH and enzymatic hydrolysis performed in shaker at a pH of 4.8 and 150 rpm for 72 h, with an enzyme loading of 25 FPU/g of total carbohydrates, the reducing sugars concentration was 99.2% (conversion of polysaccharides to monomers). On the other hand, the ethanol yield obtained from the simultaneous saccharification and fermentation treatment was 15.0 g/L, and the separate hydrolysis and fermentation was 17.1 g/L at a pH of 4.8 and 150 rpm with $1X10^7$ Cel/mL of *Saccharomyces cerevisiae* and an enzymatic loading of 25 FPU/g of total carbohydrates. When comparing the results obtained with literature data, it is concluded that this procedure is suitable to exploit the lignocellulosic wastes from the Indigenous Community of San Juan Nuevo Parangaricutiro, Michoacán, Mexico.

*Keywords: Reducing sugars; Enzymatic hydrolysis; Pine sawdust; Pinus pseudostrobus*

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## INTRODUCTION

A potential petroleum shortage in the near future, increased levels of atmospheric $CO_2$, global warming, and the pressing need to tap into readily renewable lignocellulosic material that is produced and wasted worldwide are all factors that have spurred the search for technologies that foster the production of alternative fuels for the transport sector.

A variety of studies to identify raw materials that are likely to be economically turned into ethanol of first, second, and third generation have been conducted, providing the development of new technologies that enable the use of these resources to the utmost. Some of the sources for first generation fuel production are of agricultural origin and are confirmed by the nutritional parts of plants, which have a high content of cellulose, sugars, and oils. Examples of these sources of ethanol include organic solid waste, animal fat, and residual oil and grease used in cooking and food preparation (Álvarez 2009).

Second-generation biofuels have further advantages, such as the fact that they do not compete directly with food production market and, because all the plant biomass above the ground can be used, it is possible to achieve better and more efficient land use. On the other hand, compared with first-generation fuels, second-generation fuels demand major investments (Stevens *et al.* 2004).

Third-generation fuels are produced from fast-growing non-food crops with high energetic density stored in their chemical components, called "energy crops," such as perennial grass, trees, fast-growing plants, and green and blue-green seaweed. The advantage of these biofuels is that they capture carbonic anhydride ($CO_2$) for growth, thus generating a positive greenhouse gas balance. Their disadvantage, with the exception of green seaweed, is that the growing of such energy crops would occupy farm land (Atev *et al.* 1983).

The most widely studied processes for wood biomass pretreatment include alkaline, acid, steam explosion, organosolv, and SPORL sulfite pretreatment (Zhu *et al.* 2009). Because the enzymatic procedure is limited by the presence of lignin and cellulose crystallinity (Millett *et al.* 1976; Åkerholm and Salmén 2001; McLean *et al.* 2002), several pretreatment procedures have been used to expose cellulose more easily. For instance, sodium hydroxide (NaOH) pretreatment breaks down lignin-carbohydrate bonds, partially withdraws lignin and hemicelluloses, opens the material structures, increases interface area, and reduces the polymerization degree and crystallinity (Tuor *et al.* 1995). Pretreating the biomass with organosolv pretreatment can effectively remove recalcitrant lignin from wood biomass, resulting in an enzymatic saccharification of cellulose (Pan *et al.* 2005, 2006; Pan 2008). Using $HNO_3$, which is a strong oxidizer pretreatment, results in a limited hydrolysis usually referred to as *prehydrolysis.* Prehydrolysis consists of hydrolyzing the hemicellulose fraction, while the cellulose and the lignin fractions remain with almost no alteration. Each pretreatment has specific characteristics that have been developed to maximize hydrolysis yields. The main advantage of a simultaneous saccharification and fermentation process is that it reduces the inhibition per final product that takes place in the two-stage operation because the presence of fermenting microorganisms and cellulose enzymes reduces sugar accumulation in the fermenter. The combination of both methods leads to higher rates of hydrolysis than the separate use of hydrolysis and fermentation processes, requiring a smaller amount of enzymes and obtaining higher ethanol yields (Ballesteros 2001).

The objective of this study was to optimize the pretreatment conditions with nitric acid and sodium hydroxide of pine sawdust (*Pinus pseudostrobus* L.). It has been found that the hemicellulose and lignin removal is enhanced with the consecutive use of these chemicals. Being the nitric acid a strong oxidizing agent, and the sodium hydroxide action removes part of the lignin and the remained hemicellulose after the acid pretreatment. Followed by a comparison between simultaneous saccharification and fermentation (SSF) and Separate Hydrolysis and Fermentation (SHF), to use it as raw material for bioethanol production.

## EXPERIMENTAL

### Materials

*Pinus pseudostrobus* sawdust from the Indigenous Community of San Juan Parangaricutiro, in the state of Michoacán, Mexico, was used for this study. The sawdust was dried at 80 °C for 24 h in a Novatech electric furnace (Mexico, Tlaquepaque, Jal.) and then partially reduced in size using a generic blade mill at 1500 W to obtain heterogeneous fibers. The fibers were then sieved using a 20-mesh screen (841 μm) and retained on a 40-mesh screen.

The chemical composition of this biomaterial was determined and reported in a previous work (Farías 2014). Commercial cellulase enzyme preparations of *Trichoderma reesei*, including Celluclast 1.5 L by Sigma Aldrich, were used in this study. This enzyme preparation can be used to break down cellulose into glucose as shown in the page of manufacturer. *Saccharomyces cerevisiae* strain (Instituto Tecnológico de Durango, ITD-00185, Mexico).

### Chemical Composition of Pretreated Material

The chemical features of the pretreated material determined in this study were moisture content, that was determined by Technical Association of the Pulp & Paper Industry (TAPPI) TAPPI T 210 cm-93 (1993), and ash content by TAPPI T 211 om–93 (1993). The total amount of extractive substances was determined by a sequence of Soxhlet extractions with cyclohexane, acetone, methanol, and water. Lignin by TAPPI T 222 om-88 (1998), holocellulose (Wise *et al.* 1946), and alpha cellulose by ASTM D 1103-60 (1978) contents were determined in the procedure for wood meal without extractable substances.

### Combined Pretreatment Preliminary

To evaluate the sugar yield (the response variable), a $2^3$ factorial design was performed, determining the following factors and levels: $HNO_3$ concentration (3% and 6%), temperature (50 and 100 °C), and time (30 and 60 min). In each experiment, 10 g of dry sawdust was placed in a 250-mL round-bottom flask followed by the addition of 60 mL of $HNO_3$ solution. The flask was then connected to a condenser, and the mixture was boiled for 30 min under reflux in an oil bath kept at a specific temperature. Next, the mixture was filtered and washed with 500 mL of distilled water (each experiment was done in triplicate). Finally, the three experiments was dried to 100 °C for yield determination.

The dry solids of the experiment with $HNO_3$ was put into a 250-mL round-bottom flask, followed by the addition of 120 mL of 1% NaOH solution. The residue was heated again for 30 min at reflux by means of an oil bath at 90 °C. After the chemical reactions took place, the raw material was filtered, washed with 500 mL of distilled water, and dried to 100 °C for yield determination.

### Enzymatic Hydrolysis

Samples of 1.0 g of the pretreated material ($HNO_3$, followed by NaOH) were placed into 20-mL plastic containers and mixed with 2 mL of 1% m/v sodium azide and Celluclast enzyme cocktail 1.5 L (25 FPU/g of total carbohydrates). The reaction volume was then adjusted to 10 mL using 0.1 M acetate buffer solution of a pH 4.5. All samples were incubated under enzymatic hydrolysis at 48 °C and 150 rpm.

## Optimization of the Pretreatment

A compound central design with three intermediate points was used to optimize the process and, at this point, the factors and levels were as follows: $HNO_3$ concentration (6.5% and 12%), temperature (100 and 130 °C), and time (constant time of 30 min). The content of reducing sugars in the hydrolyzed pine sawdust was the response variable in this study, was determined using the Ghose method (1987).

## Separate Hydrolysis and Fermentation (SHF)

This process was carried out with the strain *Saccharomyces cerevisiae* ITD-00185, for which the fermentation process was kept in YPG (yeast, peptone, glucose) medium at pH 5.5. Once the hydrolysis process was optimized, the fermentation of the hydrolyzates obtained with the optimal procedure was carried out.

The mixture was centrifuged at 8000 rpm for 5 min to separate the residual solids. Then, yeast extract (10 g/L) and peptone (5 g/L) were added to the centrifuged liquid. Finally, a *Saccharomyces cerevisiae* strain with a microbial charge of $1 \times 10^7$ was inoculated (the proportion of microbial culture fluid added was approximately 2% of the total volume of the fermentation).

The inoculated medium was incubated at 30 °C for 72 h, taking samples every 8 h. The ethanol content was determined by a high-performance liquid chromatography (HPLC), using a Metacarb 87 C (Agilent Technologies, Santa Clara, CA) device at 65 °C, with an isocratic flux of water and a refractive index detector at 50 °C. The working pressure was 58 bar. The reducing sugars content was determined using the Ghose method (1987).

## Simultaneous Saccharification and Fermentation (SSF)

Each experiment consisted of 2 g of the pretreated material (resulting from the optimal conditions) placed in 50-mL Erlenmeyer-flasks, and then shaken. Afterwards the contents were mixed with 15 mL of YP growing medium (10 g/L yeast extract and 5 g/L peptone). Then the final reaction volume was adjusted to 20 mL with a 0.05 M acetate buffer of pH 5.0.

The mixture was sterilized at 120 °C for 15 min and then inoculated with a 2% v/v of a culture fluid containing $1 \times 10^7$ Cel/mL of *S. cerevisiae* and 25 FPU/g of enzymatic cellulase solution (Celluclast 1.5 L, an enzyme preparation of *Trichoderma reesei*). The whole of the mixture was incubated to 150 rpm at 35 °C for 72 h, taking samples every 8 h, under aerobic conditions. In this case the temperature needs to be higher in order to allow the enzymatic activity without decrease the growing of the strain. For this reason, one can consider that this is the compromise variable. The ethanol content was determined by HPLC at 65 °C, with an isocratic flux of water and a refractive index detector at 50 °C. The working pressure was 58 bar.

## Statistical Analysis

A $2^3$ factorial design was performed, and with the results of this was done a compound central design the following factors and levels: $HNO_3$ concentration (9.25% and 12%), and temperature (100 and 130 °C). All the experiments were performed by triplicate, and the obtained data were analyzed with 95% statistical confidence using STATGRAPHICS Centurion XVI version 16.1.18 software (Warrenton, Virginia).

## RESULTS AND DISCUSSION

### Chemical Composition of Pretreated Material

The chemical composition of *Pinus pseudostrobus* sawdust was determined and reported in a previous work (Farías 2014). The chemical analysis results (Table 1) of the pretreated sawdust indicated a 5.67% lignin content and, as expected, a higher content of polysaccharides. It follows that approximately 81% of the lignin was eliminated by the pretreatment performed.

**Table 1.** Chemical Composition of the Pretreated Sawdust and Residues of the Hydrolyzed Material (%)

| | Sawdust ** | Pretreated sawdust |
|---|---|---|
| Ash | 0.19±0.06 | 0,18±0.04 |
| Extractives | 5.11±0.32 | - |
| Lignin | 28.94±0.17 | 5,67±0.18 |
| *Holocellulose | 66.53±0.85 | 94,5±0.77 |
| Hemicellulose | 23.55±0.85 | 28,74±0.77 |
| α-cellulose | 42.98±4.96 | 65,76±1.37 |
| | 100.77±1.4 | 100,35±2.36 |

* Holocellulose is made up of α-cellulose and hemicellulose content.
**Farías-Sánchez *et al.* (2015)

### Combined Pretreatment Preliminary

According to the $2^3$ experimental design applied in this study, it was observed that the pretreatment time did not significantly influence the hydrolysis process yield ($p$ = 0.8167), while the acid concentration and temperature of the process did influence the sugar yield significantly ($p < 0.0001$). It can be concluded that the best yield was obtained under the pretreatment conditions of 6% nitric acid at 100 °C, followed by 1% NaOH at 90 °C, as shown by the means graphs (Figs. 1 and 2). As time did not influence the yield significantly, it was decided to keep the time factor constant at 30 min for the composed central design.

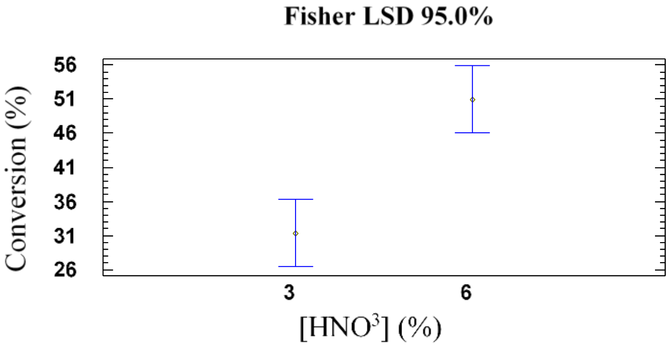

**Fig. 1.** Means of $2^3$ experimental design for acid concentration factor at 100°C

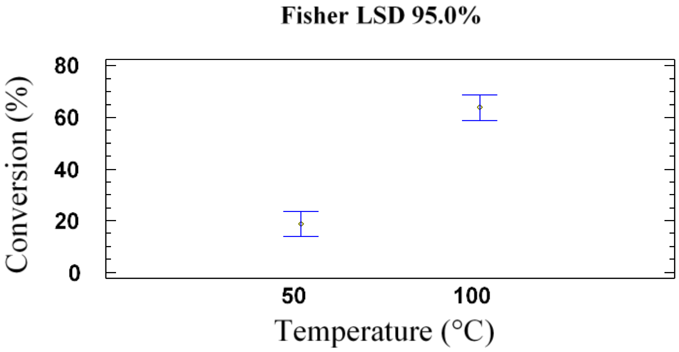

**Fig. 2.** Means of $2^3$ experimental design for temperature factor with 6% of nitric acid

## Pretreatment Optimization

Table 2 shows the optimal conditions and results obtained when applying compound central design. The conversion column indicates the amount of reducing sugars obtained by the hydrolysis with respect to the total amount of cellulose in the pretreated material. The analysis of variance performed indicates that the acid concentration ($p$ = 0.0315) and the temperature ($p$ = 0.0013) had significant effects on the yield. The average values of each experiment are shown in Table 2. The highest yield (98.61 ± 2.12%) was obtained at an acid concentration of 9.25%, temperature of 115 °C (Table 2), 30 min of pretreatment. The experimental procedure and material balance are shown in Fig. 3.

**Table 2.** Average Values of the Compound Central Design Matrix

| Run | [$HNO_3$] (%) | Temperature (°C) | Conversion (%) |
|---|---|---|---|
| 1 | 9.25 | 94 | 70.63 ± 1.80 |
| 2 | 6.25 | 100 | 73.71 ± 2.21 |
| 3 | 12.00 | 100 | 97.23 ± 1.94 |
| 4 | 5.36 | 115 | 93.67 ± 1.80 |
| 5* | 9.25 | 115 | 98.61 ± 2.12 |
| 6 | 13.13 | 115 | 92.93 ± 1.71 |
| 7 | 6.5 | 130 | 92.93 ± 1.92 |
| 8 | 12.00 | 130 | 87.23 ± 2.03 |
| 9 | 9.25 | 136 | 83.75 ± 1.82 |
| * This analysis was repeated six times, according to the compound central design. | | | |

The optimization of the acid treatment using the response surface method showed that the optimal conditions were as follows: pretreatment time of 30 min, $HNO_3$ concentration of 10.90%, pretreatment temperature of 114.32 °C, and hydrolysis time of 72 h. The graph of the response surface results is shown in Fig. 4.

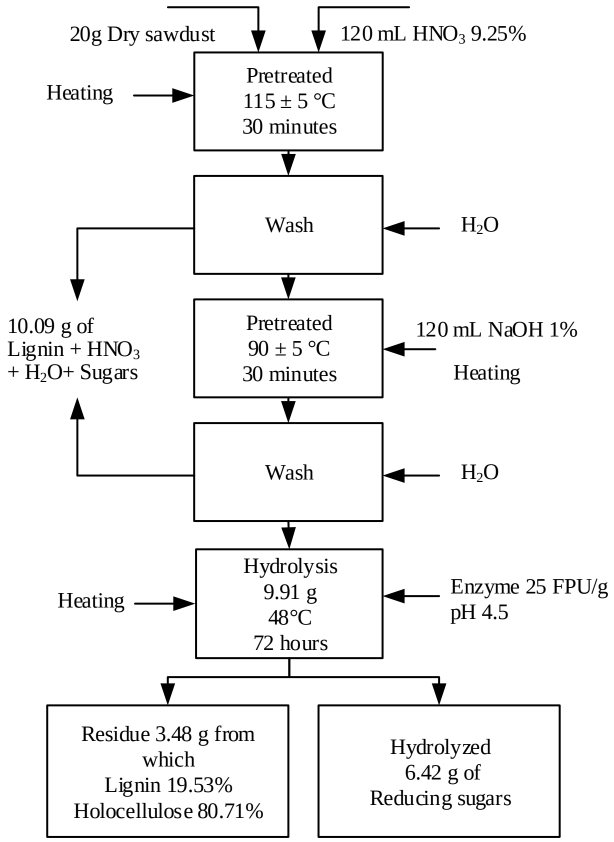

**Fig. 3.** Material balance and process flow diagram

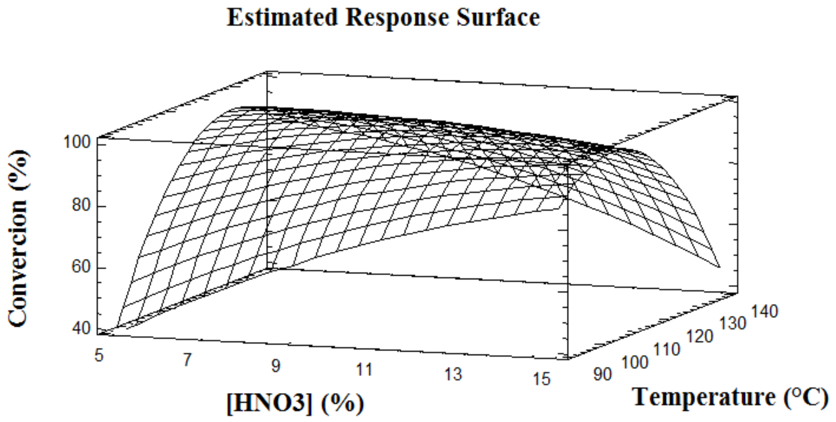

**Fig. 4.** Compound central design of pretreatment conditions optimization

Figure 4 shows the behavior of experiments. This was done by taking the data of Table 2, the columns [HNO3] (%) *vs.* Temperature (° C) *vs.* Conversion (%) of each of the experiments, which is given by the STATGRAPHICS Centurion XVI software version 1.16.18 (Warrenton, Virginia). Results can be expressed as Eq. 1, for which the coefficients, based on data regression for reducing sugar concentration, are given in Table 3.

**Table 3.** Regression Coefficients for [Conversion]

| Coefficient | Estimated |
|---|---|
| Constant | -738.52 |
| A: [$HNO_3$] | 26.065 |
| B: Temperature | 12.1695 |
| AA | -0.267105 |
| AB | -0.177061 |
| BB | -0.0447837 |

$$Y\,(\%) = Constant + A * X_1 + B * X_2 - A * X_1^2 + AB * X_1 * X_2 + BB * X_2^2 \qquad (1)$$

In Eq. 1, *Y* is the reducing sugar concentration (%), $X_1$ is [$HNO_3$] (%), and $X_2$ is temperature (°C) and constants A, B, AB, and BB are replaced by the values given by the column "Estimated".

Entering the optimized conditions for $X_1$ as acid concentration of 10.90% and $X_2$ as temperature of 114.32 °C into Eq. 1 provides a reducing sugar yield of 99.15% with relation to the total cellulose contained in the pretreated biomass. These results might lead to the development of a technology to exploit pine sawdust for the generation of an alternative fuel for the automotive industry and other value-added products.

## Separate Hydrolysis and Fermentation (SHF)

The numerical results obtained from the separate treatments of hydrolysis and fermentation are shown in Fig. 5, which is a plot based on these results.

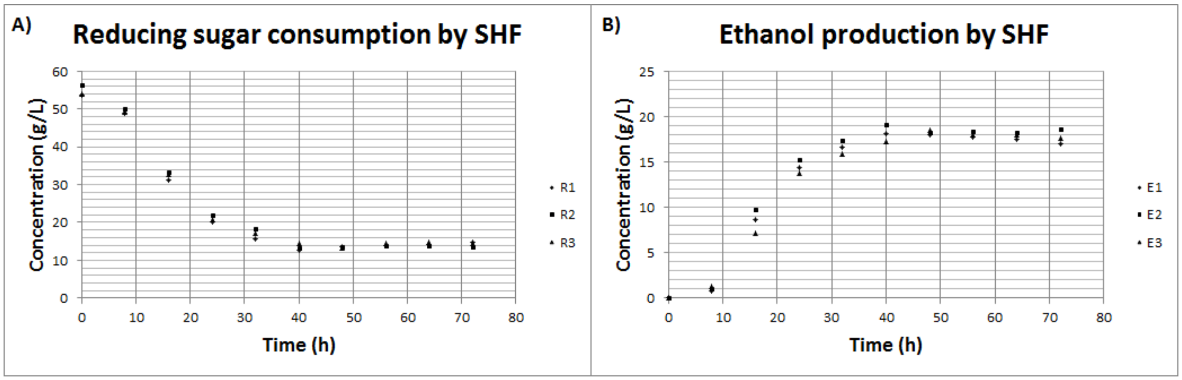

**Fig. 5**. Kinetics data for separate hydrolysis and fermentation treatments

The maximum production of ethanol was 17.01 g/L, at 40 h. This result is equal to 88% of the theoretical conversion of the alcoholic fermentation process. Also, after 40 h, the reducing sugar concentration did not show any noticeable change. Therefore, the residual sugar could be xylose, as the *S. cerevisiae* strain used in this study cannot ferment xylose.

## Simultaneous Saccharification and Fermentation (SSF)

The numerical results for the SSF treatment are shown in Fig. 6.

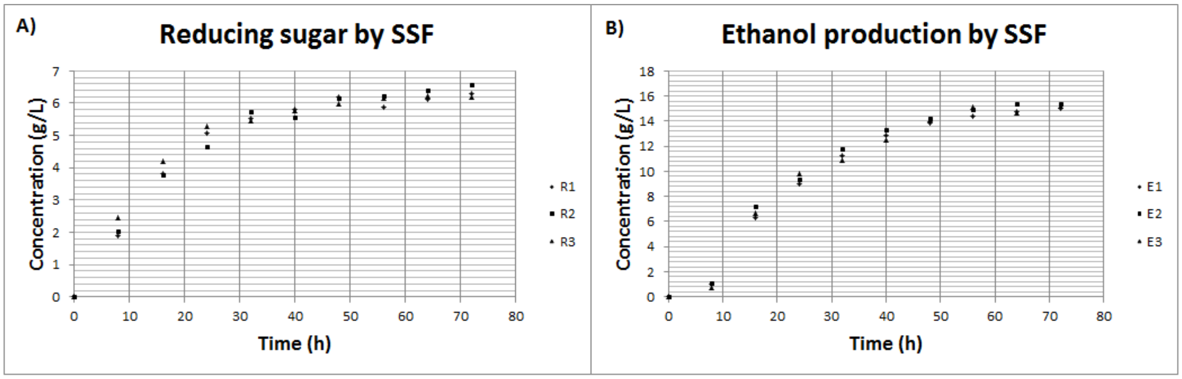

**Fig. 6.** Kinetics data for simultaneous saccharification and fermentation

The aim of the integrated hydrolysis and fermentation treatment was to improve the yield of ethanol. This is not supported by the results shown in Figs. 5 and 6, for which the concentration of ethanol was almost the same in both process configurations. In the SSF process there is not a high concentration of glucose at any time, and for this reason the ethanol production was lower than the SHF process. On the other hand, during SHF process the hydrolysis continues until the fermentation stage. So, the pentose concentration can be greater than that in the SSF process, which explains the higher value of residual reducing sugars in SHF.

For both SSF and SHF, the equivalent of 537.2 kg of reducing sugars per ton of pretreated material was transformed, resulting in 235.3 L of ethanol in the SHF and 167.0 L of ethanol in the SSF.

The results obtained in this research were relatively high when compared with the results reported in other works. Tang *et al.* (2013) obtained from corn starch a ratio of 86.9% by a polysaccharide utilization with commercial thermostable α-amylase and amyloglucosidase for saccharification and *Streptococcus thermophiles*, *Lactobacillus bulgaricus*, and *Saccharomyces cerevisiae* for SSF. Luo *et al.* (2010) obtained 69% of the theoretical ethanol yield with the SPORL procedure. In their work, these authors used 2.21% sulfuric acid gas charges and 8% sodium bisulfite on oven-dried wood, and an enzymatic load composed of Celluclast 1.5 L (15 FPU/g substrate) and Novozyme 188 (22.5 CBU/g substrate). Ballesteros *et al.* (2004) treated wheat straw and obtained an ethanol concentration of 18.1g/L and a yield of 62.5%. Vázquez-Ortega (2013) worked with bean straw and obtained an ethanol concentration of 10.50 g/L and a yield of 50.59%. Saucedo-Luna *et al.* (2010) used 2% sulfuric acid at 151 °C and a reaction time of 10 min, obtaining a yield of 48.5% and, when using *Agave tequilana* bagasse as the raw material, the ethanol concentration was 22.02 g/L. Nevertheless, the results in the present work are slightly lower than the data reported by Shuai *et al.* (2010), who reported a 77.7% yield at 180 °C using steam explosion for 30 min and a 5% sulfuric acid load on oven-dried spruce wood with a 1:5 wood-liquor ratio and an enzymatic load of 15 FPU. Velázquez-Valadez *et al.* (2016) worked with *Tequilana weber* agave bagasse using 6% w/w sodium hydroxide solution, 6% w/w hydrogen peroxide solution, 6% Cellic CTec3, and 6% Cellic HTec3 and obtained a concentration of 165.67 g/L of reducing sugars and 84.49 g/L of theoretical ethanol yield.

## CONCLUSIONS

1. Optimization of acid treatment using the response surface method showed that the optimal conditions were: pretreatment time, 30 min; $HNO_3$ concentration, 10.9%; pretreatment temperature, 114.8 °C; and hydrolysis time, 72 h. Under these conditions, a 99.2% yield of reducing sugars was obtained with respect to the cellulose contained in the pretreated biomass.
2. A higher yield of fermenting sugar was observed with the use of nitric acid and sodium hydroxide as well as a shorter fermentation time of solids when compared with previous pretreatment conditions and to sawdust without any pretreatment. It was also observed that the hydrolysis efficiency with nitric acid pretreatment was even higher than that of untreated cellulose.
3. Results suggest that combined pretreatment of the sawdust withdraws lignin and part of the hemicelluloses, making the conversion of crystalline cellulose into amorphous cellulose easier. Therefore, the combined pretreatment aids the enzymes to increase the final yield by means of a more efficient hydrolysis.
4. Finally, based on the calculated yields obtained in this research work, it may be concluded that for each ton of sawdust processed, 235.3 L of ethanol can be produced.
5. To sum up, sawdust (a potentially useful wood waste) can be used for the production of reducing sugars, and thus, for bioethanol production.

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