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Alkaline pre-treatment and hydrolysis with acid cellulase of the rice husk (*Oryza sativa*) for production of bioethanol

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Abstract. Several companies generate large amounts of agroindustrial waste, which could be used as raw material for the production of bioethanol; among them, those that process and commercialize rice, which produce a large amount of husk, a lignocellulosic material, as a by-product. These companies generally use rice husk as fuel for the ovens, generating problems to the environment due to the emission of polluting smoke. The objective of this work was to obtain bioethanol from rice husk, taking advantage of its cellulose and hemicellulose content. The lignin was removed by an alkaline pretreatment with 2.00% w/v sodium hydroxide solution. Cellulose and hemicellulose in the husk were hydrolysed by enzymatic hydrolysis with acid cellulase for 72 hours. During this period of time, the concentration of total reducing sugars was monitored by the 3,5-dinitrosalicylic acid method, in order to determine the reaction time in which the highest concentration of total reducing sugars is obtained. The sugars obtained were fermented with *Saccharomyces cerevisiae*, for the production of alcohol. During the enzymatic hydrolysis of the pre-treated rice husk, it was found that the highest concentration of total reducing sugars in the samples was 7.778 g/L, obtained in a time of 24 hours of hydrolysis. The sugars obtained were fermented with *Saccharomyces cerevisiae*, for alcohol production. A distillate was obtained with a concentration of 3.7% v/v.

1. Introduction

The treatment of solid waste that produces environmental pollution is one of the most important issues today; since they should look for suitable forms for its handle and final disposal, of such way that do not affect the environment. At present, organic materials with a high content of lignin and cellulose are no longer considered waste by-products, but have become the raw material of multiple industrial processes such as the production of fuel alcohol, although many complications occur when obtaining ethanol from lignocellulosic tissues that complicate the degradation of biomass [1]. With the passage of time, the incorporation of lignocellulosic materials in the elaboration of new products, is a trend that gains more strength, since products similar to those coming from commercial raw materials are obtained, and which are also friendly to the environment [2]. The lignocellulosic residues generated in the processing of rice, such as husk and chaff, which is the waste left in the collection site, are considered materials of little value and in some cases are a waste.

It is important to highlight the economic and social influence of rice cultivation in Colombia, since more than 28,000 farmers and their families directly depend on it. Rice cultivation is one of the few short-cycle crops that has remained stable despite the variability of Colombian agriculture [3]. The annual production of rice is more than two million tons, generating as a by product around 400000 tons of husk. In Colombia, the burning of this waste is usually a quick strategy for its elimination.



However, the negative environmental impact that is generated must not be ignored [4], due to the fact that, during the combustion process of the rice husk (RH), high levels of greenhouse gases are emitted [5].

RH has a high content of polysaccharides such as cellulose and hemicellulose, so this is considered a valuable resource in the production of sugars that can be fermented [4]. Because the national production of rice is considerable, large quantities of husk are obtained that currently have little use, therefore, it is important to find other applications, in addition to their use as fuel for the ovens, among them, their use as raw material for the production of sugars and its subsequent fermentation and distillation to obtain bioethanol.

The literature reports obtaining bioethanol from RH using *Kluyveromyces marcianus* CK8 [5]. With respect to the use of the waste generated during the production of rice, it is found the use of these residues to obtain sugars [6,7], obtaining a carbonated alcohol beverage [4] and obtaining lactic acid. [8], among others.

In San José de Cúcuta, Norte de Santander, Colombia there are several companies that process and market rice, among them Coagronorte Ltda, Arrocería Agua Blanca S.A. and Arrocería Gelvez S.A.S., among others, which generate as a by-product a large amount of husk. These companies generally use the RH as fuel for the ovens, generating problems to the environment due to the emission of polluting smoke. With this it is inferred that these companies have no established plans to use or adequately dispose of the large quantities of husk that are produced. For this reason, the proposal was made to produce bioethanol from this agroindustrial waste, taking advantage of its content of cellulose and hemicellulose, which are broken down into sugars through enzymatic hydrolysis with acid cellulase (CFB3S) and subsequent fermentation of sugars obtained for the production of alcohol.

2. Materials and methods

2.1. Sampling of rice husk

The sample of RH was supplied by the company Coagronorte Ltda. located at km 8 via to the Zulia of San José de Cúcuta. The sampling point was the pressing section of the husk, the sample was taken under the hopper, where the husk is emptied to be pressed.

2.2. Pretreatment of the sample

This stage was carried out in a Bioflo®/celliGen®115 Fermenter and Bioreactor with a maximum capacity of 5.0 L. For pre-treatment of the RH, three batches of 300.0 g of husk were weighed, each of which was mixed with 4.0 L of NaOH (Merck) solution at 2.00% w/v for two hours, the mixture was made at 70°C and 400 rpm, the method was modified in terms of temperature (85 °C) compared to what was reported by [6,7], because the maximum temperature reached by the Bioflo®/celliGen®115 is 70 °C. At the end of the process, the three batches of pre-treated husk and the mixture was washed with tap water repeatedly (12 times), was left dried for three days at room temperature, was ground in a manual disc mill and was sifted to 16 mesh.

2.3. Enzymatic hydrolysis

To carry out the enzymatic hydrolysis process, exact quantities around 10.00 g of pretreated RH were measured, these were placed in 250 ml Erlenmeyer flasks with cotton stoppers, to which were added sodium citrate buffer solution (pH 5.00), in a ratio of 10 g of RH pretreated by 100 ml of buffer [9]. The Erlenmeyer flasks were placed in a multipurpose digital shaker Orbit™ 1000, with 120 rpm shaking, this assembly was taken to a drying oven at 55 °C and when the temperature was reached, 2.5 ml volumes of acid cellulase enzyme (CFB3S) were added, the enzymatic treatment was carried out in triplicate. During the hydrolysis process, the concentration of total reducing sugars (TRS) was monitored at time intervals of 0.5, 24, 48 and 72 hours, in order to establish the adequate time for enzymatic hydrolysis.

2.4. Analysis of total reducing sugars by the dinitrosalicylic acid method

For the analysis of the TRS in the samples obtained during the process of enzymatic hydrolysis on a laboratory scale, 1.00 ml of the supernatant of each erlenmeyer was collected at times of 0.5, 5, 24, 48 and 72 hours, which were transferred to plastic vials of 1.5 ml and allowed to decant for 10 minutes for further analysis by the dinitrosalicylic acid (DNS) method (3,5-dinitrosalicylic acid). The analysis of the samples was carried out in the Dinko 2300 II spectrophotometer at a wavelength of 540 nm. The conditions for the enzymatic hydrolysis were set according to the technical sheet of the acid cellulase enzyme (CFB3S), supplied by Sunson Industry Group Co. Ltd., where they establish the optimum pH range of 4.50-5.50 and the optimal temperature between 45 and 65 °C, so the average value of the pH and temperature ranges, respectively, was used.

2.5. Fermentation of glucosed syrups

The fermentation of the glucosed syrups obtained with the enzymatic hydrolysis was carried out in two stages, first the reproduction of the yeast *Saccharomyces cerevisiae*, in order to obtain the greatest amount of biomass possible, this stage goes until the obtaining of acetaldehyde and the carbon dioxide liberation; and then alcoholic fermentation, in which acetaldehyde is reduced by the action of dehydrogenase to ethyl alcohol [10].

The reproduction stage of the biomass is very important because if the fermentation starts too early, the population will not be large enough to obtain a good conversion rate to ethanol [11]. For this reason, oxygen was supplied to the syrups obtained in the enzymatic hydrolysis, by agitation of 200 rpm, and the cell count was performed by Neubauer chamber using a Leica DM500 microscope, to plot a growth curve of *Saccharomyces cerevisiae*, in order to establish the approximate time in which biomass production ends. On the other hand, to determine the approximate time of the alcoholic fermentation, the syrups were left to rest without agitation; during this period, a daily measurement of the Brix degrees was made to the samples using an automatic digital refractometer ATAGO RX-007 α , and at the time they remained stable, the time of the alcoholic fermentation was determined.

2.6. Preparation of glucosed syrups to obtain alcohol

Once the adequate conditions were established, both to obtain the highest concentration of TRS during the enzymatic hydrolysis and the time required to carry out the alcoholic fermentation, the syrups were again prepared in triplicate starting from three samples of 30.00 g of RH pretreated with NaOH 2.00% w/v, mixed with 300 ml of sodium citrate buffer solution pH 5.00 and 7.5 ml of acid cellulase enzyme (CFB3S). After the time previously determined for enzymatic hydrolysis, the glucosed syrups were filtered. Subsequently, each syrup was added 0.7500 g of urea and 0.7500 g of ammonium hydrogen phosphate, to ensure the presence of sufficient nitrogen and phosphorus for the metabolism of the yeast [12].

Finally, the syrups were sterilized in an autoclave at 120 °C and 15 psi for 20 minutes, in this way the syrups free of microorganisms that could alter the fermentation process were obtained. Three inocula of yeast (*Saccharomyces cerevisiae*) active commercial Levapan mark were prepared, mixing in each 0.3000 g in 10.00 ml of distilled water (40-50 °C) previously sterilized, since the concentration of yeast in each syrup should be 1 g/L [12]. The cell concentration of the inocula was determined by means of the cell count by Neubauer chamber, later each inoculum of yeast was added to the respective 300 ml of syrup. After the yeast reproduction stage, the syrups were left to rest for several days in a tray with water at 28 °C \pm 2 °C, making a daily measurement of the Brix degrees, when these stabilized the alcoholic fermentation was finished. The syrups were shaken in a shaker at 200 rpm to start the yeast reproduction stage and perform the cell count by Neubauer chamber. A curve of growth of *Saccharomyces cerevisiae* was plotted, in order to establish the time of reproduction of the yeast, for this measurements were made every hour during 12 hours and some at 24 hours, taking samples of 1.00 ml of the syrups which were diluted to 10⁻¹, to perform the count under the microscope on the 40X objective. After the stage of reproduction of *Saccharomyces cerevisiae*, the syrups were left at rest for several days in a tray with water at 28 °C \pm 2 °C, the Brix degrees were measured daily using

an automatic digital refractometer ATAGO RX-007 α , and when these remained established the alcoholic fermentation was finished.

2.7. Distillation of alcohol

After the alcoholic fermentation, the alcohol was obtained by simple distillation. The alcohol content expressed in %v/v was measured with the METER TOLEDO Densito30PX densitometer.

3. Results

3.1. Pretreatment of the sample

For the chemical pretreatment, a RH sample of 300.00 g was taken, after the pretreatment process, drying to the environment, grinding and sieving, a sample quantity of 129.71 g was obtained. According to these results, the performance of the process was 43.24%.

3.2. Analysis of total reducing sugars by the DNS method

The monitoring of TRS concentration (g/L) by the DNS method during enzymatic hydrolysis was performed at times of 0.5, 5, 24, 48 and 72 hours. Figure 1 shows a curve of TRS concentration as a function of time.

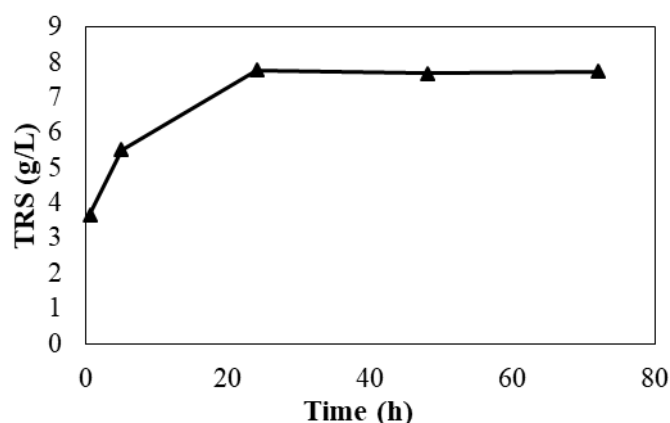


Figure 1. Concentration of TRS respect to time during enzymatic hydrolysis.

According to the results shown in Figure 1, the highest concentration of TRS in g/L in the samples hydrolysed enzymatically, was obtained in a time of 24 hours of hydrolysis (7.778 g TRS/L) and from this time the concentration tended to remain stable.

3.3. Fermentation of glucosed syrups

The fermentation stage was developed with the glucosed syrups obtained by the treatment of 2.5 ml enzyme per 10.00 g of RH. For this, three yeast inocula (*Saccharomyces cerevisiae*) were prepared so that their concentration in each syrup was 1 g/L. The cell concentration of each inoculum was determined by Neubauer chamber using the Leica DM500 microscope, with an average count of 2.62×10^9 cells/ml. This concentration of yeast was added to each of the glucose syrups, a cell count was performed every hour for 12 hours and at 24 hours, in order to estimate the time in which the yeast stopped reproducing, to establish the time of yeast reproduction. Table 1 shows the results of the cell/ml concentration of *Saccharomyces cerevisiae*.

According to Table 1, the concentration of *Saccharomyces cerevisiae* in glucosed syrups, initially (zero hours) is 1.92×10^7 cells/ml, it is observed that after one hour it increases to 2.39×10^7 cells/ml, and at six hours reached its maximum growth, with a cell concentration of 3.65×10^7 cells/ml, so it was

established that the stage of reproduction of the yeast, should be performed for six hours, therefore, the syrups should be in shaking at 200 rpm during this time.

Table 1. Concentration of *Saccharomyces cerevisiae* /ml cells with respect to time.

Time (h)	Concentration (cells/ml)
0	1.92x10 ⁷
1	2.39 x10 ⁷
2	2.38 x10 ⁷
3	2.24 x10 ⁷
4	2.32 x10 ⁷
5	2.79 x10 ⁷
6	3.65 x10 ⁷
7	3.67 x10 ⁷
8	3.50 x10 ⁷
9	3.46 x10 ⁷
10	3.55 x10 ⁷
11	3.62 x10 ⁷
12	3.68 x10 ⁷
24	3.51 x10 ⁷

After the aerobic stage, the glucose syrups were left to rest without agitation and the Brix degrees were measured daily for 7 days, in order to observe when they stabilized, in order to establish the time of the resting stage in the fermentation, to finally proceed to the alcohol distillation stage. According to the results shown in Table 2, the Brix degrees tend to remain stable after six days of rest, so this time, which is necessary to carry out the alcoholic fermentation stage, was established.

Table 2. Brix grades measured during alcoholic fermentation.

Time (days)	Brix Degrees				Std.
	(R ₁)	(R ₂)	(R ₃)	Average	
0	6.831	6.830	6.827	6.829	0.002
1	5.283	5.288	5.284	5.285	0.003
2	4.320	4.315	4.317	4.317	0.002
3	3.720	3.729	3.728	3.726	0.005
4	3.120	3.128	3.123	3.124	0.004
5	2.920	2.923	2.914	2.919	0.004
6	2.415	2.409	2.411	2.412	0.003
7	2.411	2.408	2.410	2.410	0.002

According to the results shown in Table 2, the Brix degrees tend to remain stable after six days of rest, so it was established this time, the necessary for carry out the alcoholic fermentation stage. After the alcoholic fermentation, the musts were filtered and by simple distillation volumes of 50 ml of distillate were obtained, to which the alcohol concentration (% v/v) was measured with the METER TOLEDO Densito 30PX densitometer, with an average concentration 3.7% v/v alcohol.

4. Conclusions

The pre-treatment of the RH with the NaOH 2.00% w/v solution gave a performance in the process of 43.24%, losing on average, half of the initial mass of the RH.

During the process of enzymatic hydrolysis of the pretreated RH, using the acid cellulase enzyme (CFB3S), it was found that the highest concentration of TRS in the samples was 7.788 g TRS/L, obtained in a time of 24 hours of hydrolysis.

After the alcoholic fermentation of the glucosed syrups obtained, at a laboratory scale, a distillate with a concentration of 3.7% v/v was achieved by simple distillation.

This work presents an alternative for the use of one of the residues generated in the Colombian rice industry, since from the rice husk can be obtained second generation bioethanol. This work successfully developed a methodology for obtaining bioethanol from rice husks at laboratory scale, which can be considered as a basis for the development of future work at pilot scale, also for the study of other possible forms of pre-treatment of lignocellulosic material.

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