Improvement of Enzymatic Hydrolysis of Steam-exploded Wheat Straw by Simultaneous Glucose and Xylose Liberation

M. Marcos, M. T. García-Cubero, G. González-Benito, M. Coca, S. Bolado, and S. Lucas*

Department of Chemical Engineering and Environmental Technology, University of Valladolid, Doctor Mergelina s/n, 47011 Valladolid, Spain

Original scientific paper
Received: October 4, 2012
Accepted: January 29, 2013

This work aimed at enhancing enzymatic hydrolysis of steam-exploded wheat-straw by investigating factors affecting hydrolysis. A multi-objective optimization of glucose and xylose release was performed using Celluclast 1.5L and Ultraflo-L mixtures with maximal values of 20 for enzyme/substrate ratio, 72 h for reaction time, 50 °C for temperature and 5.0 for pH. The highest sugar yields obtained were 18.9±0.4 g/100g_{DM} for glucose and 4.7±0.2 g/100g_{DM} for xylose. The addition of Ultraflo-L could increase the liberation of xylose, but has no pronounced effect on glucose release.

The effect of β-glucosidase addition to the Ultraflo+Celluclast mixture for improving sugar yield was also studied. The β-glucosidase supplementation increased the production by approximately 29.9 % for glucose and 5.9 % for xylose, when a β-glucosidase loading of 10 % $g_{\beta\text{-glucosidase}}/g_{cellulose}$ was used.

Key words:
Enzymatic hydrolysis, cellulase, β-glucosidase, RSM, multiobjective optimization

1. Introduction

Lignocellulosic biomass, for its large quantities and relatively low cost, is regarded as a potential renewable energy resource for cost-effective ethanol production.[1]

Wheat straw is one of the most abundant crop residues in European countries with a production of 170 million tonnes per year, and it seems to be one of the cheapest and the most useful raw material for ethanol production.[2] Wheat straw is composed of a mixture of cellulose and hemicellulose (45 % and 30 %, respectively) that are bound to lignin (approx. 25 % w/w DM) by hydrogen and covalent bonds.

One of the major limitations of the second generation (cellulosic and hemicellulosic) ethanol production is the sugar recovery step, where fermentable carbohydrates are released from biomass using enzymes.[3] This process step has to be further improved before commercialization of the process. There are several approaches to increase the efficiency and decrease the cost of cellulose and hemicellulose hydrolysis, i.e. better pretreatment techniques,[4,5] optimizing enzyme complex composition and operating variables of hydrolytic process[6–10] or addition of proteins, surfactants and other chemicals to enhance cellulose/hemicellulose conversion.[11]

The enzymes catalyzing the degradation of lignocellulosic material into fermentable sugars are a mixture of endoglucanases (EG), cellobiohydralases (CHB) and β-glucosidases. They act in synergism by targeting lignocellulosic material differently; endoglucanases randomly attack cellulose chains and release cello-oligosaccharides, cellobiohydrolases attack the ends of the cellulose and cleave cellobiose units off, thereby 'feeding' the cellobiohydrolases with cellulose ends and, finally, the β-glucosidases catalyze the hydrolysis of cellobiose and short chain oligosaccharides into glucose.[12] The addition of β-glucosidase greatly increases the rate and extent of hydrolysis (saccharification) by ensuring the efficient hydrolysis of cellobiose and reducing the influence of end-product inhibition.[13] The hydrolytic efficiency of a multi-enzyme mixture in the process of lignocellulose saccharification depends both on the properties of individual enzymes and their ratio in the multi-enzyme cocktail.[7,14,15]

There are many factors affecting enzymatic hydrolysis, including substrate concentration, reaction time, enzymatic activity (that depends on the temperature and pH), etc. To improve the yield of the saccharification stage, an optimization of the different processing parameters must be carried out. The aim of the optimization process is to obtain a more efficient enzymatic hydrolysis of lignocellulose (higher sugar concentrations to be converted into ethanol).[7]

*Corresponding author: Tel: +34 983184074; Fax: +34 983423616; E-mail address: susana@iq.uva.es

So far, published works on enzymatic hydrolysis of lignocellulosic materials are mainly based on the improvement of the pre-treatment step and their conditions including only a reduced number of specific parameters of hydrolysis step. The goal of these optimization works, using statistical methodologies, was to maximize the individual sugar liberation.[7,16–20]

In this work, a complete and systematic study of optimization of the enzymatic hydrolysis of steam-exploded wheat straw with recent cellulolytic and hemicellulolytic preparations, Celluclast 1.5L (main activity cellulase) and Ultraflo-L (main activity *β*-glucanase), has been carried out. Five independent variables (temperature, pH, enzyme/substrate ratio, Ultraflo/Celluclast ratio and hydrolysis time) were screened. Different experimental designs and statistical tools were used to generate an optimum experimental design and to optimize the enzymatic hydrolysis step, taking glucose and xylose concentrations as responses.

The aim of the optimization process was to find an optimal combination of operating factors that affect both glucose and xylose yields (sugars convertible into ethanol).

The effect of *β*-glucosidase supplementation to the Ultraflo+Celluclast mixture for improving the sugar yields was also analyzed. The influence of *β*-glucosidase loadings, the Ultraflo/Celluclast ratio and the hydrolysis time were tested and the optimum conditions for enhancing the release of sugars were determined.

2. Material and methods

2.1. Substrates and pretreatment

Wheat straw was kindly donated by the Castilla y León Institute of Technological Agriculture. The straw was ground in a blender and sieved to obtain a particle size of around 20 mm.

Steam explosion pre-treatment of wheat straw was carried out in a 5 L stainless steel batch reactor in which the straw was loaded at the top and heated to the previously determined optimum temperature (210 °C) with saturated steam injected directly into the reactor. When the pre-set residence time concluded (10 min.), the steam-treated biomass was released from the reactor by rapid depressurization of the vessel. The treatment results in substantial breakdown of the lignocellulosic structure, hydrolysis of the hemicellulosic fraction, depolymerization of the lignin components and defibrations.[18] After pre-treatment, the product was washed with warm water and the insoluble solids fraction was separated by filtration. The solid portion was dried in an oven at 45 °C for 24 h, stored in a freezer and used for enzymatic hydrolysis.

A complete description of the experimental setup and the optimization of the pretreatment operating conditions to maximize sugar yield have been detailed in previous works.[21–23]

The compositions (% w/w) of raw and pre-treated material were determined. For raw wheat straw, this material contains: cellulose (as glucose) 32.4 %, hemicellulose (as xylose) 19.1 %, acid insoluble lignin (AIL) 21.3 %, acid soluble lignin (ASL) 6.4 % and ash 6.9 %. In the case of pre-treated wheat straw, the composition was: cellulose (as glucose) 37.7 %, hemicellulose (as xylose) 16.8 %, acid insoluble lignin (AIL) 18.4 %, acid soluble lignin (ASL) 1.3 % and ash 4.3 %.

2.2. Enzymes

The commercial cellulase product Celluclast 1.5L has been studied in supplementation assays with Ultraflo-L and Novozyme 188 (*β*-glucosidase). These enzyme preparations and their product sheets were supplied by Novozymes A/S (Bagsvaerd, Denmark).

Celluclast 1.5L is a cellulolytic enzyme complex derived from *Trichoderma reesei*. It has cellulase activity but also hemicellulase activity provided by *β*-xylosidase, which is responsible for debranching xylobiose and short chain xylo-oligosaccharides. The enzyme mixture is likely to have high activity in the pH range 5–6 at 40–50 °C.

Ultralflo-L, derived from *Humicola insolens*, is a multi-component enzyme preparation that contains *β*-glucanase (endo-1,4-*β*-glucanase) and xylanase (endo-1,4-*β*-xylanase) as the main activities and several side activities, e.g.: cellulases, hemicellulase, pentosanase. This enzyme has optimal activity at pH 6 and temperature 40 °C.

Novozyme 188 is a commercial preparation of *β*-1,4-glucosidase (cellobiase) derived from *Aspergillus niger* that hydrolyzes cellobiose to glucose. The temperature optimum is around 50 °C and the pH stability range between 4–5.

2.3. Enzymatic hydrolysis

Enzymatic hydrolysis experiments of steam-exploded wheat straw were conducted under different operating conditions, which were studied with a design of experiments (DOE). The experimental variables considered in this work were temperature (A), pH (B), enzyme/substrate ratio (C), Ultraflo/Celluclast ratio (D) and hydrolysis time (E) when Celluclast 1,5L and Ultraflo L were employed (see Table 1).

Table 1 – *Experimental design and results obtained for enzymatic hydrolysis of steam-exploded wheat straw with Celluclast 1.5L*

Run	Experimental variables					Responses	
	temperature (°C)	pH	(E/S)[1] (w/w %)	ultraflo / celluclast (w/w %)	time (h)	glucose (g/100 g_{DM})[2]	xylose (g/100 g_{DM})[2]
1	50 (0)	5 (0)	9 (0)	25 (0)	72 (0)	13.1±0.4	4.5±0.0
2	60 (1)	4 (–1)	9 (0)	25 (0)	72 (0)	4.0±0.1	2.7±0.1
3	50 (0)	5 (0)	20 (1)	25 (0)	48 (–1)	15.2±0.2	4.0±0.1
4	40 (–1)	5 (0)	9 (0)	50 (1)	72 (0)	6.0±0.3	3.5±0.1
5	50 (0)	5 (0)	9 (0)	0 (–1)	48 (–1)	14.6±0.5	3.9±0.0
6	40 (–1)	5 (0)	9 (0)	25 (0)	48 (–1)	9.5±0.4	3.4±0.1
7	50 (0)	5 (0)	20 (1)	0 (–1)	72 (0)	19.9±0.1	4.5±0.1
8	50 (0)	4 (–1)	20 (1)	25 (0)	72 (0)	14.7±0.2	4.0±0.0
9	40 (–1)	5 (0)	9 (0)	25 (0)	96 (1)	9.9±0.2	4.1±0.1
10	50 (0)	4 (–1)	9 (0)	0 (–1)	72 (0)	12.6±0.3	4.0±0.1
11	60 (1)	5 (0)	9 (0)	25 (0)	96 (1)	8.9±0.1	3.1±0.1
12	60 (1)	5 (0)	9 (0)	50 (1)	72 (0)	5.6±0.3	2.5±0.0
13	50 (0)	4 (–1)	9 (0)	25 (0)	48 (–1)	8.1±0.2	3.9±0.0
14	50 (0)	5 (0)	9 (0)	50 (1)	48 (–1)	11.3±0.5	3.5±0.1
15	50 (0)	5 (0)	2 (–1)	50 (1)	72 (0)	3.7±0.3	2.9±0.1
16	50 (0)	4 (–1)	9 (0)	25 (0)	96 (1)	8.1±0.3	4.2±0.1
17	50 (0)	5 (0)	2 (–1)	0 (–1)	72 (0)	6.8±0.2	2.7±0.1
18	50 (0)	6 (1)	2 (–1)	25 (0)	72 (0)	3.4±0.2	2.0±0.1
19	60 (1)	6 (1)	9 (0)	25 (0)	72 (0)	2.6±0.4	1.8±0.0
20	50 (0)	6 (1)	9 (0)	50 (1)	72 (0)	8.0±0.1	4.2±0.1
21	40 (–1)	5 (0)	2 (–1)	25 (0)	72 (0)	3.1±0.1	2.9±0.1
22	60 (1)	5 (0)	2 (–1)	25 (0)	72 (0)	2.4±0.3	2.0±0.1
23	50 (0)	6 (1)	20 (1)	25 (0)	72 (0)	15.0±0.6	4.7±0.1
24	50 (0)	5 (0)	20 (1)	25 (0)	96 (1)	15.9±0.3	4.8±0.1
25	50 (0)	6 (1)	9 (0)	0 (–1)	72 (0)	11.2±0.1	3.4±0.1

[1](E/S) = (Enzyme/Substrate) where Enzyme = Celluclast + Ultraflo expressed in w/w % (g_{enzyme}/ $g_{cellulose}$)
[2]DM: Dry matter
Value in parenthesis represents coded factor levels

All the experiments were performed in 100 mL shake flasks using 3 % dry matter (DM) in a shaking incubator at 300 rpm. In these experiments, the pH was adjusted using citrate buffer.

A dry matter of 3 % w/w DM was selected for all the experiments. This value has been established to overcome end product inhibition and substrate transfer limitation that have not been taken into account in this study. Similar dry matter contents (2-5 %) have been used elsewhere in enzymatic hydrolysis of lignocellulosic materials.[10] Studies at high substrate loading are more properly tackled at pilot scale.

Once the operating conditions with Celluclast 1.5L and Ultraflo L were optimized, the enzyme *β*-glucosidase was added in order to increase the yield of sugars. These experiments were carried out under optimal conditions previously obtained, but changing the Ultraflo/Celluclast ratio and the *β*-glucosidase loading.

After the hydrolysis phase, 750 μL samples were withdrawn, passed through a 0.22 μm filter and stored for carbohydrate analysis by high-performance liquid chromatography (HPLC). Every test was conducted in triplicate and the mean value and standard deviation were calculated.

2.4. Analytical methods

Acid insoluble lignin, acid soluble lignin, cellulose and hemicellulose in the raw material were estimated using the procedure Determination of Structural Carbohydrates and Lignin in Biomass (NREL 2008).[24] On the other hand, a Bio-Rad HPX-87P ion-exclusion column was used to measure carbohydrate concentrations. The mobile phase was

water at a flow rate of 0.6 mL min^{-1} and 60 °C. The detector was based on the refraction index measurement (Waters 2414 refractive index detector).

2.5 Optimization of parameters for glucose and xylose production

Response Surface Methodology (RSM) was used for modelling enzymatic hydrolysis of pre-treated wheat straw with Celluclast 1.5L and Ultraflo L. The most commonly used methodology to determine response surfaces are full and fractional factorial designs and the more complex central composite, Box-Behnken, Doehlert and mixture designs.

2.5.1 Box-Behnken design

Response surface methodology (RSM) using the Box-Behnken design of experiments was used to determine simple response surfaces of the investigated factors and which factors do significantly affect the experimental results.

Five independent variables, namely temperature (A), pH (B), enzyme/substrate ratio (C), Ultraflo/Celluclast ratio (D) and time (E) were studied at three levels (-1 for the low level, 0 for the intermediate level, and +1 for the high level) as shown in Table 1.

Glucose and xylose productions were taken as response variables (g/100g_{DM}).

D-optimal

D-optimal designs are a type of design provided by a computer algorithm. This optimal criterion is based on minimizing the generalized variance of all parameter estimates for a pre-specified model. Given the total number of treatment runs for an experiment and a specified model, the computer algorithm chooses the optimal set of design runs from a candidate set of possible runs.

Multi-objective optimization

Multi-objective optimization is the simultaneous optimization of more than one objective. In this paper, two objectives are presented: maximization of the production of both glucose and xylose. This technique allows a common optimal point to be reached in order to maximize the two variables at the same time. MATLAB was the professional software used to determine the optimal conditions. Vectorial optimization was the mathematical method applied for the resolution of the problem.

In this study, Box-Behnken was the selected model for the design of experiments. With this experimental design, the number of runs was 46. However, when D-optimal was applied, the experimental runs were reduced to 25, as shown in Table 1. This represents a significant saving in cost and time. Both designs were performed by the professional software STATGRAPHICS Plus. Therefore, a total of 25 experiments for screening the assigned variables were carried out in triplicate.

ANOVA analysis was used to perform a statistical analysis of the experimental data. Three-dimensional surface plots were drawn to illustrate the effects of the independent variables on the dependent variables that can be described by a quadratic polynomial equation, by fitting of experimental data. Solving the regression equation, an optimum value of the selected variables was obtained using MATLAB software. A confirmatory experiment was carried out in order to verify this optimum.

3. Results and discussion

3.1. RSM design for optimization of enzymatic hydrolysis

Table 1 shows the experimental design and the responses obtained for each evaluated condition. It can be noted that the results varied strongly according to the hydrolysis conditions used. The values of glucose released vary from 2.6 to 19.9 g/100 g_{DM} and from 1.8 to 4.8 g/100g_{DM} for xylose concentration in the range of operating conditions tested.

3.1.1. Glucose release model

The Pareto chart (Fig. 1a) represents the estimated effects of the variables temperature, pH, enzyme/substrate ratio, Ultraflo/Celluclast ratio and time, affecting the glucose production response. The length of each bar is proportional to the standardized effect. Bars extending beyond the vertical line correspond to effects statistically significant at the 95 % confidence level.

The ANOVA analysis, which includes the multiple linear regression coefficients, significances (p-value) for a confidence level of 95 % and the standard error (SE) of the surface response model, is shown in Table 2a.

As may be observed from the Pareto chart and ANOVA analysis, the variables enzyme/substrate ratio, temperature, pH and Ultraflo/Celluclast ratio presented a statistical significance for the glucose concentration response. Moreover, the squared-temperature, the squared-pH and the squared-enzyme/substrate ratio are also significant interactions. The p-values for these variables and interactions were all lower than 0.05.

The enzyme/substrate ratio, temperature and pH are the most important factors affecting glucose

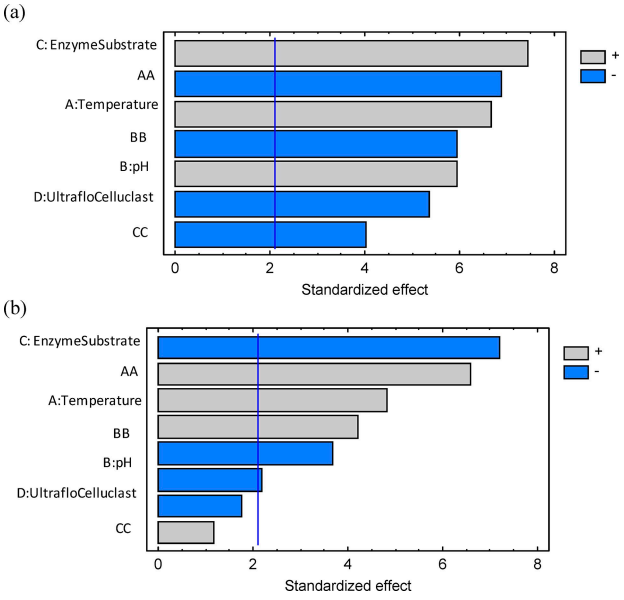

Fig. 1 – *Pareto chart for (a) Glucose production (g/100 g_{DM}) (b) Xylose production (g/100 g_{DM}). Bars extending beyond the vertical line correspond to effects statistically significant at 95% confidence level.*

liberation according to standardized effects. All of them presented a positive effect, suggesting that the use of the highest levels of these variables (E/S = 20; T = 60 °C and pH = 6) favoured the response. Similar results were reported in the literature for the response surface optimization process of enzymatic hydrolysis of *Cistus ladanifer* and *Cytisus striatus* with two commercial enzyme solutions: NS50013 (cellulose complex) and NS50010 (*β*-glucosidase).[6] This work concluded that pH, temperature and cellulase concentration were the most significant factors affecting enzymatic hydrolysis of forestry biomass.

The Ultraflo/Celluclast ratio has been demonstrated to have a lower effect on sugar liberation. The negative effect of the Ultraflo/Celluclast ratio reveals that the use of the lowest level of this variable (Ultraflo/Celluclast = 0 %) increased glucose release. This result is in agreement with a previous work where the influence of different enzyme preparations on arabinoxylan degradation was investigated.[15] As expected, treatments with Celluclast 1.5L, containing cellulase as the main activity, resulted in high glucose yields. However, treatment with Ultraflo/Celluclast = 50 % mixture did not give synergistically high levels of release of glucose at any of the reaction conditions tested (see glucose production data in Table 1).

The hydrolysis time was found to be a non-significant factor for glucose release, in a range of 48-96 h. This result may be ascribable to the long reaction times employed.

Equation 1 shows the response surface quadratic model for glucose production. This model describes the correlation between significant variables and the released glucose for enzymatic hydrolysis of steam-exploded wheat straw with Celluclast 1.5L. The R^2-value was 0.958, in good agreement with the adjusted R^2-value of 0.940. The high R^2-value 0.958 indicates that the model was well adapted to the response.

Table 2 – *Design and results of RSM design for (a) Glucose production and (b) Xylose production obtained from the enzymatic hydrolysis of steam-exploded wheat straw with Celluclast 1.5L*

Variable	(a) GLUCOSE MODEL		
	coefficient	F-ratio	P-value
Constant	–171.56		
A: Temperature	3.9223	43.91	< 0.0001
B: pH	32.421	34.93	< 0.0001
C: (E/S)	1.2177	55.11	< 0.0001
D: (Ultraflo/Celluclast)	–0.08475	28.55	0.0001
AA	–0.04000	46.95	< 0.0001
BB	–3.2290	35.07	< 0.0001
CC	–0.02783	15.96	0.0009

Variable	(b) XYLOSE MODEL		
	coefficient	F-ratio	P-value
Constant	–14.674		
A: Temperature	0.6870	43.15	< 0.0001
B: pH	1.161	1.34	0.2638
C: (E/S)	–0.1355	2.99	0.1032
E: Time	0.01265	17.51	0.0007
AA	–0.00742	51.52	< 0.0001
BB	–0.2090	4.67	0.0462
BC	0.06392	23.15	0.0002
CC	–0.00455	13.34	0.0021

$$\text{Glucose } (g/100g_{DM}) = -171.56 + 3.9223 \cdot \text{Temperature} + 32.421 \cdot \text{pH} + 1.2177 \cdot (E/S) - 0.08475 \cdot (\text{Ultraflo/Celluclast}) - 0.04000 \cdot \text{Temperature}^2 - 3.2290 \cdot \text{pH}^2 - 0.02783 \cdot (E/S)^2 \quad (1)$$

The relationship between the response and variables is visualized by the response surface plots to see the influence of the parameters.

The response surface plots for glucose release obtained for the optimization of the enzymatic hydrolysis of steam-exploded wheat straw with Celluclast 1.5L, as a function of two significant factors, are presented in Fig. 2a. These plots show the effect of (A) temperature and enzyme/substrate; (B) pH and enzyme/substrate ratio; (C) temperature and pH; (D) Ultraflo/Celluclast ratio and tempera-

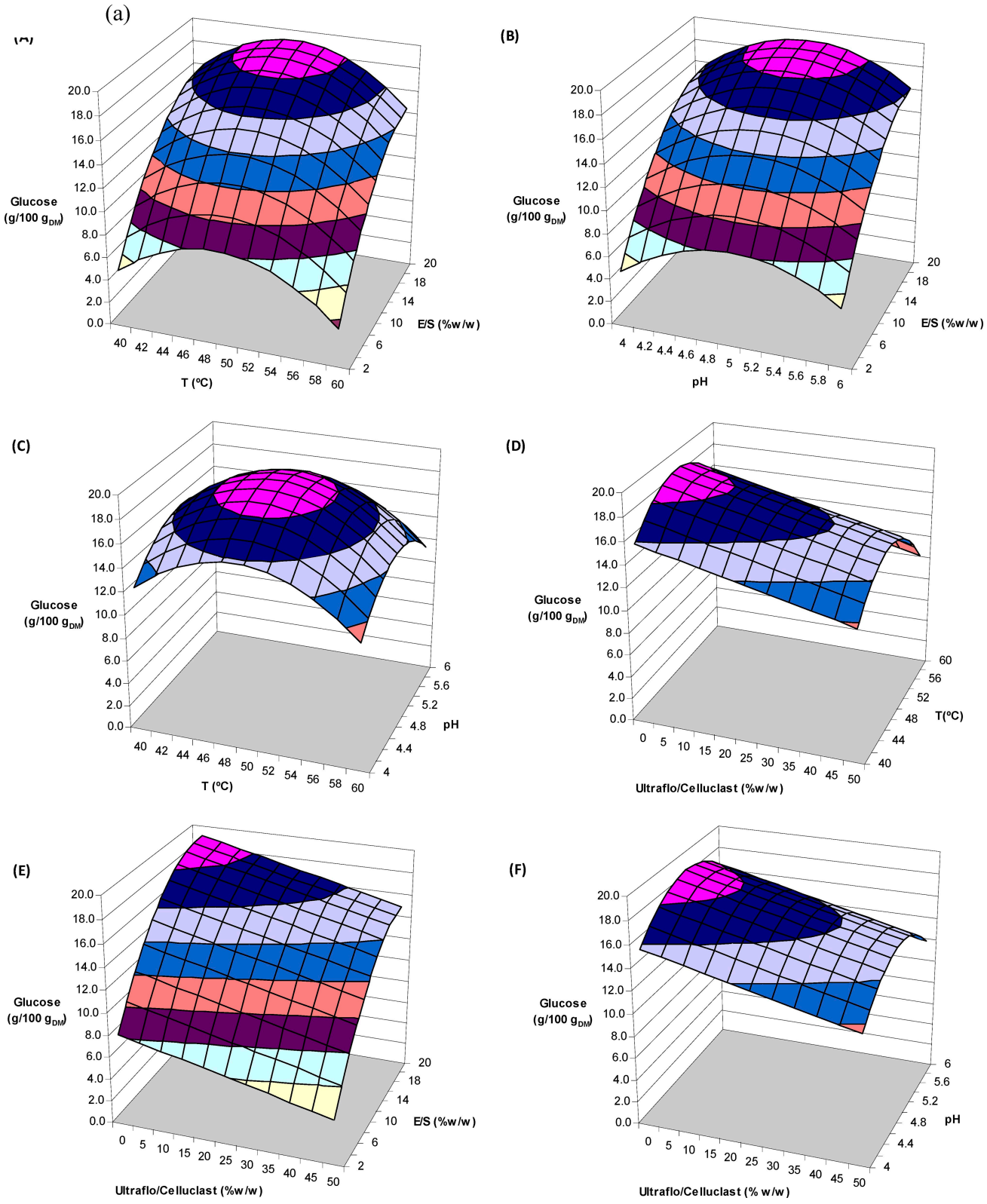

Fig. 2 – *Response surface plots of RSM design for the optimization of the enzymatic hydrolysis of steam-exploded wheat straw with Celluclast 1.5L. (a) Glucose production and (b) Xylose production.*

ture; (E) Ultraflo/Celluclast ratio and enzyme/substrate ratio; (F) Ultraflo/Celluclast ratio and pH, maintaining all other factors fixed at the optimum level.

These representations are helpful to visualize graphically the shape of a response surface and to understand how the response changes in a given direction by adjusting the design variables.

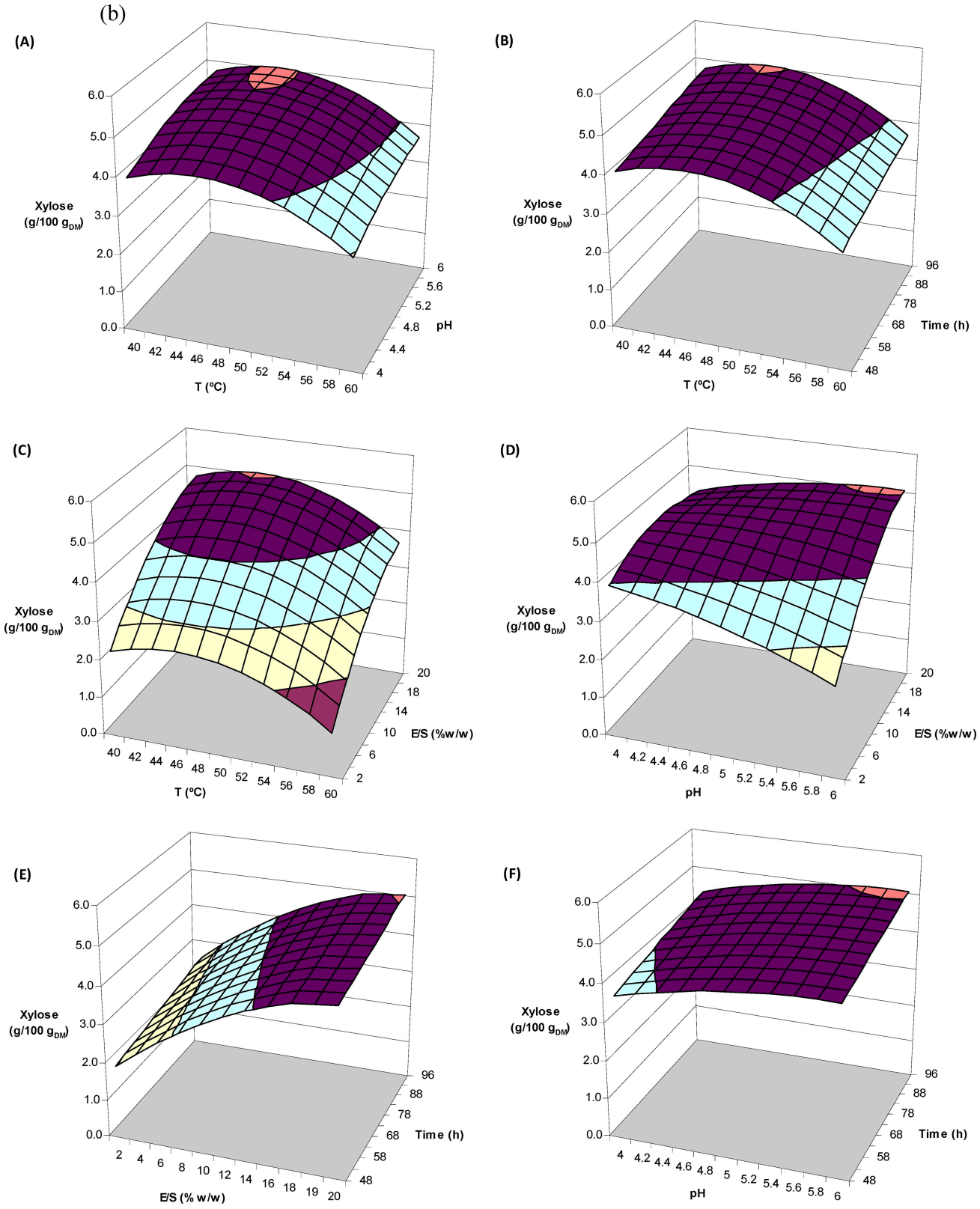

Fig. 2 – continued

3.1.2. Xylose release model

Pareto chart and ANOVA analysis for xylose concentration response are presented in Fig. 1b and Table 2b, respectively.

From these statistical tools, it could be concluded that the temperature and time variables presented a statistical significance for the xylose concentration response. Moreover, the squared-temperature, the pH-enzyme/substrate ratio, the

squared-enzyme/substrate ratio, and the squared-pH are also significant interactions.

The temperature and hydrolysis time were the most important factors affecting xylose liberation, according to standardized effects. Both of them presented positive effects, suggesting that the use of the highest levels of these variables favoured xylose release (T = 60 °C and time = 96 h). This conclusion is in agreement with that of Sørensen et al. who observed that higher enzyme dosage and temperature, and longer reaction time increased xylose yields from wheat arabinoxylan using Celluclast 1.5L, Ultraflo-L and the Celluclast 1.5L:Ultraflo-L blend.[15]

The variables enzyme/substrate ratio and pH were considered in the xylose model, as these individual factors are included in the significant interactions pH-enzyme/substrate ratio, squared-enzyme/substrate ratio and squared-pH.

The Ultraflo/Celluclast ratio has been found to be a non-significant variable for xylose liberation. However, higher values for xylose production were obtained when a ratio of Ultraflo/Celluclast = 25 % was used (see Table 1). This result suggests that there is a certain synergism between Celluclast 1.5L and Ultraflo-L in the release of xylose, as was suggested in previous works on purified wheat arabinoxylan.[15] The observed synergism between Celluclast 1.5L and Ultraflo-L is the result of positive interaction between α-L-arabinofuranosidase and endo-1,4-β-xylanase activities present in Ultraflo-L that released arabinose, xylobiose and xylotriose, and β-xylosidase activities in Celluclast 1.5L, capable of catalyzing the hydrolysis of xylobiose and xylotriose to xylose.

The response surface quadratic model for xylose release is presented in Equation 2. This model describes the correlation between significant variables and the released xylose during enzymatic hydrolysis of steam-exploded wheat straw with Celluclast 1.5L. The R^2-value was 0.960, in good agreement with the adjusted R^2-value of 0.940. The high R^2-value (0.960) and the low mean absolute deviation (0.140) indicate that the model was well adapted to the response.

$$\begin{aligned}\text{Xylose } (\text{g}/100\text{g}_{\text{DM}}) = {} & -14.674 + \\ & + 0.6870 \cdot \text{Temperature} + 1.161 \cdot \text{pH} - \\ & - 0.1355 \cdot (\text{E/S}) + 0.01265 \cdot \text{Time} - \\ & - 0.00742 \cdot \text{Temperature}^2 - 0.2090 \cdot \text{pH}^2 + \\ & + 0.06392 \cdot \text{pH} \cdot (\text{E/S}) - 0.00455 \cdot (\text{E/S})^2\end{aligned} \quad (2)$$

Response surface plots for xylose liberation obtained for the optimization of the enzymatic hydrolysis of steam-exploded wheat straw with Celluclast 1.5L, as a function of two significant factors, are presented in Fig. 2b. These graphs show the effect of (A) temperature and pH; (B) temperature and time; (C) temperature and enzyme/substrate ratio; (D) pH and enzyme/substrate ratio; (E) enzyme/substrate ratio and time; (F) pH and time when all other factors were constant at the optimum level.

3.1.3. Model validation

Mono-objective and multi-objective optimization techniques have been used to obtain the optimum values of the variables for enzymatic hydrolysis of steam-exploded wheat straw with Celluclast 1.5L, as shown in Table 3. Mono-objective optimization was used to optimize a single objective (glucose production or xylose production). On the other hand, multi-objective optimization was used to find the optimum values for the simultaneous liberation of both glucose and xylose.

To validate the model, the optimum values for both mono- and multi-objective optimization, were tested in triplicate.

The experimental responses for mono-objective optimization corresponding to the validation experiments were 19.0±0.3 g/100g_{DM} and 4.9±0.2 g/100g_{DM} for glucose and xylose release, respectively. These values are in good agreement with the predicted model values that were glucose concentration of 19.2 g/100g_{DM} and xylose concentration of 5.1 g/100$g_{DM,}$ considering a range of 95 % confidence.

For multi-objective optimization, the experimental responses were 18.9±0.4 g/100g_{DM} for glucose concentration and 4.7±0.2 g/100g_{DM} for xylose concentration. The predicted model values were 19.1 g/100g_{DM} for glucose concentration and

Table 3 – *Optimal values of the tested variables and predicted maximum with 95% confidence interval of released glucose and xylose (g/100g_{DM}). Mono-objective and multi-objective optimization.*

Variables	Interval		Mono-objective optimization		Multiobjective optimization
	low	high	glucose response	xylose response	glucose/xylose responses
A: Temperature (°C)	40.0	60.0	49.1	46.3	48.6
B: pH	4.0	6.0	5.0	5.8	5.1
C: Enzyme/substrate (w/w%)	2.0	20.0	20.0	20.0	20.0
D: Ultraflo/Celluclast (w/w %)	0.0	50.0	~ 0	24.4	~ 0
E: Time (h)	48.0	96.0	73.6	96.0	96.0
Predicted response (CL = 95%) (g/100g_{DM})			19.2	5.1	19.1 / 4.9
Experimental response (g/100g_{DM})			19.0±0.3	4.9±0.2	18.9±0.4 / 4.7±0.2

4.9 g/100g_{DM} for xylose concentration for a confidence level of 95 %. This behaviour shows a good fit between the model and the experimental results, confirming the validity and adequacy of the proposed models for glucose and xylose releases.

For simultaneous glucose and xylose optimization, it can be concluded that higher enzyme dosage level (E/S = 20) and longer reaction time (t = 96 h), led to higher glucose and xylose yields (Table 3). However, from an economic point of view, a time of 72 h is selected as the optimal value. The maximum glucose concentration was reached at 73.6 h. The increase in xylose concentration observed when reaction time was prolonged from 72 h to 96 h was not high enough (less than 3 %) to justify the operation at the longer time. This conclusion is in agreement with previous findings on forestry wastes, wheat starch fibre and purified arabinoxylan, where it was concluded that the higher the enzyme dosage with a relatively long time, the higher were the glucose and xylose concentrations obtained.[6,13,15] The optimum temperature (48.6 °C) and pH (5.1) were the intermediate values in the operating range analysed. Similar results were reported in the enzymatic hydrolysis of treated palm oil by using a combination of cellulase and *β*-1.4-glucosidase.[25] They concluded that, as pH and temperature increased, the glucose production also increased up to pH 4.8 and 50 °C (the range of pH was 4-6 and temperature 30–60 °C).

The addition of Ultraflo L could increase the liberation of xylose, but it has no pronounced effects on glucose release. The combination of the enzyme Celluclast 1.5L and Ultraflo L exhibited a strong synergistic interaction in catalyzing the release of xylose from wheat arabinoxylan, as has been reported in literature.[15]

3.2. Effect of *β*-glucosidase supplementation on enzymatic hydrolysis with Celluclast 1.5L and Ultraflo-L enzymes

In this section, the potential of *β*-glucosidase supplementation on the hydrolysis of steam-exploded wheat straw with Celluclast 1.5L and Ultraflo-L was studied. The aim of this supplementation was to enhance the enzymatic hydrolysis by increasing the overall sugar yield. The low *β*-glucosidase activity of Celluclast 1.5L may lead to the incomplete hydrolysis of cellobiose, resulting in the inhibition of the cellulase enzymes. This problem could be overcome by supplementation with extra *β*-glucosidase enzyme. The reduction of cellulase inhibition and the presence of some additional side activities contained in Novozyme 188 could result in an increase in the yield of fermentable carbohydrates.[13,25,26]

Experiments were conducted to study the effect of the *β*-glucosidase loading, the Ultraflo/Celluclast ratio, and the hydrolysis time on the yield of fermentable carbohydrates. Enzymatic hydrolysis was carried out with a 3 % DM of steam-exploded wheat straw (210 °C, 10 min). Temperature, pH and enzyme/substrate ratio were fixed at the optimum values obtained from multi-objective optimization, shown in section 3.1.4. (T = 50 °C, enzyme/substrate ratio = 20 $g_{enzyme}/g_{cellulose}$ (enzyme = Celluclast + Ultraflo) and pH = 5).

A total of 15 experiments were performed at hydrolysis times of 48 and 72 h (the optimum value without *β*-glucosidase supplementation), with *β*-glucosidase loadings of 0–10 % $g_{\beta\text{-glucosidase}}/g_{cellulose}$ and Ultraflo/Celluclast ratios ranging from 0 to 50 % $g_{Ultraflo}/g_{Celluclast}$. The set of experimental runs were performed in triplicate under the following operating conditions: Runs 1–5 (0% $g_{Ultraflo}/g_{Celluclast}$), runs 6–10 (25 % $g_{Ultraflo}/g_{Celluclast}$) and runs 11-15 (50 % $g_{Ultraflo}/g_{Celluclast}$), corresponding to *β*-glucosidase loadings ($g_{\beta\text{-glucosidase}}/g_{cellulose}$) of 0 %, 2.5 %, 5 %, 7.5 % and 10 % w/w, respectively.

Experimental results for *β*-glucosidase supplementation are presented in Fig. 3.

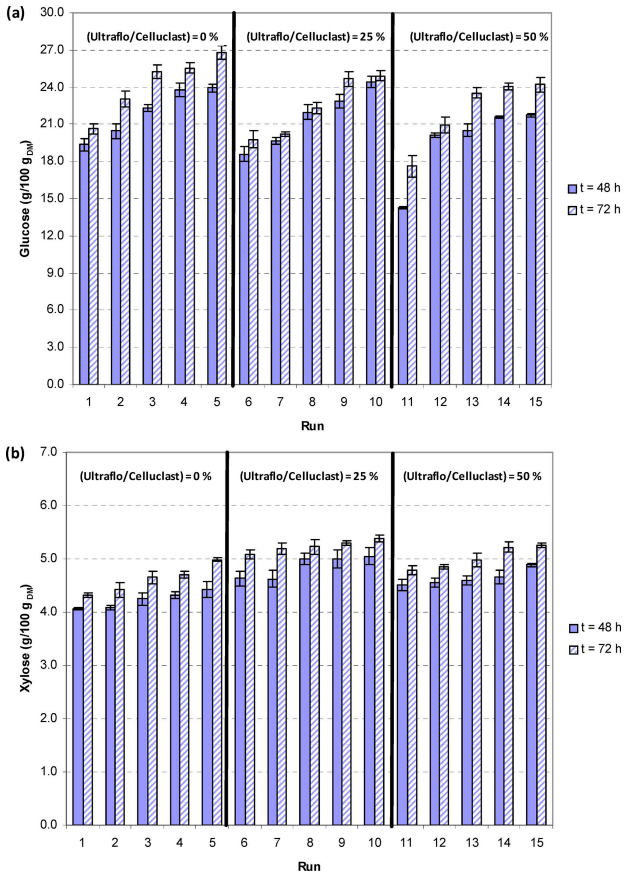

Fig. 3 – *Results for β-glucosidase supplementation on enzymatic hydrolysis of steam-exploded wheat straw with Celluclast 1.5L and Ultraflo L enzymes. Effect of β-glucosidase loading, Ultraflo/Celluclast ratio and hydrolysis time on fermentable carbohydrates released. (a) Glucose production, (b) Xylose production.*

3.2.1. Effect of β-glucosidase loading

Fig. 3 shows that β-glucosidase addition, at a fixed Ultraflo/Celluclast ratio and hydrolysis time, significantly increases sugar liberation, as compared with the control assay (hydrolysis without β-glucosidase supplementation). For glucose liberation (Fig. 3a), operating with the highest enzyme loading (run 5; 10 % $g_{\beta\text{-glucosidase}}/g_{cellulose}$), the concentration of glucose increased by 29.9 % as compared to the control assay (run 1; 0 % $g_{\beta\text{-glucosidase}}/g_{cellulose}$). For xylose release, the highest enzyme loading (run 10; 10 % $g_{\beta\text{-glucosidase}}/g_{cellulose}$) resulted in an increment of 5.9 % in xylose concentration in comparison with run 6 (0 % $g_{\beta\text{-glucosidase}}/g_{cellulose}$). This result is in agreement with previously published data on wheat starch fibre, where the addition of β-glucosidase to the Celluclast + Ultraflo mixture resulted in an increase of approximately 10 % in overall sugar yield (glucose, xylose and arabinose).[13] The significant increase in glucose concentration with β-glucosidase supplementation is due to the fact that the inhibition of the cellulase enzymes has been overcome by supplementation with extra β-glucosidase enzyme. The β-glucosidases of Novozym 188 preparation were able to degrade cellodextrins with an exo-acting approach and could hydrolyse pretreated wheat-straw to monomeric sugars when combined with Celluclast 1.5/Ultraflo-L mixtures. Moreover, the presence of some additional side activities in Novozyme 188 resulted in an increase of other sugar concentrations (p.e. xylose and arabinose).

3.2.2. Effect of Ultraflo/Celluclast ratio

From Fig. 3a, it may be observed that an increase in the Ultraflo/Celluclast ratio, at fixed β-glucosidase loading and hydrolysis time, resulted in a significant decrease in the glucose concentration. For example, comparing runs 5 and 15 (β-glucosidase loading = 10 % $g_{\beta\text{-glucosidase}}/g_{cellulose}$ and t = 72 h), the concentration of glucose decreases from 26.8±0.6 g/100g_{DM} for the Ultraflo/Celluclast ratio = 0 % $g_{Ultraflo}/g_{Celluclast}$ to 24.2±0.6 g/100g_{DM} when the Ultraflo/Celluclast ratio is 50 % $g_{Ultraflo}/g_{Celluclast}$. However, the trend of xylose concentration presented a maximum value for Ultraflo/Celluclast ratio = 25 % $g_{Ultraflo}/g_{Celluclast}$ (run 10). These results are coherent with the influence of the Ultraflo/Celluclast ratio on glucose and xylose release shown in section 3.1.2 and 3.1.3.

3.2.3. Effect of hydrolysis time

In relation to hydrolysis time, a significant increase of glucose and xylose concentration is observed when the hydrolysis is conducted at a higher reaction time (see Figs. 3a and 3b). In this sense, an average increment of 9.2 % for glucose and 8.4 % for xylose is obtained when the time is increased from 48 h to 72 h, for a fixed value of the other operating parameters.

This trend is in accordance with experimental results reported in this paper for the influence of hydrolysis time on simultaneous glucose and xylose liberation when a mixture Ultraflo + Celluclast is used (see section 3.1.4.).

4. Conclusions

In this work, the optimization of the enzymatic hydrolysis of steam-exploded wheat straw using Celluclast 1.5L and Ultraflo L has been carried out. Response surface methodology (RSM) using the Box-Behnken and the D-optimal experimental designs were used as effective tools to optimize the glucose and xylose productions when temperature (40–60 °C), pH (4–6), enzyme/substrate ratio (2–20 % w/w), Ultraflo/Celluclast ratio (0–50 % w/w) and hydrolysis time (48–96 h) were selected as independent variables.

The variables enzyme/substrate ratio, temperature, pH and Ultraflo/Celluclast ratio presented a statistical significance for the glucose concentration response. For xylose production, the main significant factors were the temperature and hydrolysis time.

The proposed models for glucose and xylose concentrations have shown good agreement with experimental data with R^2 of 95.8 % and 96.0 %, respectively.

Multi-objective optimization carried out for simultaneous glucose and xylose optimization has demonstrated that the higher the enzyme dosage level (E/S = 20) with relatively longer time (t = 72 h), the better were the obtained glucose and xylose yields. The optimum temperature and pH were the intermediate values in the operating range analyzed (T = 50 °C and pH = 5). The addition of Ultraflo L could increase the liberation of xylose, but it has no pronounced effects on glucose release. The optimum experimental sugar productions were 18.9±0.4 g/100 g_{DM} for glucose and 4.7±0.2 g/100 g_{DM} for xylose, in good accordance with predicted model responses. According to steam-exploded wheat straw characterization, cellulose and hemicellulose conversions were about 50 % and 28 %, respectively.

The effect of β-glucosidase addition to the Ultraflo + Celluclast mixture for improving sugar yields has also been studied. The influence of the β-glucosidase loadings (0-10 % $g_{\beta\text{-glucosidase}}/g_{cellulose}$), the Ultraflo/Celluclast ratio (0-50 % $g_{Ultraflo}/g_{Celluclast}$) and the hydrolysis time (48-72 h) were analyzed. The supplementation of β-glucosidase to the Cellu-

clast + Ultraflo mixture significantly increased the conversion of cellulose and hemicellulose. The production of glucose and xylose increased by approximately 29.9 % for glucose, and 5.9 % for xylose, when a β-glucosidase loading of 10 % $g_{\beta\text{-glucosidase}}/g_{cellulose}$ was used. The maximum glucose production (26.8 ± 0.6 g/100 g_{DM}) was obtained with the highest β-glucosidase loading (10 % $g_{\beta\text{-glucosidase}}/g_{cellulose}$), the highest hydrolysis time (72 h) and no addition of Ultraflo enzyme (0 % $g_{Ultraflo}/g_{Celluclast}$). For xylose, the maximum production was reached at the highest values for both β-glucosidase loading and time, and for an intermediate Ultraflo/Celluclast ratio of (25 % $g_{Ultraflo}/g_{Celluclast}$), with a value of 5.4 ± 0.1 g/100 $g_{DM.}$ For a simultaneous optimization of glucose and xylose carbohydrates with β-glucosidase supplementation is used, this investigation suggests that the addition of the Ultraflo L enzyme is not recommended.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the financial support from the Ministerio de Ciencia e Innovación (CTQ2006-15217), the Instituto Tecnológico de Castilla y León (VA-15-C2-1) and the Junta de Castilla y León (GRE-233) for their contribution to the development of the project.

References

1. *Mckendry, P.,* Bioresour. Technol. **83** (2002) 37.
2. *Tabka, M. G., Herpoël-Gimbert, I., Monod, F., Asther, M., Sigoillot, J. C.,* Enzyme Microb. Technol. **39** (2006) 897.
3. *Sun, Y., Cheng, J.,* Bioresour. Technol. **83** (2002) 1.
4. *Yang, B., Wyman, C. E.,* Biofuels, Bioprod. Biorefin. **2** (2008) 26.
5. *Alvira, P., Tomás-Pejó, E., Ballesteros, M., Negro, M. J.,* Bioresour. Technol. **101** (2010) 4851.
6. *Ferreira, S., Duarte, A. P., Ribeiro, M. H. L., Queiroz, J. A., Domingues, F. C.,* Biochem. Eng. J. **45** (2009) 192.
7. *Zhou, J., Wang, Y. H., Chu, J., Zhuang, Y. P., Zhang, S. L.,* Bioresour. Technol. **100** (2009) 819.
8. *Zhang, M., Su, R., Qi, W., He, Z.,* Appl. Biochem. Biotechnol. **160** (2010) 1407.
9. *Banerjee, G., Scott-Craig, J. S., Walton, J. D.*, Bioenerg. Res. **3** (2010) 82.
10. *Alvira, P., Negro, M. J., Ballesteros, M.,* Bioresour. Technol. **102** (2011) 4552.
11. *Sipos, B., Szilágyi, M., Sebestyén, Z., Perazzini, R., Dienes, D., Jakab, E., Crestini, C., Réczey, K.,* C. R. Biologies **334** (2011) 812.
12. *Hansen, M. A. T., Kristensen, J. B., Felby, C., Jørgensen, H.,* Bioresour. Technol. **102** (2011) 2804.
13. *Palmarola-Adrados, B., Juhász, T., Galbe, M., Zacchi, G.,* Biotechnol. Progr. **20** (2004) 474.
14. *Berlin, A., Maximenko, V., Gilkes, N., Saddler, J.,* Biotechnol. Bioeng. **97** (2007) 287.
15. *Sørensen, H. R., Pedersen, S., Viksø-Nielsen, A., Meyer, A. S.,* Enzyme Microb. Technol. **36** (2005) 773.
16. *Fang, H., Zhao, C., Song, X. Y.,* Bioresour. Technol. **101** (2010) 4111.
17. *Tu, M., Zhang, X., Paice, M., MacFarlane, P., Saddler, J. N.,* Bioresour. Technol. **100** (2009) 6407.
18. *Cara, C., Ruiz, E., Ballesteros, M., Manzanares, P., Negro, M. J. Castro, E. J.,* Process Biochem. **41** (2006) 423.
19. *Soares, I. B., Travassos, J. A., Baudel, H. M., Benachour, M., Abreu, C. A. M.,* Ind. Crops Prod. **33** (2011) 670.
20. *Maache-Rezzoug, Z., Pierre, G., Nouviaire, A., Maugard, T., Rezzoug, S. A.,* Biomass Bioenerg **35** (2011) 3129.
21. *García, M. T., Bolado, S., González, G., Catalina, I., Miranda, A.,* In proceedings of the 10th International Chemical and Biological Engineering Conference. Eugénio C. Ferreira and Manuel Mota, eds., Braga (2008) 964.
22. *García-Cubero, M. T., Marcos, M., Bolado, S., Coca, M., González-Benito, G.,* Chem. Eng. Trans. **21** (2010) 1285.
23. *Bellido, C., Bolado, S., Coca, M., Lucas, S., González-Benito, G., García-Cubero, M. T.,* Bioresource Technol. **102** (2011) 10868.
24. National Renewable Energy Laboratory (NREL). Chemical Analysis and Testing Laboratory Analytical Procedures: LAP-002, LAP-003, LAP-004. NREL, Golden, CO, USA (2008).
25. *Hamzah, F., Idris, A., Shuan, T. K.,* Biomass Bioenergy **35** (2011) 1055.
26. *Pallapolu, V. R., Lee, Y. Y., Garlock, R. J., Balan, V., Dale, B. E., Kim, Y., Mosier, N. S., Ladisch, M. R., Falls, M., Holtzapple, M. T., Sierra-Ramirez, R., Shi, J., Ebrik, M. A., Redmon, T., Yang, B., Wyman, C. E., Donohoe, B. S., Vinzant, T. B., Elander, R. T., Hames, B., Thomas, S., Warner, R. E.,* Bioresour. Technol. **24** (2011) 11115.