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FOOD AND ENVIRONMENT SAFETY

# [illegible]

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**Abstract:** *In this paper there has been studied the influence of dilute acid pretreatment upon the production of reducing sugars from wheat straws during the enzymatic hydrolysis. The acid pretreatment was led at different temperatures of 120 °C, 130 °C, 160 °C and 170 °C and at different acid concentrations (4%, 1%, 0.5% and without acid) before the enzymatic hydrolysis stage. There have been selected an optimal period of time for the pretreatment of 40 minutes and a temperature of 160 °C, combined with a temperature of 48 °C for the enzymatic hydrolysis. In optimal conditions it was achieved an increasement of the reducing sugars content of 63.37% in comparison with the control sample to which no preliminary pretreatment was applied. The results obtained indicate the fact that the dilute acid pretreatment could increase the ethanol concentration at the end of the fermentation stage. In this case the maximum ethanol concentration of 1.118% vol. was obtained in 72 hours of fermentation with S. cerevisiae.*

**Keywords:** *lignocellulose, cellulase, DNS method, reducing sugar*

## 1. Introduction

Bioethanol is a fuel derived from renewable sources of feedstock; it is an alternative fuel produced almost entirely from food crops. It represents an important, renewable liquid fuel for motor vehicles [1].
The bioethanol produced as a transportation fuel can help to the reduce of $CO_2$ buildup in two important ways: by relacing the use of fossil fuels, and by recycling the $CO_2$ that is released when it is combusted as fuel. An important advantage of crop-based bioethanol is its GHG benefits [2].
Bioethanol can be produced from different kinds of raw materials: sucrose-containing feedstocks (e.g. sugar cane, sugar beet, sweet sorghum and fruits), starch materials (e.g. corn, wheat, rice, sweet potatoes and barley) and lignocellulosic materials (e.g. wood, straw and grasses).
Currently, a focus is on bioethanol production from crops, such as corn, wheat, sugar cane, as well as on highly abundant agricultural wastes [1].
The challenge in producing ethanol from cellulose is the difficulty in breaking down cellulosic matter to sugars [3].
Chemical composition of lignocellulosic materials is a key factor affecting efficiency of biofuel production during conversion processes. The structural and chemical composition of lignocellulosic materials is highly variable because of

genetic and environmental influences and their interactions [4].

Lignocelluloses consist mainly of cellulose, hemicellulose and lignin; these components build up about 90% of dry matter in lignocelluloses, with the rest consisting of extractive and ash [5].

Ethanol production from lignocellulosic biomass comprises the following main steps: hydrolysis of cellulose and hemicellulose, sugar fermentation, separation of lignin residue and, finally, recovery and purifying the ethanol to meet fuel specifications [6, 7].

Pretreatment of lignocellulosic biomass is an essential step for obtaining potentially fermentable sugars in the hydrolysis step. The aim of the pretreatment is to break down the lignin structure and disrupt the crystalline structure of cellulose for enhancing enzyme accessibility to the cellulose during hydrolysis step [8].

There are several different ways of pretreating biomass depending on its type and composition, as well as the processing technology that will be applied [9].

The goal of this study was to investigate the possibilities for increasing the yield of reducing sugars and of ethanol by applying a dilute acid pretreatment in the wheat straws bioethanol production using the SHF (separate hydrolysis and fermentation) procedure.

It was studied the efficiency of the dilute acid pretreatment upon the lignocellulosic structure disintegration and upon the destruction of the crystalline cellulosic structure to favourize the accessibility of the enzyme in the enzymatic hydrolysis process.

It was also studied the influence of the saccharification temperature upon the content of the reducing sugars.

## 2. [illegible]

### 2.1. [illegible]

The wheat straws (named Triticum aestivum) used in this study were obtained from Vrancea area farmers.

The wheat straws were cut into pieces with a lenght of 5 cm, dried at air and grinded in a hammer mill until they got dimensions of 2 mm.

### 2.2. [illegible]

In order to destroy the crystalline structure of the cellulose and to increase the lignocellulosic material susceptibility to the enzyme attack, the wheat straws were submitted to a preliminary pretreatment. So, a dilute acid pretreatment was applied to the wheat straws using sulfuric acid of concentrations between 0.5 – 4%. 5 g of wheat straws grinded in the hammer mill and 100 ml sulfuric acid were mixed and pretreated at different temperatures between 120 –170 ºC, for 30 – 60 minutes.

The working data in the case of the pretreatments applied were the following:

- pretreatment P1: $H_2SO_4$ 4% at a temperature of 120 ºC, for 60 minutes;
- pretreatment P2: $H_2SO_4$ 1% at a temperature of 130 ºC, for 50 minutes;
- pretreatment P3: $H_2SO_4$ 0.5% at a temperature of 160 ºC, for 40 minutes;
- pretreatment P4: water, at a temperature of 170 ºC, for 30 minutes;

After pretreatment, the solid fraction was separated from the liquid fraction by filtration.

The wash was realized to eliminate the inhibitors resulted after the pretreatment (furfural, HMF) and the resulted glucose that inhibits the enzymatic activity of the cellulase used in the following step [10].

### 2.3. [illegible]

The raw material (wheat straws) used in the study was analyzed according to the analytical procedures.

The water content of the raw material was determined by drying 5g of wheat straws at 105 ºC for 2 hours in drying vials. The samples were cooled then in a desiccator weighted and introduced again at 105 ºC until a constant weight [11].

The ash content was determined by calcination of 1g of sample at 575 ºC for 3 hours. The samples were cooled in the desiccator, weighted and introduced again in the calcination oven at 575 ºC until a constant weight [12, 13].

The wheat straws cellulose content was determined by using the method described by Ishtiaq et al. [11].

The lignin content was determined according to the method described by NREL [14].

The quantification of the reducing sugars resulted in the enzymatic hydrolysis phase was realized using the DNS method [15].

The enzymatic hydrolysis of the solid fraction resulted after the pretreatment was realized in a buffer solution of $Na_2HPO_4$ 0.2 M – citric acid 0.05 M with pH = 4.8 for 72 hours at temperatures between 40 – 50 °C. The enzymatic mixture used in this stage of the study was the *ONOZUKA R - 10* cellulase obtained from *Trichoderma viride* produced by Merck Company. The added quantity of enzyme was established according to the initial cellulose content as being of 15 unit/g cellulose. The reducing sugars production was evaluated at periods of 12 hours.

## 2. [illegible]

After enzymatic hydrolysis, 1 $cm^3$ of suspension of 10% *Saccharomyces cerevisiae* yeast and 2 ml nutrient for the yeast that contained 0.5 g/L $(NH_4)_2HPO_4$, 0.025 g/L $MgSO_4$, 0.1 M $NaH_2PO_4$ [16] were inoculated in each sample and the fermentation was realized at 30 °C for 72 hours in recipients with a capacity of 1L. At each recipient there were coupled BlueSens gas sensors to monitor the ethanol, oxygen and $CO_2$ content during the fermentation process.

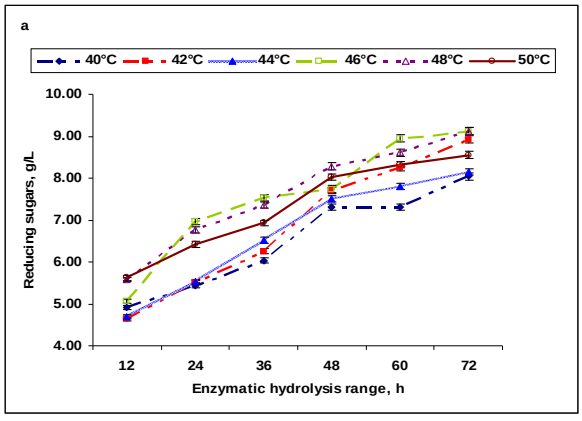
## 3. [illegible]

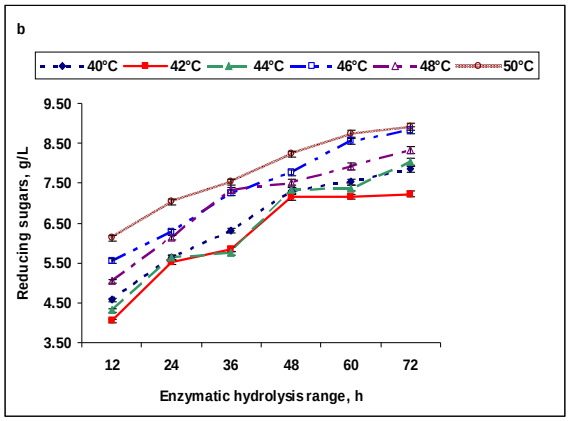
### 3.1. [illegible]

The composition of the wheat straws used in this study is: 38.09% cellulose, 13.82% acid insoluble lignin, 1.78% acid soluble lignin, 92.98 % dry substance and 6.82% ash.

### 3.2. [illegible]

The reducing sugars content resulted from the enzymatic hydrolysis of the solid fraction from the pretreatment in different temperature conditions is shown in the figures 1 a-d.



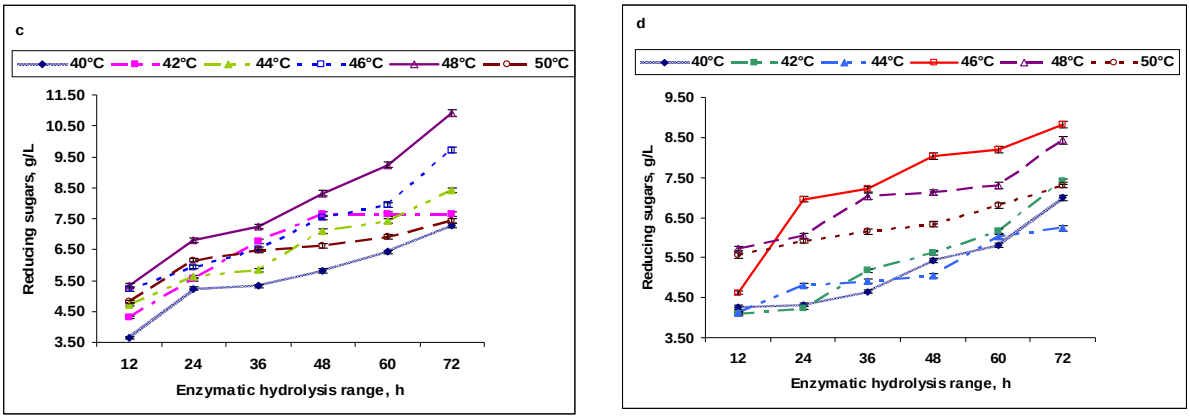



**Figure 1. The reducing sugars content in mash during enzymatic hydrolysis: a- Pretreatment with $H_2SO_4$ 4%, for 60 minutes at 120 °C, enzymatic hydrolysis with *ONOZUKA* between 40-50 °C, b- Pretreatment with $H_2SO_4$ 1%, for 50 minutes at 130 °C, enzymatic hydrolysis with *ONOZUKA* between 40-50 °C, c- Pretreatment with $H_2SO_4$ 0.5%, for 40 minutes at 160 °C, enzymatic hydrolysis with *ONOZUKA* between 40-50 °C, d- Pretreatment with water, for 30 minutes at 170 °C, enzymatic hydrolysis with *ONOZUKA* between 40-50 °C.**

Figure 1-a indicates the evolution of the reducing sugars content resulted after the enzymatic hydrolysis of the wheat straws pretreated with $H_2SO_4$ 4% at a temperature of 120 °C for 60 minutes. The enzymatic hydrolysis was done at temperatures between 40 – 50 °C. The reducing sugars content was determined at periods of 12 hours using the DNS method. It can observe an increase of the reducing sugars content after each 12 hours of enzymatic hydrolysis. The hydrolysis temperature is also an important factor that favours the reducing sugars accumulation. It can be observed that at the temperatures of enzymatic hydrolysis of 46 °C and 48 °C a higher content of reducing sugars was obtained (9.12 g/L).

In figure 1-b it is presented the evolution of the reducing sugars at the enzymatic hydrolysis of the wheat straws pretreated with $H_2SO_4$ 1% at a 130 °C for 50 minutes. During the 72 hours of enzymatic hydrolysis a higher accumulation of reducing sugars was registered at the temperatures of 46 °C (8.83 g/L) and of 50 °C (8.92 g/L). Figure 1-c shows the evolution of the reducing sugars content at the enzymatic hydrolysis of the biomass resulted after the pretreatment with $H_2SO_4$ 0.5 % at a temperature of 160 °C for 40 minutes. They can observe significant increases of the reducing sugar at the hydrolysis temperature of 46 °C (9.71 g/L) and 48 °C (10.92 g/L).

Figure 1-d indicated the reducing sugars content resulted during the enzymatic hydrolysis of the wheat straws pretreated with water at 170 °C for 30 minutes. It can observe that the temperatures of 46 °C and 48 °C led to higher quantities of reducing sugars (8.82 g/L and 8.43 g/L). It can say that the applied pretreatments with $H_2SO_4$ 0.5 % at 160 °C for 40 minutes combined with a enzymatic hydrolysis temperature of 46 °C and 48 °C led to the obtaining of the highest reducing sugars quantity (9.71 g/L and 10.92 g/L). In figure 2 it is presented the content of the reducing sugars after 72 hours of enzymatic hydrolysis according to the applied pretreatment and temperature of hydrolysis in comparison with the control sample (which the enzymatic hydrolysis was done without a preliminary pretreatment).

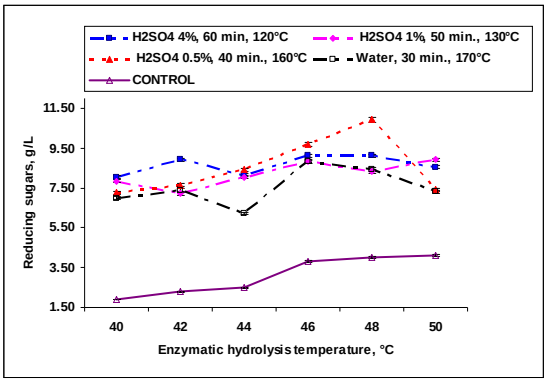



**Figure 2. The reducing sugars content after 72 hours of wheat straws enzymatic hydrolysis**

Figure 2 indicates that the pretreatment of the lignocellulosic material plays an important role in the production of the reducing sugars facilitating the enzyme accessibility to the cellulosic layer by destroying the crystalline structure of the cellulose. In optimal conditions, it was reached an increase of the reducing sugars content of 63.37% in comparison with the control sample that was not submitted to any preliminary pretreatment.

## 3 Fifth h

The process of fermentation of the wheat straws mash was done separately from the enzymatic hydrolysis phase, in fermentation cells with a capacity of 1 L. During the 72 hours of fermentation, the ethanol, oxygen and $CO_2$ content were monitored with the BlueSens gas sensors.
The figure 3 presents the ethanol content resulted after the fermentation of the hydrolyzed mashes at temperatures between 40 – 50 °C. Figure 3 indicates that the best yield of reducing sugars conversion in ethanol was evaluated in the pretreated samples with $H_2SO_4$ 0.5% at a hydrolysis temperature of 46 °C and 48 °C (85.12% reported to the theoretical one).

Also there was obtained a good yield of conversion for the sample pretreated with $H_2SO_4$ 4% at a hydrolysis temperature of 42 °C (81.59% reported to the theoretical one) and for the sample pretreated with $H_2SO_4$ 1% at a hydrolysis temperature of 50 °C (81.59% reported to the theoretical one).

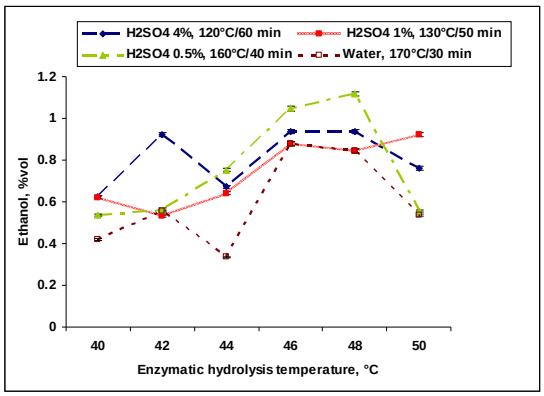



**Figure 3. The ethanol content resulted after the fermentation of the hydrolyzed mashes at temperatures between 40–50 °C**

## 4 Data

Data model regarding the prediction of sugar content, sugar content after fermentation, sugar content consumed at fermentation and ethanol obtained after fermentation using lignocellulosic materials in function of different factors (pretreatment temperature, pretreatment time, sulfuric acid concentration, hydrolysis temperature and hydrolysis time, the latter factor has been used only for sugar content prediction) has been made using a 3rd grade polynomial equation with 4 or 5 variables (using Design Expert 6.0 trial version). The measured and predicted values have been compared to see the suitability of the model. The equation 1 of the model is:

$$A = b_0 + \sum_{i=1}^{n} b_i x_i + \sum_{i=1}^{n} b_{ii} x_i^2 + \sum_{i=1}^{n} b_{iii} x_i^3 + \sum_{i<j<k}^{n} b_{ijk} x_i x_j x_k + \sum_{i<j}^{n} b_{ij} x_i x_j + \sum_{i<j}^{n} b_{ij} x_i^2 x_j + \sum_{i<j}^{n} b_{ij} x_i x_j^2 \quad (1)$$

Where A is the parameter predicted, $b_0$ is a constant that fixes the response at the central point of the experiments, $b_i$ – regression coefficient for the linear effect

terms, $b_{ij}$ – interaction effect terms, $b_{ii}$ – quadratic effect terms and $b_{iii}$ – cubic effect terms.
The operating region and the levels of the design variables (key factors) are given in actual and coded values as it is shown in table 1.

**Table 1**

**Correspondence between actual and coded values of design variables**

| **Design variables** | **Symbol** | **Actual values of coded levels** | |
|---|---|---|---|
| | | -1 | +1 |
| Pretreatment temperature, °C | $X_1$ | 120 | 170 |
| Pretreatment time, min | $X_2$ | 30 | 60 |
| Sulfuric acid concentration, % | $X_3$ | 0 | 4 |
| Hydrolysis temperature, °C | $X_4$ | 40 | 50 |
| Hydrolysis time, min | $X_5$ | 12 | 72 |

In table 2 are presented the predicted models for the experimental data of lingocellulose samples. The coefficients of regression were superior of 0.903, and all models were significant ($P<0.01$). In figure 4 a–d are presented the predictability of the proposed models for the parameters.

**Table 2**

**Predicted models for the experimental data of lignocelluloses samples**

| **Parameter** | **Equation** | **$R^2$** |
|---|---|---|
| Sugar, g/L | $Sugar = 6.82 + 1.59 \cdot x_1 + 2.60 \cdot x_2 - 0.44 \cdot x_3 + 1.59 \cdot x_4 + 1.58 \cdot x_5 - 0.39 \cdot x_4^2 - 0.17 \cdot x_5^2 - 0.4 \cdot x_1 \cdot x_4 + 0.86 \cdot x_1 \cdot x_5 - 0.62 \cdot x_2 \cdot x_4 + 1.35 \cdot x_2 \cdot x_5 + 0.04 \cdot x_3 \cdot x_4 - 0.25 \cdot x_3 \cdot x_5 - 0.17 \cdot x_4 \cdot x_5 - 1.10 \cdot x_4^3 + 0.22 \cdot x_5^3 - 2.38 \cdot x_1 \cdot x_4^2 + 0.22 \cdot x_1 \cdot x_5^2 - 2.17 \cdot x_2 \cdot x_4^2 - 0.34 \cdot x_2 \cdot x_5^2 - 0.09 \cdot x_3 \cdot x_4^2 + 0.30 \cdot x_3 \cdot x_5^2 - 0.41 \cdot x_4^2 \cdot x_5 - 0.03 \cdot x_4 \cdot x_5^2 + 0.49 \cdot x_1 \cdot x_4 \cdot x_5 + 0.76 \cdot x_2 \cdot x_4 \cdot x_5 - 0.23 \cdot x_3 \cdot x_4 \cdot x_5$ | 0.903 |
| Sugar after fermentation, g/L | $Sugar\ after\ fermentation = 0.46 - 0.34 \cdot x_1 - 0.59 \cdot x_2 + 0.29 \cdot x_3 - 0.04 \cdot x_4 - 0.03 \cdot x_4^2 + 0.12 \cdot x_1 \cdot x_4 - 0.01 \cdot x_2 \cdot x_4 + 0.09 \cdot x_3 \cdot x_4 + 0.06 \cdot x_4^3 + 0.03 \cdot x_1 \cdot x_4^2 - 0.04 \cdot x_2 \cdot x_4^2 + 0.02 \cdot x_3 \cdot x_4^2$ | 0.956 |
| Sugar consumed at fermentation, g/L | $Sugar\ consumed\ at\ fermentation = 7.84 + 4.15 \cdot x_1 + 5.77 \cdot x_2 - 1.1 \cdot x_3 + 1.59 \cdot x_4 - 0.46 \cdot x_4^2 + 0.52 \cdot x_1 \cdot x_4 + 1.15 \cdot x_2 \cdot x_4 - 0.69 \cdot x_3 \cdot x_4 - 1.39 \cdot x_4^3 - 4.46 \cdot x_1 \cdot x_4^2 - 5.09 \cdot x_2 \cdot x_4^2 + 0.68 \cdot x_3 \cdot x_4^2$ | 0.937 |
| Ethanol, %vol | $Ethanol = 0.39 + 0.30 \cdot x_1 + 0.04 \cdot x_2 - 0.05 \cdot x_3 + 0.21 \cdot x_4 - 0.08 \cdot x_4^2 - 0.03 \cdot x_1 \cdot x_4 + 0.01 \cdot x_2 \cdot x_4 - 0.05 \cdot x_3 \cdot x_4 - 0.19 \cdot x_4^3 - 0.39 \cdot x_1 \cdot x_4^2 - 0.40 \cdot x_2 \cdot x_4^2 + 0.01 \cdot x_3 \cdot x_4^2$ | 0.955 |

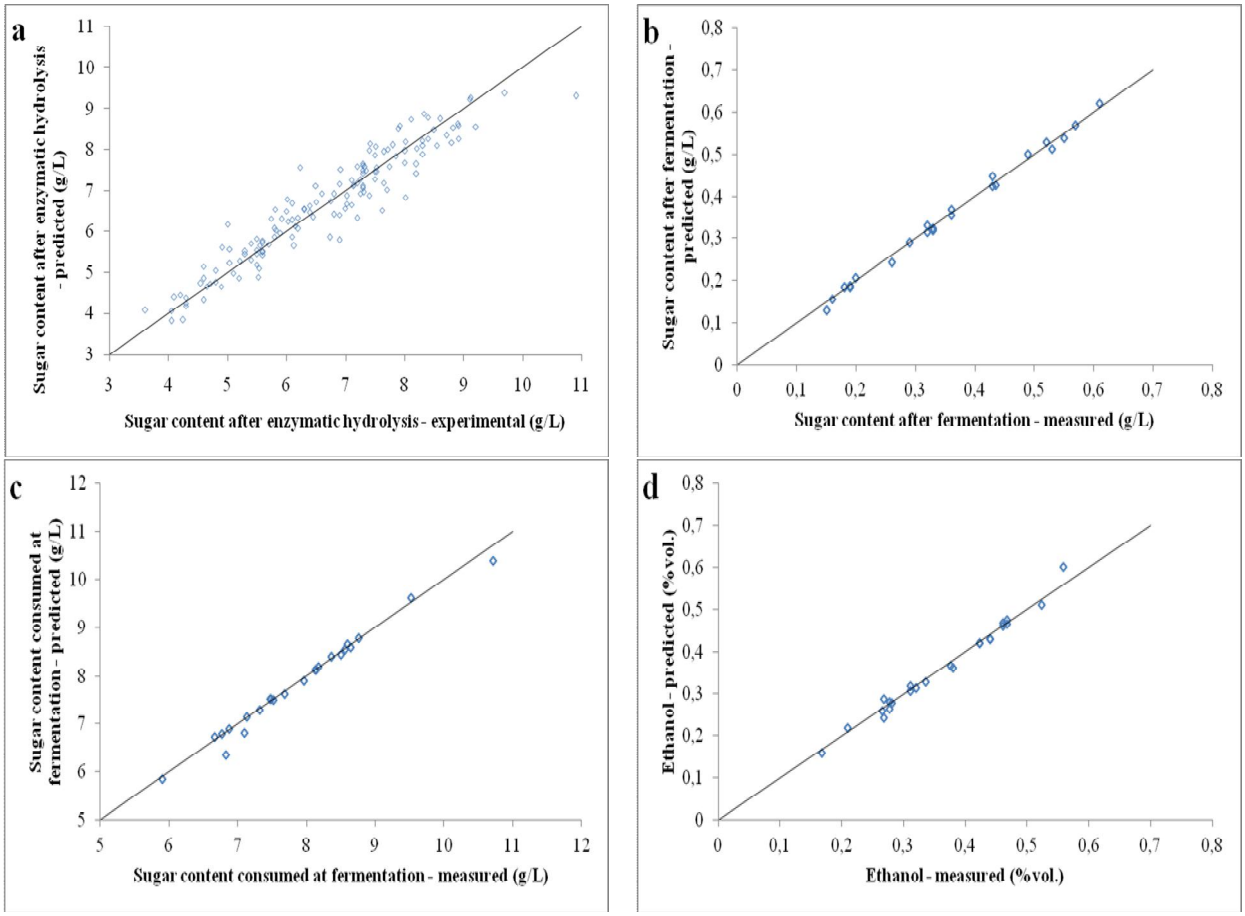



**Figure 4. The predictability of proposed models: a –sugar content after hydrolysis, g/L, b – sugar content after fermentation, g/L, c – sugar content consumed at fermentation, g/L, d – ethanol, %vol.**

The optimization of parameters simultaneously is very important for product quality. The desirability function approach is used to optimize the multiple characteristics concurrently. In the desirability function approach, first each characteristics, $y_i$, is converted into an individual desirability function, $d_i$, that varies over the range,

$$0 \le d_i \le 1 \qquad (2)$$

If the characteristic $y_i$ is at its target, then $d_i = 1$. If the characteristic is outside an acceptable region, then $d_i = 0$. Finally, the design variables can be chosen to maximize the overall desirability:

$$D = \left(d_1 x d_2 x ... x d_n\right)^{1/n} \qquad (3)$$

Where n is the number of characteristics. When the target (T) for the characteristic y is a maximum value and the lower limit is noted by, L,

$$d_i = \begin{cases} 0 & y < L \\ \left(\dfrac{y-L}{T-L}\right)^r & L \le y \le T \\ 1 & y > T \end{cases} \qquad (4)$$

When the target (T) for the characteristic y is a minimum value and the upper limit is denoted by U [17].

$$d_i = \begin{cases} 0 & y < T \\ \left(\dfrac{U-y}{U-T}\right)^r & T \le y \le U \\ 1 & y > U \end{cases} \qquad (5)$$

In this study, the desirability value of n was calculated by eq. 5, while desirability values of other characteristics were calculated by eq. 4, as other authors [18]. The exponent r is referred to the weight

and specified as 1. Overall desirability, which represents, the desirability of all characteristics simultaneously (D), was calculated by eq. 3. Ten of lignocelluloses combinations yielding the largest D values and predicted characteristic values are given in table 3 for sugar content after enzymatic hydrolysis, and in table 4 sugar content after fermentation, sugar content consumed at fermentation, sugar content and ethanol. Making tradeoffs between these parameters were possible.

**Table 3**

**Desirability values and predicted characteristic values of ten combinations for sugar content after enzymatic hydrolysis**

| No. | $X_1$ | $X_2$ | $X_3$ | $X_4$ | $X_5$ | Sugar after enzymatic hydrolysis, g/L | Desirability |
|---|---|---|---|---|---|---|---|
| 1 | 0.72 | 0.94 | -0.58 | 0.11 | 0.43 | 12.21300 | 1.000000 |
| 2 | 0.98 | 0.03 | -0.78 | -0.14 | 0.94 | 11.00950 | 1.000000 |
| 3 | -0.04 | 0.99 | 0.73 | 0.26 | 0.86 | 11.37950 | 1.000000 |
| 4 | 0.83 | 0.53 | 0.85 | 0.56 | 0.98 | 11.49220 | 1.000000 |
| 5 | -1.00 | -0.88 | -0.48 | 1.00 | -1.00 | 10.01700 | 0.877667 |
| 6 | -1.00 | 1.00 | -1.00 | 0.20 | 0.87 | 9.94548 | 0.867874 |
| 7 | 1.00 | -0.28 | 1.00 | 0.07 | 1.00 | 9.77736 | 0.844843 |
| 8 | -0.72 | -1.00 | -0.55 | 1.00 | -1.00 | 9.65469 | 0.828040 |
| 9 | -1.00 | -1.00 | 0.37 | 1.00 | -0.58 | 9.63380 | 0.825178 |
| 10 | 0.99 | -0.41 | 1.00 | 0.41 | 1.00 | 9.25547 | 0.773352 |

The parameters presented in the table 3 have been predicted keeping into account that the sugar content after hydrolysis should be maximum.

**Table 4**

**Desirability values and predicted characteristic values of ten combinations for sugar after fermentation, sugar consumed at fermentation and ethanol**

| No. | $X_1$ | $X_2$ | $X_3$ | $X_4$ | Sugar after fermentation, g/L | Sugar consumed at fermentation, g/L | Ethanol, %vol. | Desirability |
|---|---|---|---|---|---|---|---|---|
| 1 | 0.18 | 1.00 | 0.92 | 0.65 | 0.11725 | 12.0294 | 0.624413 | 1 |
| 2 | 0.83 | -0.12 | -0.66 | 0.36 | 0.060924 | 11.6928 | 0.644662 | 1 |
| 3 | 0.21 | 0.99 | 1.00 | -0.52 | 0.042227 | 10.7233 | 0.594715 | 1 |
| 4 | -0.03 | 0.91 | 0.67 | -0.15 | 0.127307 | 11.7495 | 0.674899 | 1 |
| 5 | -0.08 | 0.91 | 0.54 | 0.25 | 0.103458 | 12.4897 | 0.721032 | 1 |

The parameters presented in the table 4 have been predicted keeping into account that the sugar content after fermentation should be minimum, sugar consumed at fermentation and ethanol should be maximum.

According to the optimization (presented in the table 4), the highest content of ethanol (0.721032%) can be obtained in the next condition: pretreatment temperature (143 °C), pretreatment time (58.65 min), sulfuric acid concentration (3.08%) and hydrolysis temperature (46.25 °C).

This study has analyzed the feasibility of the diluted acid pretreatment to enhance the release of reducing sugars during the enzymatic hydrolysis process using as a lignocellulosic material the wheat straws. At the same time it was pursued an increase of the yield of ethanol by applying the hydrolysis and fermentation systems separately (SHF).

The pretreatment with $H_2SO_4$ 0.5% at 160 °C for 40 minutes increased efficiently the reducing sugars content during the enzymatic hydrolysis phase that was realized at 48 °C/72 hours (with 63.37% in comparison with the control sample that was not preliminary pretreated).

Therefore this study shows that the pretreatment with $H_2SO_4$ 0.5% at 160 °C for 40 minutes enhanced the ethanol production in the fermentation phase with *S. cerevisiae.*

The maximum ethanol concentration of 1.118% corresponds to a yield of 85.12% from the theoretical one (0.51 g/g).

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