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OPTIMIZATION OF ENZYMATIC SACCHARIFICATION OF WHEAT STRAW IN A MICRO-SCALE SYSTEM BY RESPONSE SURFACE METHODOLOGY

OPTIMIZACIÓN DE LA SACARIFICACIÓN ENZIMÁTICA DE PAJA DE TRIGO EN MICROESCALA A TRAVÉS DE LA METODOLOGÍA DE SUPERFICIE DE RESPUESTA

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Abstract

This paper studies the combined effects of temperature, pH and enzyme-substrate ratio (E/S_R) on hydrolysis yield and specific reaction rate (S_{RV}) in a microscale system in order to maximize enzymatic hydrolysis of pretreated wheat straw (WS). The WS was pretreated by alkaline-peroxide. Enzymatic complex Accellerase 1500^{TM} was used for hydrolysis assays. Using response surface methodology, optimal parameter values were determined. A complete enzymatic kinetic of the hydrolysis reaction was obtained in 10 h. The optimal value of reducing sugars concentration (RS_C), given by the model, was 5.97 mg/mL and the corresponding yield was 61.73%. The maximum yield for the WS hydrolysis was 61.73% and was achieved at a temperature of 52.0°C, pH 4.6, and a E/S_R of 2.1 mL of Accellerase 1500^{TM}/g of cellulose. The S_{RV} was 4.80 U/mg and was obtained with the following conditions: pH 5.0, temperature of 48.5°C and an E/S_R of 0.19 mL/g. A quadratic polynomial equation for predicting the hydrolysis yield was developed. The confirmation experiment showed a final value for RS_C of 5.98 ± 0.81 mg/mL. This result indicates a % error of 0.33. The experimental results were in good agreement with predicted value.

Keywords: enzymatic saccharification, wheat straw, response surface methodology, microscale system.

Resumen

El presente trabajo estudia la hidrólisis de paja de trigo utilizando un sistema de microreacción. El Método de Superficie de Respuesta se utilizó para estudiar los efectos combinados de la temperatura, el pH y la relación enzima-sustrato ($R_{E/S}$) sobre la hidrólisis enzimática y la velocidad específica de reacción V_{ER}. El sustrato fue paja de trigo pretratada de forma alcalino-oxidativa. El extracto enzimático utilizado fue Accellerase 1500^{TM}. El tiempo de obtención de una cinética de la hidrólisis enzimática completa fue de 10 h. El valor óptimo de la concentración de azúcares reductores (C_{AR}) arrojado por el modelo fue de 5.97 mg/mL y el rendimiento correspondiente fue de 61.73%. Estos valores fueron obtenidos con una temperatura de 52.0°C, pH 4.6 y una $R_{E/S}$ de 2.1 mL de Accellerase 1500/g de celulosa. La V_{ER} óptima fue de 4.80 U/mg y fue obtenida con una temperatura de 48.5°C, pH 5.0 y una $R_{E/S}$ de 0.19 mL/g. Se realizó un ensayo de confirmación en el que el valor predicho de C_{AR} por el modelo fue de 5.96 mg/mL y el valor obtenido experimentalmente fue de 5.98 ± 0.81 mg/mL, indicando un error de 0.33%.

Palabras clave: hidrólisis enzimática, paja de trigo, metodología de superficie de respuesta, micro-escala, accellerase 1500^{TM}.

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1 Introduction

Lignocellullosic materials can be considered as suitable feedstock for the production of bioethanol of second generation (2G) due to their availability worldwide, whose estimated production is around 10-15 billion tons per year (Alfaro *et al*., 2009). In addition, these resources are considered renewable with a low carbon footprint (Areque *et al*., 2008). The production of 2G bioethanol using biochemical platforms is considered as a strong alternative for commercial production because the current maturity of process technologies (IEA Biofuels for transport, 2004). A biochemical platform comprises four fundamental steps: a) pretreatment, b) enzymatic hydrolysis, c) fermentation and d) downstream processes (Poonam and Anoop, 2011). Several investigations have been conducted in the last decade to maximize 2G bioethanol production, and one of the stages in which the investigations have focused the attention is on the saccharification step. Many authors have reported the enzymatic hydrolysis of a variety of substrates as rice straw (Ma *et al*., 2009), wheat straw (Benkun *et al*., 2009), perennial grass (Karthika *et al*., 2012), corn cobs (Primo *et al*., 1995) and stover (Chen *et al*., 2014), wheat bran (Lequart *et al*., 1999), eucalyptus (Martín-Sanpedro *et al*., 2012), cedar (Toyokazu *et al*., 2012), straw bean (González-Rentería *et al*., 2011) and even synthetic biomass which was formed by the three main components of lignocellulose (Lin *et al*., 2010) such as cellulose, hemicellulose and lignin using an specific enzyme or an enzyme complexes extracted from microorganisms (Lin *et al*., 2010; Grande and De Maria, 2012; Kawai *et al*., 2012; Song *et al*., 2012).

Enzymatic hydrolysis of cellulose to glucose is carried out by cellulase enzymes which are highly specific catalysts. The increase of hydrolysis yield to reducing sugars (RS) is well accepted to be a central issue to achieve commercial 2G ethanol production. Considerable attention has been given to the enzymatic hydrolysis process in order to maximize the production of RS (Saha *et al*., 2005; Benkun *et al*., 2009). Response surface methodology (RSM) has been used by Lin *et al*., (2010) in order to assay the saccharification of a synthetic biomass composed of cellulose, hemicellulose and lignin in order to understand the different behaviors of three biomass components in hydrolysis and their potential interaction. Benkun *et al*., (2009), showed the optimization of the hydrolysis of pretreated WS by alkaline-peroxide using a RSM with a central composite design (CCD) in order to develop a useful tool to predict and optimize the hydrolysis process of WS. Furthermore, many reports have shown that the operating variables that affect the hydrolysis process are pH, enzyme loading, substrate concentration, incubation time, and agitation speed (Brudecki *et al*., 2012; Jeya *et al*., 2012; Liu *et al*., 2010; Ma *et al*., 2009).

All the works mentioned above have been carried out at laboratory scale with test volume of 20 mL or higher, nevertheless, only few works have shown microscale saccharification in volume smaller than 1 mL. Microscale methods represent a technical advance to select the most appropriate combinations since many different cellulase complexes can be evaluated rapidly (Berlin *et al*., 2005; Chundawast *et al*., 2008). Furthermore, when reducing the volume assay, the amount of enzyme required for the tests is very little. Since recombinant enzymes obtained at lab-scale are often produced in small quantities, micro-scale methods are appropriate to evaluate the enzymatic performance of novel enzyme mixtures (Kim *et al*., 2009). Also, a great quantity of experiments can be realized simultaneously in a controlled system. Moreover, shaking of microwell plates is the simplest and most efficient way of promoting liquid mixing, and a rapid and efficient mixing during liquid addition to individual wells underpins the reproducibility of all bioprocess studies, in addition the loss of heat and mass are minimized allowing study purely kinetic aspects (Micheletti and Lye, 2006). Characterization of the engineering environment in micro-well systems will ultimately underpin their use in bioprocess studies, ensuring the generation of reproducible, quantitative and, above all, scalable bioprocess information (Micheletti and Lye, 2006). Enzymatic microscale have been developed to evaluate the performance of different enzymes on enzymatic hydrolysis of wheat straw and the results obtained in enzymatic microscale were comparable with those from standard procedures in shake flasks (Alvira *et al*., 2010).

The main objective of this research is to maximize the reducing sugar production using a microscale system. Furthermore, an experimental design of microscale saccharification in a volume smaller than 1 mL was not reported before for the hydrolysis of wheat straw and this is the contribution of this study. A CCD was used for the analysis of hydrolysis of pretreated WS by alkaline-peroxide using an enzymatic complex Accellerase 1500^{TM}. This enzymatic complex has been used by other

authors in the hydrolysis of a variety of substrates as rice straw, wheat straw, eucalyptus, cedar and even in a mix of the principal components of lignocellulosic as cellulose, hemicelulose and lignin (Grande and de Maria 2012, Kawai *et al*., 2012, Lin *et al*., 2010, Pessani *et al*., 2011, Song *et al*., 2012), obtaining good results.

2 Material and methods

2.1 Substrate and enzyme

WS was obtained from local farmers in San Francisco del Rincón, Guanajuato, México. It was cut to 3 mm in length, washed with distilled water to remove some impurities, and then dried at 70°C in an oven (Gravity convection Oven, ThermoFisher Scientific, Waltham, Massachusetts, USA).

The commercial enzymatic complex Accellerase^{TM} 1500 was employed in this research. Accellerase^{TM} 1500 was a gift from Genencor International Incorporation. Accellerase^{TM} 1500 (referred to as Cellulase) is an enzyme complex which contains a mixture of cellulase, hemicellulase and *β-glucosidase*. The RS_C, was performed using different substrates to determine single activities. Overall cellulase activity was determined using Avicel and Carboximeticelullose (Ghose, 1987). Xylanase activity was determined on xylan birch and xylan oat (Bailey and Nevalainen, 1981). An experiment with WS whit out pretreatment was realized according to Ghose (1987), in order to compare the effect of enzymatic complex over this not pretreated substrate.

2.2 Pretreatment of WS by alkaline peroxide

Before enzymatic hydrolysis, the WS was pretreated by an alkaline peroxide method according to Pattel and Bhatt (1992), with a modified concentration of NaOH. The pretreatment was accomplished in a stirred flask at 150 rpm using a stirred and hot plate (Stirring hot plate, Corning®, Model PC-420D, Tewksbury, USA), with an alkaline-oxidative (AO) solution with a solid-liquid ratio of 1:20. The solution was prepared with distilled water; pH was adjusted to 11.5 with a 10 M NaOH solution. Then, the WS was added and mixed. Then, hydrogen peroxide was added to the suspension until a concentration of 2% (v/v) was reached. The suspension was stirred at a temperature of 60°C for 6 h. The solid residue was filtered under vacuum, and washed with distilled water until pH 8 was reached. The pH was adjusted with a diluted solution of acetic acid until pH 7 was reached. The solid was dried at 70°C in a gravity convection oven until the weight remained constant for subsequent enzymatic hydrolysis. The chemical composition of pretreated WS is shown in Table 1.

2.3 Enzymatic Hydrolysis

2.3.1. Microscale procedure

Microscale enzymatic hydrolysis experiments were performed in sample conical tubes of 1.5 mL, with an effective reaction volume of 1 mL containing 1% (v/v) of pretreated WS, Accellerase^{TM} 1500 and 50 mM citric acid/citric sodium buffer according to Benkun *et al*., 2009. The tubes were incubated in a thermomixer at 700 rpm, and this was called microscale system. Samples of 50 μL, were taken from the reaction mixture at different times during a period of 600 minutes. All samples were heated to 100°C immediately for 5 min to denature the enzymatic complex and cooled to room temperature. The experimental conditions were established according to dosage guidelines described in the product information for Accellerase^{TM} 1500. The ranges of the values for the three factors were pH 4 to 6, temperature from 45 to 65ºC, and 0.6 to 1.8 mL of Accellerase 1500^{TM} per gram of cellulose as shown in Table 2.

Table 1. Cellulose, hemicellulose and lignin content before and after pretreatment of WS. % of dry biomass (% DB).

	Cellulose	Hemicellulose	Lignin
Wheat straw (Raw)	54	18.2	15.17
Wheat straw (pretreated)	60.9	21.6	5.37

Table 2. Values of the Variables for the Central Composite Design

Variable	Actual values of natural levels				
	$-\sqrt{2}$	−1	0	1	$\sqrt{2}$
pH	4	4.3	5	5.7	6
T (°C)	45	48	55	62	65
E/S_R (ml/g)	0.1	0.33	0.9	1.46	1.8

2.3.2. Flask assay procedure

Experiments in 500 mL scale were performed in 500 mL flask (Proculture Spinner Flasks, 500 mL, Corning®, Tewksbury, MA, USA) placed over a hot plate equipped whit a temperature controller (Stirring hot plate, Corning®, Model PC-420D, Tewksbury, USA). A 50 mM citric acid/citric sodium buffer was used according to Benkun *et al*., 2009. The percentage of pretreated WS was 1%. Samples were withdrawn at different times (0, 2, 4, 6, 8, 10, 12, 24 and 48 h), and analyzed by DNS method (Miller, 1959).

2.4 Analytical methods

The composition of WS with respect to cellulose, hemicellulose and lignin was determined using the *Goering and Van Soest method* (Goering and Van Soest, 1970). Specific activity evaluation of the enzyme was based on the determination of the protein released by bovine serum albumin following the *Folin-Lowry* technique (Lowry, 1951). The RSC was determined using the 3, 5-dinitrosalycilic acid DNS (Sigma-Aldrich, 10 g L^{-1}), following the Miller method (Miller, 1959). The yield of enzymatic hydrolysis was calculated following the procedure of (Delgado *et al*., 2009): Considering a complete reaction, where the molecular weight (MW) of cellulose is 162*n (where n is the number of molecules of glucose in a molecule of cellulose) and the n molecules of glucose release have a MW of 180, the ratio of stoichiometric factors is 180/162 of released glucose per gram of cellulose. Then, the maximum concentration of RS that can be obtained can be calculated as following:

$$MC_{RS} = \frac{180}{162} X_{WS} C_{WS} \tag{1}$$

where MC_{RS} is the maximum concentration of RS that can be obtained (mg/mL), C_{WS} is dried WS concentration in the enzymatic hydrolysis media (mg/mL), and X_{WS} is the fraction of cellulose + hemicellulose in the dried substrate. Then, the yield can be calculated as follows:

$$\text{Enzimatic hydrolysis yield} = \frac{RS_C}{MC_{RS}} \times 100 \tag{2}$$

where RS_C is the RS concentration in a specific experiment. In our study, C_{WS} was 10 mg/mL and X_{WS} was 0.825 mg/mg, consequently the maximum concentration of RS that can be obtained is 9.16 mg/mL.

The specific reaction velocity (S_{RV}) is the amount of RS produced in micromoles per gram of protein per minute (U/mg).

$$S_{RV} = \frac{RS_P}{p\ t} \tag{3}$$

Where RS_P is the amount of RS production in one hour, P is the amount of protein in mg present in the enzymatic complex, and t is the time in minutes.

3 Theory/calculation

A CCD was used to study the effects of pH, T and E/S_R on the hydrolysis yield. The experiments were carried out in triplicate as independent experiments in order to take into account the non-adjustable data and allow the calculations of the analysis of variance (ANOVA). The ranges and levels of independent input variables are shown in Table 2. The model was built with five central points; the design must include center runs to provide reasonably stable variance of the predicted response. Generally, three or five center runs are recommended (Montgomery, 2009). The RSP curves were obtained over the time course of a batch experiment. Table 2 shows the experimental parameters and experimental CCD levels used.

Three significant independent variables pH, temperature, and E/S_R were included in this study, the mathematical relationship between the response of these variables and the independent variables can be presented by second-degree quadratic polynomial equation (Montgomery, 2009):

Table 3. Experimental design and summary of results for dependent variables

Experiment no.	Independent variables			Dependent variables		
	X_1	X_2	X_3	RSC (mg/ml)	Yield (%)	S_{RV} (U/mg)
1	4.3	48	0.33	6.02 ± 0.31	63.18 ± 2.57	5.37 ± 0.60
2	4.3	48	1.46	6.09 ± 0.80	65.52 ± 5.04	3.64 ± 0.72
3	4.3	62	0.33	2.11 ± 0.21	21.82 ± 2.19	9.95 ± 1.58
4	4.3	62	1.46	4.24 ± 0.42	43.89 ± 4.41	1.91 ± 0.17
5	5.7	48	0.33	4.99 ± 0.16	51.57 ± 1.69	8.67 ± 0.31
6	5.7	48	1.46	4.55 ± 0.47	61.12 ± 2.97	1.38 ± 0.36
7	5.7	62	0.33	0.54 ± 0.07	5.61 ± 0.78	0.58 ± 0.06
8	5.7	62	1.46	0.78 ± 0.06	8.11 ± 0.63	0.16 ± 0.03
9	4	55	0.9	2.98 ± 0.36	32.26 ± 1.23	4.04 ± 0.43
10	6	55	0.9	2.94 ± 0.12	30.41 ± 1.28	2.11 ± 0.33
11	5	45	0.9	2.64 ± 0.13	27.31 ± 1.30	1.98 ± 0.20
12	5	65	0.9	1.33 ± 0.07	14.17 ± 0.72	0.90 ± 0.04
13	5	55	0.1	5.06 ± 0.45	39.17 ± 2.66	0.07 ± 0.01
14	5	55	1.8	4.32 ± 0.12	48.29 ± 4.01	1.67 ± 0.09
15	5	55	0.9	5.44 ± 0.02	55.29 ± 2.45	4.12 ± 0.77
16	5	55	0.9	4.85 ± 0.61	54.40 ± 2.24	3.91 ± 0.59
17	5	55	0.9	4.52 ± 0.69	57.57 ± 5.53	4.39 ± 0.99
18	5	55	0.9	4.54 ± 0.46	59.82 ± 4.16	5.17 ± 1.51
19	5	55	0.9	4.71 ± 0.64	51.93 ± 6.17	4.36 ± 0.36

$$Z = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \varepsilon \quad (4)$$

where Z is the predicted value, in this case RS_C or S_{RV}, β_0 is the intersection term, X1 is the pH, X2 is the temperature, X3 is the enzyme substrate ratio, β_1, β_2 and β_3 are the linear coefficients, β_{11}, β_{22} and β_{33} are the quadratic coefficients, β_{12}, β_{13} and β_{23} are the interactive coefficients and ε is the total error. The accuracy and general ability of the above polynomial model were evaluated by the coefficient of determination R^2 and the adjusted R^2. The optimal values were obtained solving the regression Eq. 4 by the standard least square method and analyzing the response surface contour. The analysis of the response surface, the ANOVA and the optimal conditions were obtained using JMP 10.0.2 software. The significant effects on dependent variables were determined by *t*-test with a probability value (*p*-value) smaller than 0.05.

4 Results and discussions

4.1 Effect of enzymatic complex on pure substrates and not pretreated WS

The effect of enzymatic complex on pure substrate and WS without pretreatment after 40 minutes are shown in Figure 1 and it is possible observe that the quantity of RS is higher when the enzymatic complex was used on CMC, avicel, xylan of oat and xylan of birch compared with WS without pretreatment after 40 min of reaction. The result founded after 240 min of reaction is even much lower than that founded with pure substrates. Is worth noting that the trial realized with not pretreated WS contained 5 times more enzymatic complex than that done with pure substrates. This result showed the need of a pretreatment prior to enzymatic assay. Lin *et al*., 2010, realized experiments with different composition of the principal components of lignocellulose (cellulose, hemicellulose and lignin), the results shown that a less content of lignin increases the enzymatic hydrolysis yield.

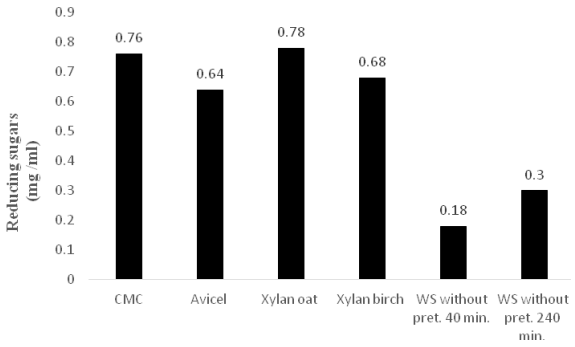


Fig. 1. Reducing sugars production after 40 min of hydrolysis reaction of pure substrates (CMC, Avicel, Xylan oat and birch) and WS without pretreatment. Assay temperature 50°C. The enzymatic complex was 5 times higher for WS without pretreatment.

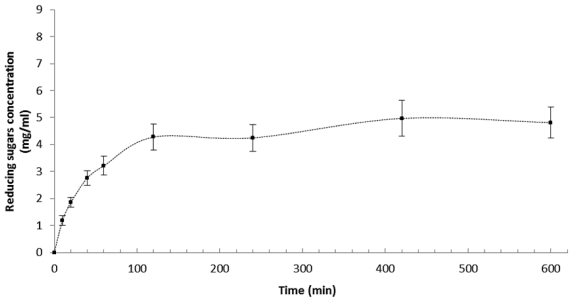


Fig. 2. Representative plot of the enzymatic hydrolysis in microscale assay, treatment 15 to 19.

4.2 Pretreatment of WS by alkaline peroxide.

WS was analyzed for chemical components after alkaline-peroxide pretreatment. The cellulose, hemicellulose and lignin before and after pretreatment are shown in Table 1. As can be seen in Table 1, the percent cellulose and hemicellulose content of pretreated WS were increased from 54% to 60.9% and from 18.9% to 21.6% respectively. In contrast, the percent lignin content after alkaline peroxide pretreatment decreased from 15.17% to 5.37% thus removing 64.60% of lignin from the raw material. The percentage of delignification is similar to that obtained by Benkun *et al*., (2009), which reached a 65.97% of delignification with 1.50% (w/v) of NaOH, at 150 rpm, 50 °C, for 6 h, using a solid:liquid ratio of 1:25 of WS pretreated by alkaline peroxide. Also, the work presented by Sun *et al*., (2000), showed that rye straw pretreated by alkaline peroxide (2% H_2O_2 at pH 11.5 and 50 °C for 12 h) showed dissolution of 83.1% of original lignin and 70.0% of original hemicelluloses, which is higher percentage of delignification compared whit this work, neverthelees, the time used in this research is low and the percentage of delignification is acceptable according whit Benkun *et al*., (2009). In addition, Patel *et al*., (1992), optimized alkaline peroxide pretreatment for the delignification of raw straw obtaining a 62% lignin solubilization which also, is according to our research. It is important to mention, that an increase in cellulose content in pretreated WS would increase the level of reducing sugars obtained from enzymatic saccharification, while a decrease in hemicellulose and lignin content could improve the efficiency of enzymatic hydrolysis of lignocellulosic materials (Mussatto *et al*., 2007).

4.3 Kinetic studies of enzymatic hydrolysis in microscale system

Table 2, shows the experimental data basis for the model fitting, which consists of the experimental design and the summary of the results. The experiments were conducted in a random order. Experimental results are averages of three independent experiments and their respective standard deviation. Figure 2 shows the kinetic behavior, with error bars, of the saccharification process for the five central points of the CCD (experiments 15 to 19), with a X_1 = 5, X_2 = 55°C and X_3 = 0.9 mL/g respectively. The average of RSC at 600 min was 4.81 ± 0.57 mg/mL, with the corresponding yield of 49.69%. The other treatments show similar behavior, although rates of the various parameters measured and their maximum concentrations were different in each case. Figure 2 shows, that was possible to obtain a complete hydrolysis kinetic in 10 h and not in 72 h like the conventional experiments developed by other authors (Benkun *et al*., 2009, Fujii *et al*., 2009), which is advantages in order to obtain kinetic data for the study of the enzymatic hydrolysis in a relative short time. Alvira *et al*., 2010 obtained a hydrolysis time of 72 h for microscale system and flask assays using Acellerase 1000. Our results showed that is possible obtained relative short time for enzymatic hydrolysis in micro assays. The difference in the hydrolysis time in this research can be attributed to the use of a different enzymatic complex.

Table 4. Analysis of Variance (ANOVA) for the Quadratic Model RS_C

Source	DF	SS	MS	F value	Probability (P) > F
model	9	13190.3	1465.6	11.0	< 0.001*
Error	47	6257.1	133.1		
Lack of fit	5	5306.0	1061.2	46.8	
pure error	42	951.0	22.6		
C. Total	56	19447.3			

Table 5. Significance of the coefficients of Regression for RS_C

Model term	Parameter estimate	Standard error	F value	p-value
Model constant	-98.1	21.76	-4.37	<0.0001*
pH	17.3	5.19	3.33	0.0017*
T	2.3	0.55	4.22	0.0001*
E/S_R	1.0	4.45	0.22	0.8288
pH*T	-0.09	0.05	-1.91	0.0622
pH* E/S_R	-0.38	0.57	-0.66	0.5154
T* E/S_R	0.04	0.06	0.67	0.5043
pH*pH	-1.3	0.45	-2.87	0.0061*
T*T	-0.02	0.01	-4.26	<0.0001*
E/S_R * E/S_R	-0.30	0.64	-0.47	0.6436

4.4 Effects of the variables on the hydrolysis yield

The statistical treatment combinations of the test variables along with the measured response values, expressed as hydrolysis yield corresponding to each combination, are summarized in Table 3. The RS_C response as a function of pH, temperature and E/S_R was evaluated and Table 3 shows a summary of the results including the corresponding yield. The application of RSM yielded the following regression equation, which was an empirical relationship between reducing sugars concentration and the test variables:

$$RS_C = -98.10 + 17.29X_1 + 2.31X_2 + 0.96X_3 - 0.1X_1X_2 - 0.04X_1X_3 + 0.04X_2X_3 - 1.28X_1^2 - 0.02X_2^2 - 0.29X_3^2 \quad (5)$$

An ANOVA test was carried out to verify the statistical significance of the model. The results are shown in Table 4. The model F value of 11.0 and values of probability (P) > F (< 0.0001) showed that the model terms are significant. The R^2 value is always between 0 and 1. The closer the R^2 is to 1.0, the stronger the model and the better it predicts the response. Normally, a regression model with an R^2 higher than 0.90 is considered to have a very high correlation (Haaland, 1989). The adjusted R^2 value corrects the R^2 value for the sample size and for the number of terms. A high value of adjusted determination coefficient (adjusted R^2 = 0.95) advocates for a high significance of the model. If there are many terms in the model and the sample size is not very large, the adjusted R^2 may be noticeably smaller than R^2 (Montgomery, 2009).

Table 5 shows the F test and the corresponding p-value along with the parameter estimate. While the p-value is smaller than 0.05 the significance of the corresponding coefficient is bigger. The parameter estimates and the corresponding p-values suggest that, the independent variables, pH and T, have significant effects on hydrolysis yield. The quadratic terms of pH and T also have significant effects on the hydrolysis yield. A statistically significant model only with significant terms can be written as follows:

$$RS_C = -98.10 + 17.29X_1 + 2.31X_2 - 0.10X_1X_2 - 1.28X_1^2 - 0.02X_2^2 \quad (6)$$

The response surfaces were plotted to analyze the

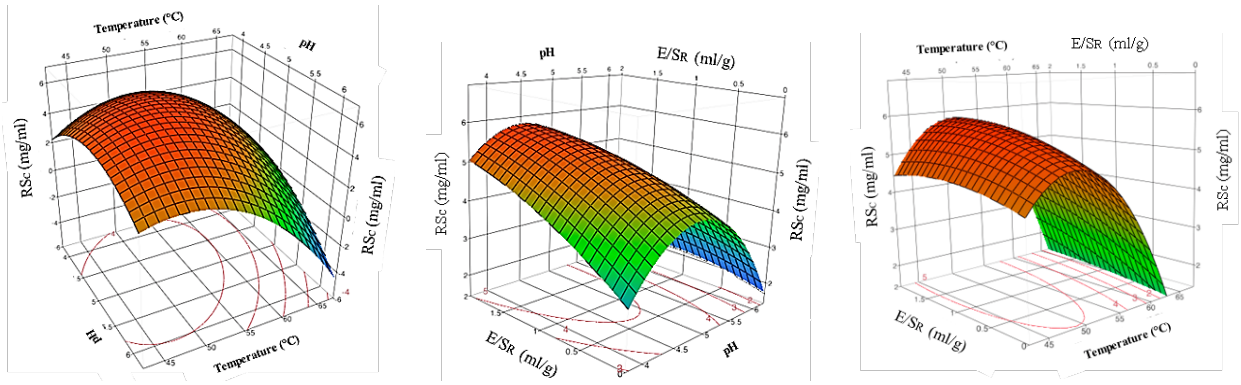
effects of the interactions between the variables and for the determination of the optimum RSC of the saccharification of WS in a microscale system. The optimal value of RSC, given by the model, was 5.97 mg/mL and the corresponding yield was 61.73%. It was achieved with an X_1 = 4.6, X_2 = 52°C and X_3 = 2.1 mL/g substrate.

The effect of temperature and pH on the hydrolysis yield of WS, when E/S_R is at center point, is shown in Figure 3a. When the value of pH is low and T is increased from 45°C to 52°C, the value of RSC will increase until it reaches the maximum value, nevertheless when the temperature increases from 52°C to 65°C the RSC decreases. Sun *et al*., (2002) and Tabka *et al*., (2006), have reported that the increase in temperature from 37 °C to 50 °C improved the hydrolysis of cellulose in the enzymatic treatment. Several works have shown the use of a temperature of 50°C (Benkun *et al*., 2009; Lin *et al*., 2010), and is well known that enzymes usually operate under mild conditions: pH from 4 to 9 and T from 24 to 71°C (Fogler, 2001).

The effect of pH over the yield is also shown in Figure 3a, and it is possible observe that the yield increase when the pH increases from 4 to 4.5 and decrees from 4.5 to 6 and the temperature is held at a low level. Benkun *et al*., (2009), founded that the optimal yield of the process was at pH 4.8, also other authors have used pH 5 for all them experiments of enzymatic hydrolysis (Lin *et al*., 2010; Alvira *et al*., 2010). These values of pH are closer of the value of pH founded in this research.

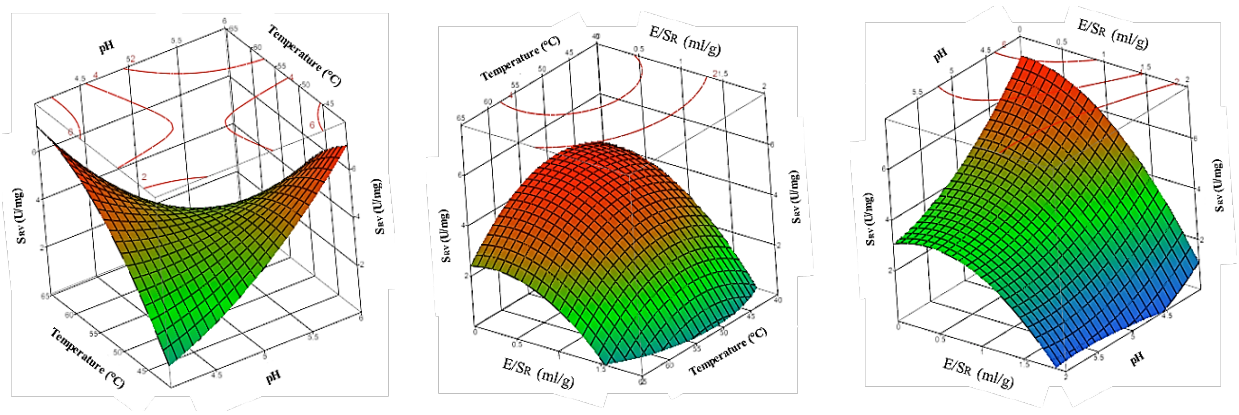
Figure 3b, shows the effect of temperature and E/S_R when pH is the center point. Figure 3b shows that RS_C was increased when E/S_R increased from 0 to 2 mL/g. This behavior was observed by Benkun *et al*., (2009) when increased the enzyme loading from 10 FPU/g to 50 FPU/g. The enzyme loading used by (Benkun *et al*., 2009), in filter paper units (FPU), was in the range of 10 to 50 FPU/g substrate. Our results showed that the increases of E/S_R permit increases the hydrolysis yield. Also, the yield reached in this research with a E/S_R of 28.5 FPU/g substrate (61.73%) was higher than the founded by Xu *et al*., (2007), who performed enzymatic hydrolysis of ammonia liquor pretreated soybean straw using an enzyme loading of 50 FPU/g substrate and obtained a maximum hydrolysis yield of 51.22% at 5% (w/v) substrate concentration for 36 h.

Figure 3c, also shows that the production of RS increases with increasing the reaction temperature from 45 to 52°C and after this range the production decreases. It is also notes that the E/S_R affect positively the RS production when it increases from 0 to 2 mL/g.



a) b) c)

Fig. 3: a) Response surface plot and contour plot of the combined effects of pH and temperature on reducing sugars concentration. b) Response surface plot and contour plot of the combined effects of pH and E/S_R on reducing sugars concentration. c) Response surface plot and contour plot of the combined effects of temperature and E/S_R on reducing sugars concentration.



a) b) c)

Fig. 4: a) Response surface plot and contour plot of the combined effects of pH and temperature on specific reaction velocity. b) Response surface plot and contour plot of the combined effects of enzyme-substrate ratio and temperature on specific reaction velocity. c) Response surface plot and contour plot of the combined effects of enzyme-substrate ratio and pH on specific reaction velocity.

4.5 Effects of the variables on the specific reaction velocity

Effects of pH, temperature and E/S_R on S_{RV} during hydrolysis of pretreated WS were examined. The observed values of SRV are presented in Table 3. A regression analysis was applied on the data in Table 3 and the obtained second-order polynomial equation could well explain the S_{RV} as follows:

$$S_{RV} = -70.60 + 7.14X_1 + 2.38X_2 - 3.53X_3 - 0.31X_1X_2 + 0.78X_1^2 - 0.01X_2^2 - 1.54X_3^2 \quad (7)$$

The statistical significance of the above equation was checked by the F test, and the analysis of variance (ANOVA) for the response surface quadratic model is shown in Table 6. The model F value of 53.51 and values of probability (P) > F (<0.0001) showed that the model terms are significant. A high value of adjusted determination coefficient $R^2 = 0.93$ advocates for a high significance of the model. The parameter estimates and the corresponding p-values suggest that temperature and the interaction between pH and temperature has significant effects on S_{RV} (Table 7).

In this case the maximum predicted provided by the model, Eq. (7), for S_{RV} was 4.80 U/mg and was obtained with the following conditions: pH 5.0, temperature of 48.5°C and an E/S_R of 0.19 mL/g. The response surface plots base on Eq. (7) with one variable being kept constant at their optimum values and variations of the other 2 variables within the experimental range are depicted in Figure 4. Fig. 4a-c were plotted with pH and temperature, pH and E/S_R and temperature and E/S_R, respectively, being kept constant. Results indicated that the solution is a maximum. In Figure 4a, is possible observe that exist two points whit high value of the SRV and are positioned when pH is low and T high, and when pH is high and T is low. A logic behavior of the system is found when the pH is proximate to pH 4, and is well know that a high temperature accelerate enzymatic reactions. The Figure 4b shows the effect of T and E/S_R on S_{RV}. Is possible observe in Figure 4b that a low level of E/S_R increase the S_{RV}.

Table 6. Analysis of Variance (ANOVA) for the Quadratic Model of S_{RV}

Source	DF	SS	MS	F value	p-value
model	8	11398.6	1045.30	10.0	< 0.001*
Error	39	5934.3	98.50		
Lack of fit	4	4632.0	834.87	42.17	
pure error	34	893.0	19.80		
C. Total	47	16923.6			

Table 7. Significance of the Coefficients of Regression for S_{RV}

Model term	Parameter estimate	Standard error	F value	p-value
Model constant	-70.868	41.976	-1.69	0.098
pH	7.187	10.011	0.72	0.476
T	2.384	1.056	2.26	0.0287*
E/S_R	-3.506	8.582	-0.41	0.685
pH*T	-0.309	0.089	-3.45	0.0012*
pH* E/S_R	0.651	1.110	0.59	0.560
T* E/S_R	0.017	0.111	0.16	0.873
pH*pH	0.775	0.863	0.9	0.374
T*T	-0.008	0.008	-1	0.324
E/S_R * E/S_R	-1.578	1.226	-1.29	0.204

The Figure 4c shows the effect of pH and E/S_R. When E/S_R was at a low level, an increase of pH resulted in a high increase in S_{RV}.

5 Validation of the model

5.1 Microscale validation model

In order to confirm the validity of the statistical experimental strategies of microscale system, one experiment with 10 reproductions was made. For this experiment the conditions were pH 4.6, a temperature of 52°C and an E/S_R of 1.8 mL/g substrate. The experiment with error bars is shown in Figure 5. The experiment showed a final value for RS_C of 5.98 ± 0.81 mg/mL. The predicted value obtained with the RS_C second order equation was 5.96 mg/mL. This result indicates a % error of 0.33. Usually, it is important to check the adequacy of the model to ensure that it provides maximum approximation on the relationships between factors and response. The residual from the least squares is an important tool for judging model adequacy (Li *et al.*, 2007). Normal probability was checked by plotting the normal probability plot of residuals. The normality assumption is satisfactory as the normal residual falls along a straight line as shown in Figure 6. In the Figure 7 is the plot of internally studentized residuals versus predicted response. The residual plots of the model are randomly distributed without any trends. This result indicates good predictions of maximum response along with constant variance and adequacy of the quadratic models (Mu *et al.*, 2006).

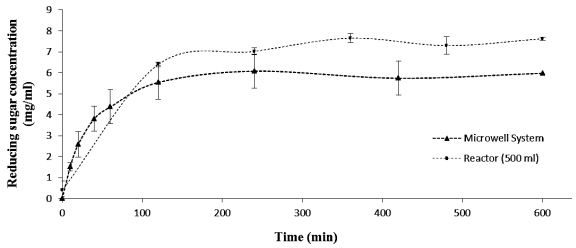


Fig. 5. Comparative plot between microscale system and 500 ml scale using pH 4.6, T 52°C and an E/S_R of 1.8 ml/g.

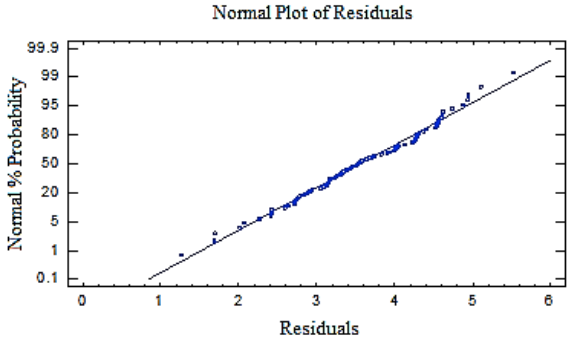


Fig. 6. Normal probability of residuals for WS hydrolysis.

5.2 Effect of volume assay

An experiment was performed in triplicated on a scale of 500 mL using the same conditions tested in microscale previously shown in Section 5.1. The trajectory of enzymatic hydrolysis of microscale and 500 mL scale are compared in Figure 5. It is observed that the kinetics of enzymatic hydrolysis of both scales is similar, and the difference in the amount of RS is not

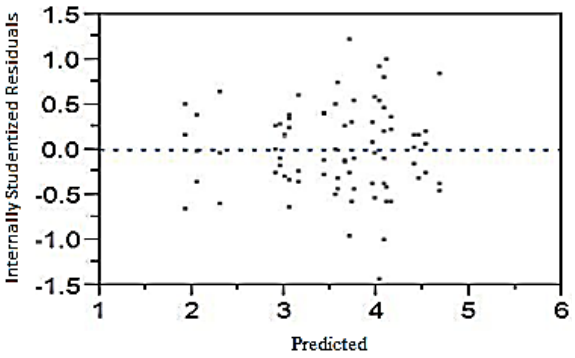


Fig. 7. Plot of internally studentized residuals-vs-predicted response.

significant statistically ($p > 0.05$). It is also observed that the full trajectory of enzymatic hydrolysis for both scales was reached at 10 h of operation. This was confirmed considering that the RS_C in the time of 48 h (8.02 mg of RS/mL) is only 4% higher than that found at 10 h (7.7 mg/mL). Alvira *et al*., 2010, showed a comparison between microscale and 20 mL scale. Found that the development of the trajectory of the enzymatic hydrolysis in two scales was similar, however, the complete enzymatic trajectory was only reached after 72 h of operation.

Conclusion

The efficiency of pretreatment of WS by alkaline peroxide was similar to that showed in similar works, obtained a percentage of delignification of 64.60%. The decrease in the content of lignin could facilitate the process of enzymatic hydrolysis. The RSM was performed to investigate the enzymatic hydrolysis of pretreated WS for production of reducing sugars. The analysis of variance showed that pH and temperature and the square value of pH and temperature have a significant effect on the enzymatic hydrolysis yield. Specific reaction velocity was affected significantly by pH and temperature and the interaction between pH and temperature. The hydrolysis of pretreated WS can be studied in a short time, since the reaction time was just 10 h. The enzyme kinetic hydrolysis for microscale and 500 mL scale was similar until reached 4 h of reaction. The regression equation predicted properly the value of the confirmation experiment. This reaction system enables one to carry out a large number of experiments at a controlled temperature in an economic way.

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Acronyms

MW	molecular weight
RS	reducing sugars
MC_{RS}	maximum concentration of RS
WS	wheat Straw
X_{WS}	fraction of cellulose + hemicellulose in the dried substrate
C_{WS}	dried WS concentration in the hydrolysis media
RS_C	RS concentration
S_{RV}	specific reaction velocity
RS_P	RS production in one hour
P	protein
t	time
T	temperature
E/S_R	enzyme substrate ratio
RSM	response surface methodology

References

Articles:

Alfaro, A., Rivera, A., Perez, A., Yáñez, R., García, J. C., and López, F. (2009) Integral valorization of two legumes by autohydrolysis and organosolv delignification. *Bioresourse Technology 100*, 440-445.

Alvira, P., Negro, M. J., Sáez, F., & Ballesteros, M. (2010). Application of a microassay method to study enzymatic hydrolysis of pretreated wheat straw. *Journal of Chemical Technology & Biotechnology 85*, 1291-1297.

Araque, E., Parra, C., Freer, J., Contreras, D., Rodriguez, J., Mendonca, R., Baeza, J. (2008). Evaluation of organosolv pretreatment for the conversion of Pinus radiate D. Don to ethanol. *Enzyme Microbiology Technology 43*, 214-219.

Bailey MJ, Biely P and Poutanen K, (1991). Interlaboratory testing of methods for assay of xylanase activity. *Journal of Biotechnology 23*, 257-270.

Benkun Q., Xiangrong C., Fei S., Yinhua W. (2009). Optimization of enzymatic hydrolysis of wheat straw pretreated by alkaline peroxide using response surface methodology. *Industrial and Engineering Chemistry Research 48*, 7346-7353.

Berlin A, Maximenko V, Bra R, Kamg KY, Gilkes N and Saddler J, A rapid microassay to evaluate enzymatic hydrolysis of lignocellulosic substrates. *Biotechnology and Bioengineering 93*, 880-886 (2005).

Brudecki, G., I. Cybulska, K. Rosentrater & J. Julson. (2012). Optimization of clean fractionation processing as a pre-treatment technology for prairie cordgrass. *Bioresourse Technology 107*, 494-504.

Chen J., Zhang W., Zhang H., Zhang Q., Huang H. (2014). Screw extrude steam explosion: A promising pretreatment of corn stover to enhance enzymatic hydrolysis. *Bioresource Technology 161*, 230-235.

Chundawat SPS, Balan V and Dale BE, (2008) High-throughput microplate technique for enzymatic hydrolysis of lignocellulosic biomass. *Biotechnology and Bioengineering 99*, 1281-1294.

Delgado R., Castro A.J., Vázquez M. A. (2009). Kinetic assessment of the enzymatic hydrolysis of potato (Solanum tuberosum). *LWT - Food Science and Technology 42*, 797-804.

Fujii, T., X. Fang, H. Inoue, K. Murakami & S. Sawayama. (2009). Enzymatic hydrolyzing performance of Acremonium cellulolyticus and Trichoderma reesei against three lignocellulosic materials. *Biotechnology for Biofuels 2*, 24.

Ghose TK, Measurement of cellulase activities. (1987). *Pure and Applied Chemistry 59*, 257-268.

González-Rentería S., Soto-Cruz N., Rutiaga-Quiñones O., Medrano-Roldán, Rutiaga-Quiñones J. and López-Miranda J. (2011). Optimization of the enzymatic hydrolysis process of four straw bean varieties. *Revista Mexicana de Ingeniería Química 10*, 17-28

Grande, P. M. and de María P. D. (2012). Enzymatic hydrolysis of microcrystalline cellulose in concentrated seawater. *Bioresource Technology 104*, 799-802.

Jeya, M., D. Kalyani, S. S. Dhiman, H. Kim, S. Woo, D. Kim & J. K. Lee. (2012). Saccharification of woody biomass using glycoside hydrolases from Stereum hirsutum. *Bioresource Technology 117*, 310-6.

Karthika K., Arun A.B., Rekha P.D. (2012). Enzymatic hydrolysis and characterization of lignocellulosic biomass exposed to electron beam irradiation. *Carbohydrate Polymers 90*, 1038-1045

Kawai T., Nakazawa N., Ida H., Okada S., Tani J., Sumitani T., Kawaguchi W., Ogasawara Y., Kobayashi Y. (2012). Analysis of the saccharification capability of high-functional cellulase JN11 for various pretreated biomasses through a comparison with commercially available counterparts. *Journal of Industrial Microbiology and Biotechnology 39*, 1741-9.

Kim ES, Lee HJ, Bang WG, Choi IG and Kim KH, Functional characterization of a bacterial expansin from Bacillus subtilis for enhanced enzymatic hydrolysis of cellulose. *Biotechnology and Bioengineering 102*, 1342-1353 (2009).

Lequart C., Nuzillard J., Kurek B., Debeire P. (1999). Hydrolysis of wheat bran and straw by an endoxylanase: production and structural characterization of cinnamoyl-oligosaccharides. *Carbohydrate Research 319*, 102-111

Li, Y., Cui, F., Liu, Z., Xu, Y., Zhao, H. (2007). Improvement of xylanase production by Penicillium oxalicum ZH-30 using response surface methodology. *Enzyme and Microbial Technology 40*, 1381.

Lin, L., Yan R., Liu Y., & Jiang W. (2010). In-depth investigation of enzymatic hydrolysis of biomass wastes based on three major components: cellulose, hemicellulose and lignin. *Bioresource Technology 101*, 8217-23.

Liu, Q., Cheng K., Zhang J., Li J., Wang G. (2010). Statistical optimization of recycled-paper enzymatic hydrolysis for simultaneous saccharification and fermentation via central composite design. *Applied Biochemistry and Biotechnology 160*, 604-12.

Lowry, O., Rosebrough, N., Farr, A. Lewis and Randall, Rose J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry 193*, 265-275.

M.G. Tabka, I. Herpoël-Gimbert, F. Monod, M. Asther, J.C. Sigoillot, Ma, H., Liu W., Chen X., Wu Y., Yu Z. (2009). Enhanced enzymatic saccharification of rice straw by microwave pretreatment. *Bioresource Technology 100*, 1279-84.

Martín-Sampedro R., Eugenio M.E., García J.C., López F., Villar J.C. Diaz M.J. (2012). Steam explosion and enzymatic pre-treatments as an approach to improve the enzymatic hydrolysis of Eucalyptus globulus. *Biomass and Bioenergy 42*, 97-106

Micheletti M., and Lye G. J. (2006). Microscale bioprocess optimization. *Current Opinion in Biotechnology 17*, 611-618.

Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry 31*, 426-428.

Mu, Y., Wang, G., Yu, H. Q. (2006). Response surface methodological analysis on biohydrogen production by enriched anaerobic cultures. *Enzyme and Microbial Technology 38*, 905.

Mussatto, S. I., Fernandes, M., Milagres, A. M. F., Roberto, I. C. (2007). Effect of hemicellulose and lignin on enzymatic hydrolysis of cellulose from brewer's spent grain. *Enzyme and Microbial Technology 43*, 124.

Patel, M., Bhatt, R. (1992). Optimization of the alkaline peroxide pretreatment for the delignification of rice straw and its applications. *Journal of Chemical Technology and Biotechnology 53*, 253.

Poonam S. and Anoop S. (2011). Production of liquid biofuels from renewable resources. *Progress in Energy and Combustion Science 37*, 52-68.

Primo E., Gil C.I., García F.J. (1995). Hydrolysis of corn-cob lignocellulosic residue from pentose preparation. *Bioresource Technology 52*, 1-4.

Saha BC, Iten LB, Cotta MA, Wu YV. (2005). Dilute acid pretreatment enzymatic saccharifiaction and fermentation of wheat Straw to etanol. *Process Biochemistry 40*, 3693-700.

Song, L., Siguier B., Dumon C., Bozonnet S., O'Donohue M. (2012). Engineering better biomass-degrading ability into a GH11 xylanase using a directed evolution strategy. *Biotechnology for Biofuels 5*, 3.

Sun, R., Fang, J., Tomkinson, J. (2000). Delignification of rye straw using hydrogen peroxide. *Industrial Crops and Products 12*, 71.

Sun, Y., Cheng, J. (2002). Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresource Technology 83*, 1.

Toyokazu M., Seung-Hwan L., Seiichi I., Takashi E. (2012). Combined pretreatment using ozonolysis and wet-disk milling to improve enzymatic saccharification of Japanese cedar. *Bioresource Technology 126*, 182-186.

Xu, Z., Wang, Q., Jiang, Z., Yang, X.-X., Ji, Y. (2007). Enzymatic hydrolysis of pretreated soybean straw. *Biomass and Bioenergy 31*, 162.

Books chapters:

Fogler Scott. (2001). *Fundamentos de reacciones enzimáticas, en Elementos de Ingeniería de las Reacciones Químicas*. Prentice Hall, México. P. 383.

Goering, H. K., Van Soest, P. J. (1970). Forage fiber analysis (apparatus, reagent, procedure and some applications). In *Agricultural Handbook* no. 379; US Department of Agriculture: Washington, DC, p 1.

Haaland, P. D. (1989). Separating signals from the noise. In *Experimental Design in Biotechnology*. Marcel Dekker: New York, p 61.

Montgomery D.C. (2009). Response surface methods and designs, in: *Design and Analysis of Experiments*, 7th ed., Wiley, USA. Pp. 417-480.

Internet:

IEA Biofuels for transport-an international perspective. International Energy Agency (IEA) (2004) http://www.iea.org/textbase/nppdf/free/2004/biofuels2004.pdf

Accellerse 1500^{TM}. Product information (2014). http://accellerase.dupont.com/fileadmin/user_upload/live/accellerase/documents/DUP-00413_ ProdSheet_ 1500_ web.pdf