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HYDROLYSIS OF SUGARCANE BAGASSE TO OBTAIN ETHANOL USING NATIVE AND COMMERCIAL ENZYMES

HIDRÓLISIS ENZIMÁTICA DEL BAGAZO DE CAÑA PARA LA OBTENCIÓN DE ETANOL EMPLEANDO ENZIMAS NATIVAS Y COMERCIALES

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ABSTRACT

In present work, enzymatic hydrolysis of sugarcane bagasse was carried out from a cocktail of enzymes produced by a bacterium native of Ecuador of the genus *Bacillus sp.*, which is mixed with a commercial enzyme produced by Sigma. With this mixture eight tests were performed changing six initial conditions. With the Plackett Bürman method, the factors that did not influence the process were discarded from the results of the glucose yield coefficients, thus leaving agitation speed and Tween 80 surfactant as the least significant factors in the levels studied. With this new adjusted model, the Box-Hunter complete factorial design of optimization of 2^{4-1} was used and it was concluded that the enzymatic cocktail of native and commercial enzymes generated glucose concentration results of 2.63 mg/ml and glucose yield of /100 g of bagasse. This is important because the possible substitution of a percentage of commercial enzymes for native enzymes reflected as an advantage in the operating costs.

Key words: enzymes; enzyme cocktail; ethanol; *Bacillus sp.*; degradation; glucose.

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RESUMEN

En el presente trabajo se realizó la hidrólisis enzimática de bagazo de caña de azúcar a partir de un coctel de enzimas producidas por una bacteria nativa de Ecuador del género *Bacillus sp.*, la cual es mezclada con una enzima comercial producida por Sigma. Con esta mezcla se realizaron ocho ensayos variando seis condiciones iniciales. Con el método de Plackett Bürman, a partir de los resultados de los coeficientes de rendimiento de glucosa fueron descartados los factores que no influyeron en el proceso, quedando así velocidad de agitación y tensoactivo Tween 80 como las menos significativas en los niveles estudiados. Con este nuevo modelo ajustado se utilizó el diseño factorial completo de optimización de Box-Hunter de 2^{4-1} y se concluyó que el coctel enzimático de enzimas comerciales y nativas generó resultados de concentración de glucosa de 2,63 mg/ml, y rendimiento de glucosa en /100g de bagazo de 15,64, lo cual resulta interesante por la posible sustitución de un porcentaje de las enzimas comerciales por las enzimas nativas reflejado como una ventaja en los costes de operación.

Palabras clave: enzimas; coctel de enzimas; etanol; *Bacillus sp.*; degradación; glucosa.

1. INTRODUCTION

The use of cellulose biomass continues to be a topic of global interest in view of the rapid depletion of oil (Piñeiros, 2014) and the high chemical energy potential of this raw material.

Due to this problem, fuels that are generated from renewable sources have been sought, with ethanol being the one that has occupied an important place within these types of fuel. Ethanol produced by sources of lignocellulosic material has become an alternative to take advantage of the waste generated in the sugarcane industry, which is a process with high accessibility to the raw material, since there is no significant use of it and the cost of bagasse is relatively low (Guarnizo et al., 2009).

For the production of ethanol with lignocellulosic materials to be considered commercially viable, it is important for the process to be economically sustainable. The stages that contribute most to the total cost are enzymatic hydrolysis and pretreatment. Pretreatment is necessary to increase the accessibility of cellulose by enzymes in enzymatic hydrolysis (Mesa, 2010).

The greatest potential for the production of ethanol from biomass is found in the enzymatic hydrolysis of cellulose. The cellulase enzyme replaces the sulfuric acid in the hydrolysis stage and the temperatures are from 30ºC to 50ºC, which reduces the degradation of the sugars (Viñals et al., 2012).

The enzymatic hydrolysis reaction is characterized by an insoluble substrate (cellulose) and a soluble catalyst (enzymes). Thus, the structural characteristics of the cellulose and the mode of action of the enzymes influence the reaction rate (Albernas et al., 2015). The susceptibility of cellulose to the enzymatic attack is determined by the accessibility of the binding sites for the cellulose, which determines the subsequent absorption of the enzyme on the solid substrate (Abril and Navarro, 2012).

There are numerous commercial enzyme preparations that contain mainly cellulolytic activity (Chacón, 2005). These enzymatic preparations are obtained from

microorganisms of fungal and bacterial origin, which mainly come from the microorganisms *Penicillium sp. Trichoderma* and *Aspergillus,* being these the most exploited source of celluloses and hemicelluloses (Mesa et al., 2015). Many of the research papers focus on the study of new enzymes, stemming from genetic manipulation or new sources of microorganisms (Gomashe and Bezalwar, 2013). When dealing with the production of enzymes with autochthonous bacteria, an interesting proposal is to replace a percentage of commercial enzymes, with enzymatic crudes produced in Ecuador, in order to obtain a mixture that minimizes the cost of the process of enzymatic hydrolysis of bagasse.

A suitable way of analyzing the processes of degradation from enzymes is the experimentation with the support of experimental plans, in which the ideas presented on the combination of experimental tests have been proposed, and this increases the efficiency of the research. Defining those ideas as the amount of useful information that can be obtained per unit of experimentation cost (González et al., 1986). It is logical to think that the statistical methods of designing experiments have an undeniable validity since their two main objectives are: to minimize the number of tests and obtain the greatest amount of information.

The following study has the overall aim: to evaluate the conditions that influence the process of enzymatic hydrolysis of a cocktail of native and commercial enzymes in Ecuador.

2. MATERIALS AND METHODS

The conception of understanding part of the work scheme in which 50% of commercial enzymes will be replaced by 50% of native enzymes is assumed. For doing this, the conditions of the block diagram for the production of ethanol, is shown in Figure 1.

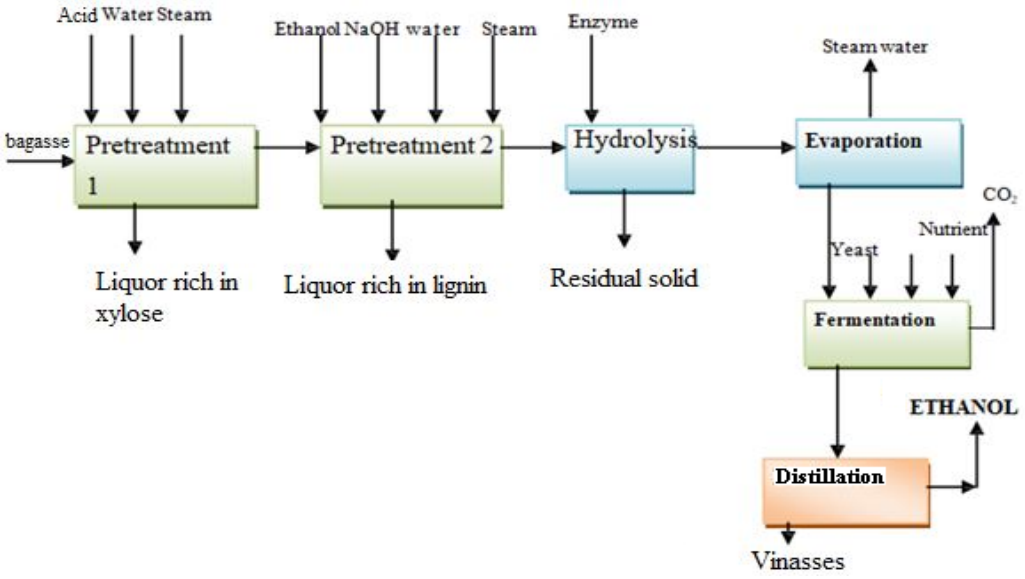

Figure 1. Block diagram for the production ethanol

2.1. Experimental design

To discard the factors that did not influence the laboratory-scale process, the Plackett-Bürman method (Isaacson, 1970) was used for multivariable systems with partial

recesses for polynomials of the first degree of the method of Box and Hunter (1961). The combination of the Plackett-Bürman method with partial fractional designs has been applied in several works with satisfactory results that have served for the identification of some stages and systems of the process (González et al., 1986).

The method of Plackett-Bürman (Isaacson, 1970), is based on a highly fractional factorial design that studies all the possible variables that affect the system, and determine the most influential ones. It is considered an initial program to study the processes that have 5 variables and the research should be continued with a more rigorous plan, in order to find a more adequate model.

The method is applicable with partial factorial designs, that "to shorten the number of tests, the vector of the column belonging to a matrix interaction that can be neglected, could be assigned to a factor" (González et al., 1986).

According to this method, it is possible to study up to (n-1) variables with N tests; however, Isaacson recommends additional experiments to estimate the standard error and variance, due to experimental errors, interactions or quadratic effects. That is why false variables are included in the experimental plan.

3. RESULTS AND DISCUSSION

In agreement with the proposed procedures, the best conditions of enzymatic hydrolysis of an enzymatic cocktail were determined starting from the similar experience reached by (Bussamra et al., 2015), including as response variables or dependent variables those listed below in Table 1.

The glucose yield is the one that most affects the evaluation of the process and responds to how the hydrolysis process was carried out. This is because it is the measure that is directly related to the amount of cellulose that is degraded and because high performance liquid chromatography is used to measure it, which makes it sensitive and specific in relation to the other glucose measurement techniques such as DNS method (Safari and Emtiazi, 2006).The dependent response or variable parameters are described below:

3.1. Dependent Variables

Y_1:Yield of glucose grams per 100 grams of raw material.

This is done in order to take advantage of the cellulose composition consisting of a polysaccharide formed by β-1,4glucosidic bonds (Cuervo et al., 2009).

Y_2:Protein in liquor

The objective here is to measure the peptides present in the hydrolysate, determined according to the Biuret assays, using 40 mg/l as standard of the concentration of fetal bovine serum (Membrillo et al., 2010).

3.2. Independent variables

X_1: Temperature (35ºC -50ºC).

Enzymatic hydrolysis is carried out at the optimum temperature of the commercial enzyme, around 50 °C, and decreases to 35 °C because it has been shown that these enzymes have greater activity in that temperatures range (Mesa, 2010).

X_2: Units of filter paper (UFP: 10-25 UPF/g)

A cellulose concentrate (10 UFP / g dry base) has a positive effect on hydrolysis, to obtain better glucose yields. In addition, the literature indicates that an enzyme load of 25 UFP / g is the most indicated to achieve better glucose yields (Peñuela et al., 2007).

X_3: Agitation, in rpm (150-200 rpm).

It has been described that enzymes work better with agitation, projecting higher glucose yield results (Mesa, 2010).

X_4: Enzymatic reaction time (15-24 hours).

Preliminary studies found good yields in a time of 24 hours. In addition, (Lin et al., 2010), states that the best glucose yields are obtained in tests carried out between 8 and 72 hours.

X_5: Solid in percentages (5 and 8%).

According to theoretical studies, this is best executed in a semi-solid state. A study carried out in the analytical laboratory of renewable energy procedures gives results of glucose yields using a 2% w / v solids load (Zhang et al., 2017). A load of solids was maintained between 6 and 10% to achieve high yields (Peñuela et al., 2007). Other authors confirm a very acceptable yield using 5% w/v (Buaban et al., 2010); (Rodrigues et al., 2014).

X_6: Use of Tween 80 surfactant, (0.1 and 0.2 g).

The literature cites that the incorporation of commercial surfactants in the enzymatic hydrolysis stage increases glucose yields in enzymatic hydrolysis up to about 20% (Mesa, 2010).

The selection of the values of the variables that affect the system was generated from the saturated Plackett-Bürmann partial fractional design (PB). Next, Table 1 is presented showing the experimental matrix of Plackett -Bürman (Isaacson, 1970) with the objective of determining the significance of each of the variables:

Table 1. Plackett-Burman experimental matrix

Variables	X_1	X_2	X_f	X_3	X_4	X_5	X_6
Tests							
1	+	+	+	-	+	-	-
2	+	+	-	+	-	-	+
3	+	-	+	-	-	+	+
4	-	+	-	-	+	+	+
5	+	-	-	+	+	+	-
6	-	-	+	+	+	-	+
7	-	+	+	+	-	+	-
8	-	-	-	-	-	-	-

In carrying out this design, the test that had the highest glucose yield is 1, as shown in Table 2. This experiment corresponds to the use of 50 ° C, 25 UFP, 150 rpm, 5% solid

and 0.1 g of Tween 80. This is possibly due to the effect that the commercial enzyme has on the enzyme crudes of the bacteria, because these data coincide with those recommended by SIGMA and that have been found in other studies with commercial enzymes (Mesa, 2010).

For experimental plans of 8 tests, the first row of the matrix is: + + + + - + -- (Plackett-Bürman, 1946).

Table 2. Glucose concentration and yield for the enzymatic cocktail

Experiments	*Concentration of glucose (mg/ml)*	*Glucose yield /100 grams of bagasse*
1	2.63	15.64
2	1.64	10.46
3	0.47	2.36
4	0.41	6.27
5	0.80	6.90
6	0.21	9.64
7	0.10	2.16
8	1.37	6.36

From the data in Table 2, the performance coefficients detailed below were calculated in Table 3, selected from the Plackett-Bürman Matrix and the corresponding equation was applied, see Equation 1. From it was found that the responses, E_3 and E_6 corresponding to agitation speed and Tween 80 were not statistically significant in the ranges evaluated on the response parameters in the cocktail hydrolysis under study.

Table 3. Glucose yield coefficient for the enzymatic cocktail

E_1	E_2	E_f	E_3	E_4	E_5	E_6
2,73	2,31	-0,04	-0,36	4,32	-6,10	-0,58

These results are possibly because the agitation speed does not allow hydrolysis to obtain a state of homogenization due to the physical characteristics of solidity shown by the bagasse cake under laboratory conditions or because the levels measured were not adequate. Regarding Tween 80, despite the fact that several studies recommend its use, it has been found (Aswathy et al., 2010), that by increasing the enzymatic load of cellulose up to 16 UFP/ g of pretreated substrate, the effect of Tween 80 on the enzymatic hydrolysis decreases.

The experimental results allow determining the Equation 1 of the Plackett-Bürman method.

$$Y = Eo + \frac{1}{2}\left[E_1X_1 + E_2X_2 + E_3X_3 + E_4X_4 + E_5X_5 + E_6X_6\right] \quad (1)$$

The Plackett-Bürman method allowed discarding variables X_3 and X_6 because they were not significant in the range studied. For the disintegration, the effects of the false variable were considered according to what is recommended by (Isaacson, 1970), where:

The standard error is obtained by calculating the effect of false variables, estimated identical as, in the case of real variables, thus

$$SE = \sqrt{\frac{\sum(Ef.)^2}{No.of falsevariables}} \quad (2)$$

The significance of each effect is verified by comparing the tabulated value of the student t to F /number of the false variables and the calculation of the expression 3.

$$t = \frac{E(I)}{S.E.} \quad (3)$$

Then, if the calculated value is greater than the tabulated one, it means that the effect of the level variation of the independent variable really causes variations in the response parameter. And this is not due to experimental errors, which depends on the degree of significance of the variable, it is obtained as it is significant: P = 80, 85, 90, 95% (González et al., 1986).

Once the variables were obtained, the tests were adjusted as proposed in an alternative combination of experimental designs by González and collaborators (González et al., 1986) using a plan by Box and Hunter (1961), as a second design in its strategy of approaching the region of optimal results.

The Factorial Design 2^{4-1} was proposed as observed in Table 4, with conditions adjusted to the experimental data obtained in the enzymatic hydrolysis experiments of the commercial enzyme cocktail and native Ecuadorian enzymes (See Table 4). A confusion factor of $X_5 = -X_1X_2$ was used for this purpose.

This will generate a relationship of

$1 = -X_1X_2X_5$ (4)

where:

$b_1 = \beta_1 - \beta_{25}$, (5)

$b_2 = \beta_2 - \beta_{15}$, (6)

$b_4 = \beta_4 - \beta_{14}$,

$b_5 = \beta_5 - \beta_{12}$, (7)

$b_{24} = \beta_{24} - \beta_{145}$,

$b_{45} = \beta_{45} - \beta_{124}$ (8)

Table 4. Box-Hunter for commercial enzyme cocktail and native enzymes

Test	***Order***	X_1	X_2	X_4	X_5
1	8	+	+	+	-
2	2	+	+	-	-
3	4	+	-	-	+
4	5	-	+	+	+
5	7	+	-	+	+
6	1	-	-	+	-
7	3	-	+	-	+
8	6	-	-	-	-

The experimental results are shown in Table 5.

Table 5. Factorial design 2^{4-1} for cocktail of commercial and native enzymes

Test	*Y´*	*Y´´*	*Difference to the square*	*Y Average*
1	14.87	16.43	2.45	15.65
2	9.93	10.98	1.09	10.45
3	2.24	2.48	0.05	2.36
4	5.95	6.58	0.39	6.27
5	6.56	7.25	0.47	6.90
6	9.17	10.13	0.93	9.65
7	2.05	2.27	0.04	2.16
8	6.03	6.66	0.40	6.34

For the Box-Hunter model, Equation 9 was used, which is found below:

$$Y = b_0 b_1 \cdot X_1 b_2 \cdot X_2 b_4 \cdot X_4 b_5 \cdot X_5 b_{24} \cdot X_2 X_4 b_{45} X_4 X_5 , \quad (9)$$

The glucose yield coefficients of the enzymatic hydrolysis for the mixture of commercial enzyme with the enzymatic cocktail of bacterium *Bacillus sp.* were evaluated using the Box-Hunter model, which are observed in Table 6.

Table 6. Glucose yield coefficients of the enzymatic hydrolysis for the commercial enzyme mixture with the enzymatic cocktail of bacterium *Bacillus sp*

b_1	b_2	b_4	b_5	b_{24}	b_{45}
1.36	1.15	2.13	-3.05	0.18	0.01

It was found that the factors Time (b_4) and Percentage of solid (b_5) are more significant than Temperature and Enzymatic load. Similar results were obtained by other authors when studying the effect of cellulose concentration (Celluclast CCN and Novozym TN 188) on the hydrolysis of 2% (w / v) of hydrothermally pretreated poplar in which they did not observe large differences in the percentages of conversion when they used concentrations between 15 and 25 UPF/g of cellulose. Similar results were obtained by (García, 2007).The experimental results and the estimates of the Box-Hunter model are shown in Table 7.

Table 7. Factorial design $2^{4-1}$for cocktail of commercial and native enzymes

Test	*Yp Average*	*Ye Equation*	*Difference to the square*	*Adequacy variance Sad=(Yp - Ye)2/GL*
1	15.65	15.19	0.21	0.44
2	10.45	10.91	0.21	
3	2.36	2.49	0.01	
4	6.27	6.35	0.007	
5	6.90	6.77	0.017	
6	9.65	10.14	0.23	
7	2.16	2.07	0.007	
8	6.34	5.86	0.23	
Reproducibility variance $S(Y)^2 = \Sigma(Y' - Y'')2/8$				0.53

According to these results, the calculated Fisher's test, Fc, 2.8 will be = 0.44/0.5354 = 0.82, which is smaller than the Tabulated F2.4 = 1.86 (Perry and Chilton, 1975) and it is considered that the model is adequate to predict the results.

3.3. Fermentative stage

3.3.1. Results of separate hydrolysis and fermentation (SHF).

The SHF fermentation step was carried out with the results of the test 1 which has the highest value in terms of glucose yield in 100g of bagasse. The test was carried out at 50 ° C, with an enzymatic load of 25 UPF, at 150 rpm, with 5% solids and 0.1% Tween 80. The hydrolysis was carried out in a period of 15 hours and the fermentation in a period of 24 hours.

The glucose concentration values obtained in the selected test at 15 hours of enzymatic hydrolysis were 15.64 g per 100 g of bagasse, and based on the theoretical yield of 0.51 g of ethanol / g of glucose (Mesa, 2010), it was obtained that there is 7.9 g of ethanol in 100g of bagasse.

4. CONCLUSIONS

1. It is shown that, E_3 and E_6 corresponding to agitation speed and Tween 80 were not statistically significant since they showed low values, so it can be said that they did not interfere in the experiment.
2. The factors Time (b_4) and Percentage of solid (b_5) had more significance compared to the Temperature and Enzymatic load, indicating that the Time and the Percentage of solid are those that most influenced the results of the experiment, being this fact decisive when carrying out this process at an industrial level.
3. The model is adequate to predict the results obtained in Fisher's test = 0.8, which is lower than Tabulated F2.4 = 1.86.
4. These preliminary results have generated information that shows the feasibility of using the cocktail mixture in the bagasse hydrolysis to obtain ethanol because the mixture produces a glucose yield of 15.65 in 100 grams of bagasse.

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