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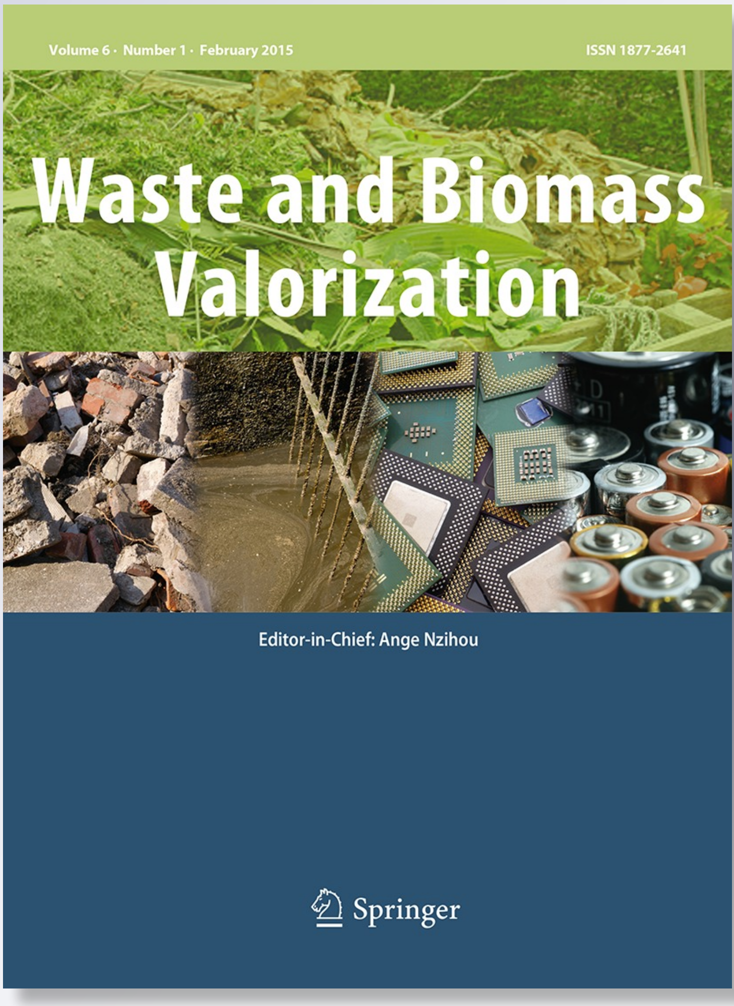

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ORIGINAL PAPER

Acid Pretreatment of Two Phase Olive Mill Waste to Improve Bioavailable Sugars: Conditions Optimization Using Response Surface Methodology

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Abstract Two phase olive mill waste (TPOMW) is the semisolid waste generated for olive oil production. The organic fraction of this waste includes compounds that serve as a source of carbon and other nutrients for microorganisms, a feature that makes it potentially attractive for bioethanol production. To increase the bioavailability of cellulose and hemicellulose, no soluble carbohydrates, the application of pretreatments is required. This paper presents the results of the optimization of the sulfuric acid pretreatment variables of TPOMW applied to improve sugars bioavailability, using the response surface methodology. First, the Plackett–Burman method was applied to find the relevant variables of the process, using total sugars content as response variable. The results showed that temperature, acid concentration and solid:liquid ratio are significant for this process. Subsequently, these variables were optimized to maximize total sugars content, using the Box–Behnken method, an experimental array based on an incomplete three-level factorial design. The results indicate that the optimum working conditions were 10.10 % w/w for H_2SO_4 concentration, 95.2 °C for temperature, and 0.17 g dry solid TPOMW per ml of acid solution, obtaining 67 % of sugars content increase.

Keywords Residual biomass · Acid pretreatment · Sugars · Optimization

Introduction

Biofuels global production has grown rapidly in the last decade, but the sustainability of many first-generation biofuels, which are mainly produced from food crops such as cereals, sugar cane and vegetable oils, has been increasingly questioned. This has increased the attention focused on the production of the so called second generation biofuels, which are those obtained from lignocellulosic materials. This production route has many advantages, including the ability to use waste materials from other economic activities, such as agroindustrial activities [1, 2].

Biomass is mainly composed of cellulose, hemicellulose and lignin, with small amounts of other components such as pectins, proteins and ash. The proportion of these components varies from one plant species to another and also with age, stage of growth and other plant conditions [3].

Two phase olive mill waste (TPOMW) is the semisolid waste generated in olive oil production by a two-phase extraction system, which represent an important environmental problem in Argentine, where it is generated in huge quantities in short periods of time, and may have a great impact on land and water environments because of their high phytotoxicity [4]. In San Juan about 50,000 tons of this highly polluting waste (COD 230–240 g/kg and BOD 90 g/kg, approximately) are produced annually, whose treatment and disposal is a serious environmental problem.

There are few alternatives for its reuse and some of them are still in experimental stages. Among them, its use for the production of second generation biofuels is particularly attractive. An important advantage of this alternative is to

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produce biofuels with no additional land requirements or impacts on food production. On the contrary, high transport costs are associated with its semisolid consistency and the large volumes that must be taken to the processing plants.

Cellulose is the major constituent of plant cell walls and takes the form of an organized fibrous structure. The hemicellulose and lignin cover these fibrils. Cellulose is present mainly in its crystalline form and only a small percentage is amorphous cellulose, which is more susceptible to be enzymatically degraded [5]. Hemicellulose is a complex carbohydrate structure, composed by monomers such us pentoses (xylose, arabinose), hexoses (mannose, glucose and galactose) and acid sugars. Lignin is an amorphous heteropolymer consisting of three different phenylpropane units (p-coumaryl, coniferyl and sinapil alcohol) linked together by various types of bonds. It gives support, impermeability and resistance to microbial attack to the plant. It is insoluble in water and optically inactive and its degradation is very difficult.

The conversion process of biomass to ethanol comprises cellulose transformation into simple sugars by hydrolysis, and the subsequent sugars fermentation for ethanol production. The first step can be performed by acid or enzymatic hydrolysis [6]. The presence of lignin and hemicellulose in lignocellulosic materials hinder the accessibility of the acid or enzymes to cellulose, reducing the efficiency of the hydrolysis [7]. Therefore, the application of pretreatments to these materials is unavoidable, in order to alter the size, structure and composition of the biomass, so that the hydrolysis of the carbohydrate fraction to monomeric sugars can be accomplished quickly and with high efficiency. These treatments are focused on the breakdown of lignin molecules present in this kind of material.

Pretreatment methods can be divided into: physical (grinding), physicochemical (pyrolysis, steam treatment, hydrothermolysis, wet oxidation), chemicals (alkalis, acids, oxidizing agents and organic solvents), biological, electrical or their combination. Chemical pretreatment with dilute sulfuric acid, is effective and easy to implement [8]. Variables, such as temperature and acid concentration, have a marked influence on the processes involved in the pretreatment and it is necessary finding their optimal values in order to obtain maximum yields.

One of the most important steps in the development of an efficient and economically viable method is the optimization of the pretreatment conditions. The response surface methods are an effective optimizing tool when several factors and their interactions affect the response. The response surface methodology (RSM) has been used by other authors in different environmental area [9–11].

Among these, the Box–Behnken method has been widely used and successfully applied to the pretreatment of biomass by other authors [12, 13].

This paper addresses the problem of TPOMW valorization in order to improve the cellulose hydrolysis releasing the maximum amount of soluble sugars. Firstly, a characterization of the olive mill waste to determine their composition was carried out. Afterwards, the Plackett–Burman method was applied to find the relevant variables of the acid pretreatment process. Finally, these variables were optimized to maximize total sugars content, using an experimental array based on an incomplete three-level factorial design, the Box–Behnken method. Such information is very useful for the researches on integrated process of the ethanol production from biomass.

Materials and Methods

Materials

Fresh TPOMW, provided by SOLFRUT S. A., was kept at −15 °C in order to preserve the starting conditions. For the material characterization, a portion of it was dried in an oven at 105 °C until constant weight.

Sulfuric acid used for acid pretreatment and total sugar determination was analytical grade with purity of 98 %. Phenol used for total sugar determination was analytical grade with purity above 99 %.

Characterization

The moisture content was obtained following AOAC 930.15 [14]. The volatile matter, ash and fixed carbon contents were determined with Shimadzu DTG-60 thermogravimetric equipment, applying ASTM E-1131 [15].

The content of cellulose, hemicellulose and lignin was determined by neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid-insoluble lignin (AIL).

The cellulose and hemicellulose content were estimated by the difference between NDF–ADF and ADF–AIL respectively. Acid insoluble lignin content was determined by a gravimetric method, stated in ASTM D 1106-96 norm [16], in which the sample is treated with acid in order to hydrolyze the carbohydrates, making them soluble and leaving an insoluble residue, which corresponds to the lignin content.

Phenol–sulfuric method was used to determine total sugar content [17]. This method is based on the formation of a colored compound by the reaction between sugars, phenol and sulfuric acid, which was determined in a Hach DR-2010 spectrophotometer. This determination was performed on the aqueous extract of the sample, obtained by adding a volume of water to it, shaking for 30 min and then, liquid–solid separation by filtration and centrifugation.

Pretreatment

The selected method consisted in treating the TPOMW with sulfuric acid at the operating conditions fixed by the experimental design explained below. Samples of TPOMW were contacted with sulfuric acid solutions at different concentrations in a stainless steel reactor of 1 l with automatic temperature control.

Experimental Design

RSM is a set of mathematical and statistical techniques that are useful for modeling and analyzing problems in which a response of interest is influenced by several variables and the objective is to optimize this response [12]. Many variables may potentially affect the efficiency of the process. In this study, a Plackett–Burman design was used to identify the relevant variables of acid pretreatment process and Box–Behnken method was employed to determine the effects of independent variables on the response and the optimal value these variables.

Variables Screening Experimental Design Plackett–Burman design screens and evaluates the significance of the variables in the pretreatment process. This design does not consider the interaction effects among variables. In the present work, four assigned variables were screened in eight experiences. Each variable was examined at two levels: −1 for low level and +1 for high level. The ratio of mass of glucose released to dry biomass was taken as response. The factors that were included in the screening experiment and their settings are given in Table 1. Table 2 presents the experimental design of the Plackett–Burman method, where the levels adopted for the variables in each assay are shown. All tests were carried out stirring at 220 rpm and at the vapor pressure corresponding to the temperature of each.

Relevant factors identification was carried out by calculating their effect and t value. Factor effects were calculated as $Ex = \frac{\sum Z(+) - \sum Z(-)}{N/2}$, where E_X is the effect of factor X, $\sum Z(+)$ and $\sum Z(-)$ the sums of the responses where factor X is at (+) or (−) level, respectively, and N is the number of experiments.

t values were calculated as the ratio effect/standard error and compared with the *t* Student value obtained from tables at a confidence level.

Table 1 Variables and set levels for pretreatment

Variables	Levels	
	−	+
H_2SO_4 concentration (w/w %)	1	10
Temperature (°C)	20[a]	120
Time (minutes)	10	90
S/L ratio (g/ml)	1/10	1/5

S/L solid/liquid

[a] Room temperature

Experimental Design for Optimization of the Relevant Variables The optimal conditions for the acid pretreatment of TPOMW were determined through a three factors and three levels Box–Behnken experimental design. Three central points (Co = 3) were taken and the resulting design involved a total of 15 experiments. The ranges for the variables taken as the center points are presented in Table 3 and the experimental design is showed in Table 4.

The response variable selected to evaluate the effect of different conditions in acid treatment was the total sugars content, determined on the aqueous extract of treated TPOMW and performed by triplicate.

The software Design-Expert 8.0.7.1-Stat-Ease™ was used for experimental design, data analysis, and quadratic model building. Both linear and quadratic effects of the three variables under study were calculated, as well as their possible interactions on released total sugar mass. Their significance was evaluated by variance analysis (ANOVA).

Three replicates at the center point were carried out to calculate the sum of squares of pure error. Experiments were randomized to maximize the effects of unexplained variability in the observed responses because of extraneous factors.

Three-dimensional response surface plots were drawn to illustrate the effects of the independent variables on the dependent variable, being described by a quadratic polynomial equation obtained by multiple regression. The fit of the model was evaluated by the determination of R-squared and adjusted R-squared coefficients.

Results and Discussion

Material Characterization

The TPOMW is a material with high moisture content (78 %). The results of the physicochemical characterization are presented in Table 5, obtained according to the mentioned techniques. Determinations were performed on dry sample.

For comparison purposes, the composition of different materials used to produce ethanol by other researchers is presented in Table 6. It can be observed that the ash content of TPOMW is low, which is an advantageous feature for ethanol production. In the other hand, a low content of cellulose and hemicellulose is observed. Besides, the lignin

Table 2 Plackett–Burman experimental design

Assay	[H_2SO_4]	Temp	Time	S/L	Dummy	Dummy	Dummy
1	+	−	−	+	−	+	+
2	+	+	−	−	+	−	+
3	+	+	+	−	−	+	−
4	−	+	+	+	−	−	+
5	+	−	+	+	+	−	−
6	−	+	−	+	+	+	−
7	−	−	+	−	+	+	+
8	−	−	−	−	−	−	−

Table 3 Variables levels set for the Box–Behnken design

Variables	Range	Central points
Temperature (°C)	20–200	110
H_2SO_4 concentration (% w/w)	5–20	12.50
S/L ratio (g/ml)	0.05–0.2	0.13

Table 4 Box–Behnken: optimization experimental design

Trial	[H_2SO_4] (% w/w)	Temperature (°C)	S/L (g/ml)
1	−	−	0
2	+	−	0
3	−	+	0
4	+	+	0
5	−	0	−
6	+	0	−
7	−	0	+
8	+	0	+
9	0	−	−
10	0	+	−
11	0	−	+
12	0	+	+
13	0	0	0
14	0	0	0
15	0	0	0

to cellulose content ratio is higher in this residue. For these reasons, pretreatment of this material is a very important step in order to release all its sugar content for subsequent fermentation for bioethanol production.

Variables Screening

Plackett–Burman design, used as a screening method, served to determine which of four factors (see Table 1) considered influential, affect significantly the process.

Table 5 TPOMW physicochemical characterization

Parameters	Value
Ash (% w/w)	4.9
Volatile matter (% w/w)	80.0
Fixed carbon (% w/w)	15.1
Cellulose (% w/w)	14.4
Hemicellulose (% w/w)	11.1
Lignin (% w/w)	14.6
Total sugars (mg/g)	90.6

This is achieved by simultaneously shifting variables from a low to a high value. The levels for the variables were chosen from preliminary assays results (data not shown). In Plackett–Burman design, the ratio of released sugars mass by dry biomass varied between 70.11 and 192.36 mg/g. The values of the variables and their combinations in each test are shown in Table 7.

The t values calculated for each factor were compared with the *t* Student value (3,143) obtained from tables at a 99 % confidence level. Figure 1 shows the calculated t value for each variable and the dotted line indicates the tabulated one. All those variables whose calculated t value is higher than the tabulated one are considered significant for this pretreatment process. In this case, the relevant variables were: H_2SO_4 concentration, temperature and solid/liquid Ratio.

Relevant Variables Optimization

The application of the Box–Behnken method yielded the optimal values of the relevant variables.

Table 8 shows the levels of the variables adopted for each test and value of response variable obtained for each one. The time, which is not significant for the process, was set at 30 min.

Table 6 Characterization of different materials used to produce bioethanol

Material	Lignin (%)	Cellulose (%)	Hemicellulose (%)	Ash (%)	References
Corn stover	17.2	36.1	21.4	7.1	Kim and Lee [18]
Wheat straw	17.0	39.0	38.7	1.8	Varrone et al. [19]
Rice straw	13.3	31.1	22.3	14.5	Chen et al. [20]

Table 7 Placket–Burman design and obtained responses

Trial	$[H_2SO_4]$ (% w/w)	Temperature (°C)	Time (min)	S/L (g/ml)	Total sugars (mg/g)[a]
1	10	20	10	1/5	85.2
2	10	120	10	1/10	192.4
3	10	120	90	1/10	177.8
4	1	120	90	1/5	73.5
5	10	20	90	1/5	69.7
6	1	120	10	1/5	86.4
7	1	20	90	1/10	70.1
8	1	20	10	1/10	61.7

[a] mg total sugars/g dry TPOMW

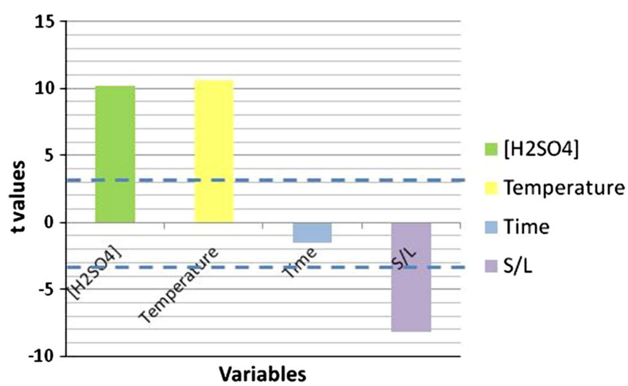

Fig. 1 Calculated (*bars*) and tabulated (*dotted lines*) *t* Student values of the studied variables of TPOMW acid pretreatment

Response surface analysis (RSA) of the data showed in Table 8 demonstrates that the relationship between total sugars content and temperature, acid concentration and S/L ratio is quadratic. The mathematical model representing total sugars yield as a function of the independent variables within the region under investigation was expressed by the equation:

$$Y = -51.97189 + 1.67113 \times A + 2.63703 \times B + 1117.88704 \times C + 0.019222 \times AB + 18.72444 \times AC - 1.71259 \times BC - 0.32619 \times A^2 - 0.013384 \times B^2 - 3463.62963 \times C^2 \quad (1)$$

where Y is the total sugars content predicted in mg of total sugars/g of dry TPOMW, A is the H_2SO_4 concentration (% w/w), B is the temperature (°C), and C is the solid to liquid ratio (S/L, g/ml).

The experimental data were statistically analyzed by analysis of variance (ANOVA), and the results are shown in Table 9. The ANOVA of the quadratic regression model indicated that it was significant, since the p value (0.0404) is below 0.05, which corresponds to a confidence level of 95 %. Also the lack of fit was not significant relative to the pure error, supporting the fitness of the model.

Table 10 reports relevant statistical values for the model fit. The simplified response surface model developed in this study for predicting the sugars yield was adequate. Gutierrez and Salazar [21] suggested that, for a good fit of a model, adjusted R^2 should be at least 0.70. This parameter was, in the present work, 0.7347, indicating that the regression model explained the variables interaction well. The fit degree of the model was high enough to explain 73.47 % of the results. Thus, this model can be applied to predict total sugars content (Y) within the experimental setting range.

Adequate precision is a measure of the contrast in predicted response relative to its associated error, in other words a signal to noise ratio. Its desired value should be four or more, according with the software prescriptions. In this case a value of 5.824 indicates a suitable signal; therefore this model can be used to navigate the design space.

The relationship between total sugars yield and significant variables for this pretreatment are shown in response surfaces graphs (Fig. 2). The three dimensional response

Table 8 Box–Behnken design with total sugars content as response variable

Assay	[H_2SO_4] (% w/w)	Temperature (°C)	S/L (g/ml)	Total sugar content (mg/g)[a]
1	5	20	0.13	84.63
2	20	20	0.13	42.89
3	5	200	0.13	5.31
4	20	200	0.13	15.47
5	5	110	0.05	120.23
6	20	110	0.05	54.10
7	5	110	0.20	176.84
8	20	110	0.20	152.84
9	12.5	20	0.05	42.51
10	12.5	200	0.05	17.75
11	12.5	20	0.20	77.25
12	12.5	200	0.20	6.25
13	12.5	110	0.13	209.00
14	12.5	110	0.13	129.85
15	12.5	110	0.13	152.65

[a] mg glucose/g dry alperujo

Table 9 ANOVA results for TPOMW acid pretreatment

Source	Sum of square	*df*	Mean square	F-value	*p* value (prob. > F)	
Model	56789.04	9	6,309.89	5.31	0.0404	Significant
A-Conc.	1,851.67	1	1,851.67	1.56	0.2673	
B-Temp.	5,125.78	1	5,125.78	4.31	0.0925	
C–S/L	3,986.80	1	3,1986.80	3.35	0.1266	
AB	673.40	1	673.40	0.57	0.4856	
AC	443.73	1	443.73	0.37	0.5680	
BC	534.53	1	534.53	0.45	0.5323	
A^2	1,243.00	1	1,243.00	1.05	0.3535	
B^2	43,395.02	1	43,395.02	36.50	0.0018	
C^2	1,401.54	1	1,401.54	1.18	0.3272	
Residual	5,945.18	5	1,189.04			
Lack of fit	2,625.21	3	875.07	0.53	0.7066	No significant
Pure error	3,319.96	2	1,659.98			
Cor. total	62,734.22	14				

Table 10 Indicators of accuracy of the model fit

R^2	0.9052
Media	85.84
Adjusted R^2	0.7347
Adequate precision	5.824

surfaces graphs are constructed using Eq. 1 and show the influence of variables on the total sugar content, keeping one constant at its optimum level, while the other two are varied within the experimental range selected [13].

The optimum conditions that maximize the response, determined by the software via numerical optimization method, were: temperature: 95.2 °C, solid/liquid ratio: 0.17 g/ml, H_2SO_4 concentration: 10.10 % w/w. The total sugar predicted was 174.32 mg glucose/g dry TPOMW.

To confirm the model adequacy for predicting maximum total sugars content, experiments by triplicate at the optimum conditions were carried out. An average maximum total sugars content of 151.53 mg glucose/g dry TPOMW was obtained, differing only in 13 % below with the predicted value. The good agreement between the predicted and experimental results verified the validity of the model and reflected the existence of an optimal point.

The total sugar content of untreated TPOMW was 90.60 mg/g of dry material. This means that applying a pretreatment with sulfuric acid at the optimum conditions increased 67 % the total sugars yield. This results in a

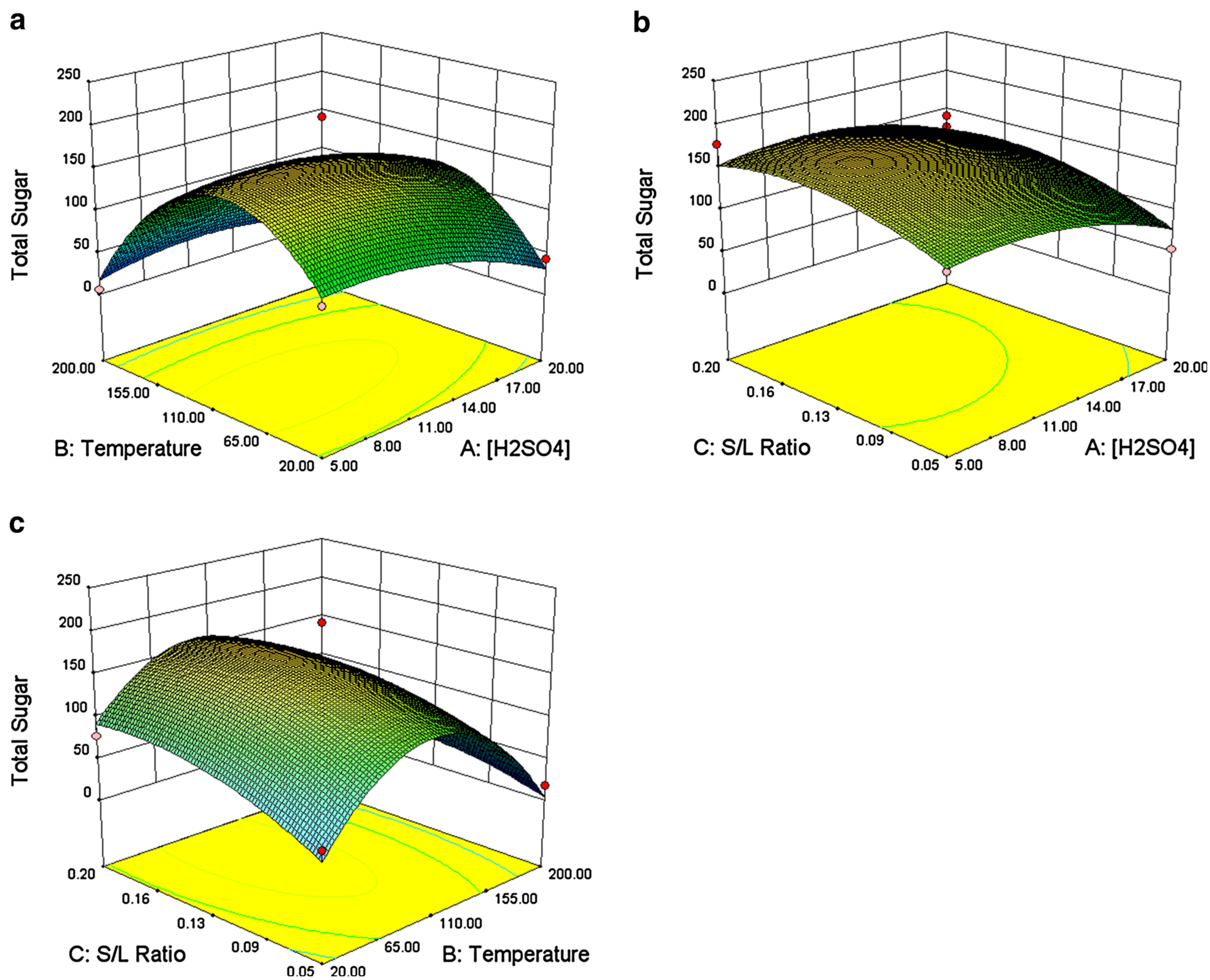

Fig. 2 Response surfaces showing the interaction effect of significant variables on total sugar yield (mg/g): **a** temperature (°C) and H_2SO_4 concentration (% w/w); **b** H_2SO_4 concentration (% w/w) and S/L ratio (g/ml); **c** temperature (°C) and S/L ratio (g/ml)

greater ability of the material to be subsequently subjected to a biological treatment for bioethanol production.

Conclusions

In this work the application of statistical methods to obtain the optimal values of the relevant variables of the acid sulfuric acid pretreatment of TPOMW, in order to improve the cellulose hydrolysis, are presented.

A Plackett–Burman design was used to identify the relevant variables in the acid pretreatment process applied for maximizing sugar bioavailability. It was found that temperature, sulfuric acid concentration and solid to liquid ratio have significant influence on the process.

The Box–Behnken design, based on RSM, was successfully employed to optimize TPOMW pretreatment conditions. A second-order polynomial model gave a satisfactory description of the experimental data. Optimal conditions were: 95.2 °C, 10.10 % (w/w) H_2SO_4 and 0.17 g/ml S/L rate. Good agreement was found between the value predicted and those determined experimentally.

Under optimal conditions total sugars content after pretreatment was 151.53 mg total sugar/g dry TPOMW. This means an increase of 67 % over the initial sugar content of this residue.

The model could be applied to optimize the conditions of acid pretreatment of any TPOMW from other provinces of Argentina and other parts of the world may differ in the optimal conditions. For future industrial application of the model should be tested on a larger scale.

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