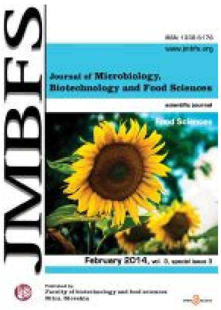

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OPTIMIZATION OF LIME PRETREATMENT FOR ENZYMATIC SACCHARIFICATION OF WHEAT STRAW

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ABSTRACT

The aim of this work was optimization of lime pretreatment parameters such as temperature, time and reaction ratio to maximization of reducing saccharide yields occurred by enzyme hydrolysis of pretreated plant material (wheat straw). Pretreatment conditions were optimized using response surface methodology. The optimal conditions were chosen to promote reducing saccharide yields following enzymatic digestion and they were temperature 91.5 °C, time 2.4 hours and reaction ratio 19.7 mL to 1 g of treated wheat straw. The experimental values agreed with predicted within a 95 % confidence interval. The computed model of wheat straw pretreatment by lime can be used for the effective utilization of secondary products obtained in agriculture sector.

Keywords: optimization, lignocellulose, enzyme hydrolysis, lime pretreatment

INTRODUCTION

Wheat straw is an abundant agricultural residue with low commercial value. During harvesting of grain, straw is collected and used as bedding material. Straw produced might be left on the field, plowed back into the soil or burned. Wheat straw is mainly composed by cellulose, hemicelluloses and lignin and a small amount of extractives and ash. Structure and composition of wheat straw provides the possibility of using as substrate for fermentation production various products such as alcohols, acids, enzymes and flavors **(Francis *et al.*, 2003**; **Taherzadeh and Karimi, 2008**; **Couto, 2008**; **Mussato *et al*., 2008)**.

For effective utilization of wheat straw by various organisms in fermentation process, lignocellulose structure may be disrupted by specific methods of pretreatment. These methods are based on the physical and chemical changes. Whereas the physical treatment caused mainly increase the specific surface, chemical pretreatment is used for disruption of chemical bonds which linked polymers of lignocelluloses **(Szczodrak and Fiedurek, 1996**; **Gable and Zachci, 2007**; **Olofsson *et al*., 2008)**.

Chemical methods of lignocellulose material pretreatment include using of acid, alkaline or oxidative agents **(Carvalheiro *et al.*, 2008**; **Taherzadeh and Karimi, 2008**; **Hendriks and Zeeman, 2009)**. Selection of chemical pretreatment agent depends on the aim of treatment. In the case of saccharide hydrolysis, the acids are using, but for disruption of chemical bonds of lignin and other phenolic compounds from lignocellulose, alkaline and oxidative agents are preferred. Disruption of chemical bonds linked the saccharides with lignin is the key step for saccharide release which in this form are more available for enzyme hydrolysis **(Wang, 2009**; **Alvira *et al.*, 2010)**. From the alkaline pretreatment agents, sodium hydroxide and calcium hydroxide (lime) are especially used. Whereas sodium hydroxide is aggressively pretreatment agent and by their application can be formed inhibition compounds, lime provides satisfactory results without formed the inhibitors. From our previously results, application of lime pretreatment to wheat straw caused releasing higher amount of reducing saccharides from lignocellulose by enzyme hydrolysis in comparison with sodium hydroxide pretreatment **(unpublished data)**. Moreover, the advantage of lime pretreatment is that their application is cheaper and more environmental friendly in comparison with other alkaline treatments **(Mosier *et al.*, 2005**; **Carvalheiro *et al*., 2008**; **Wang, 2009)**.

The aim of this work was optimization of lime pretreatment parameters such as temperature, time and reaction ratio to maximization of reducing saccharide yields occurred by enzyme hydrolysis of pretreated wheat straw.

MATERIAL AND METHODS

Material

Post-grain harvested wheat straw (*Triticum aestivum*) was sourced from the plains in the area of Male Karpaty, Slovakia. The wheat straw was dried at 40 °C for 72 h. Thereafter, it was ground in hammer mill and passed through a 0.5 mm screen. Extractives compounds were removed by Soxhlet extraction for 6 hour with methanol. After remove the extraction solvent, wheat straw was stored in dark powder flask at laboratory temperature.

Selection of experimental ranges

For the selection of experimental ranges, 1 g of extracted wheat straw was mixed with 1% (w/v) water suspension of calcium hydroxide in various reaction ratios (5, 10, 20, 30 and 40 ml to 1 g of dry wheat straw). This suspension was incubated at various temperatures (60, 80 and 100 °C) during 1, 2, 3 or 4 hours. Thereafter, reaction mixture was centrifuged at 4000 RPM for 5 minutes. Sediment was washed by 1 mol.L^{-1} water solution of acetic acid and after by distillated water to neutral reaction. After this treatment, sediment was hydrolyzed by technical enzymes.

Experimental design for the response surface methodology (RSM)

The RSM used a three-factor and central composite design in three blocks consisting of 17 experimental runs at the centre point. In all experiments, the extractions were done in experimental microtubes and stopped by a rapid decantation of extracts. The design variables were the temperature (50, 60, 75, 90, 100 °C; X_1), the time (1, 1.4, 2, 2.6, 3 hours; X_2) and the reaction ratio (10, 14, 20, 26, 30 mL of 1 % (w/v) suspension of calcium hydroxide to 1 g of dry wheat straw; X_3) (Table 1). The experimental design of described independent variables in their original and coded form is shown in Table 2. The dependent variable was reducing saccharide yields obtained by enzymatic hydrolysis of pretreated wheat straw.

Experimental data were fitted to the following second-order polynomial model (Eq. 1) and regression coefficients were obtained.

$$Y = b_0 + \sum_{i=1}^{k} b_i X_i + \sum_{i=1}^{k} b_{ii} X_i^2 + \sum_{\substack{i=1 \\ i<j}}^{k-1} \sum_{j=2}^{k} b_{ij} X_i X_j$$

where X_1, X_2, ..., X_k are the independent variables affected the response (dependent variable; Y), b_0, b_i (i = 1, 2,.., k), b_{ii} (i = 1, 2, ..., k) and b_{ij} (i = 1, 2,...k

– 1; j = 2, 3,..., k) are regression coefficients for intercept, linear, quadratic, and interaction terms, respectively, k is the number of variables.
The optimum conditions of lime straw pretreatment were calculated using Statgraphic 5.1 (Statpoint technologies, INC., Warrenton, Virginia, USA). The verification of the validity and adequacy of the predictive extraction model was carried out in these optimum conditions of temperature, time and reaction ratio. Three experimental replicates were performed at the optimized conditions and the experimental and predicted values were compared.

Table 1 Independent variables and their coded and actual values used for optimization

Factors	Coded levels						
	-1.682	-1	0	1	1.682	Step	Mean
Temperature [°C]	50	60	75	90	100	15	75
Time [hour]	1	1.4	2	2.6	3	0.6	2
Reaction ratio [mL.g^{-1}]	10	14	20	26	30	6	20

Analyses of the response variables

Enzymatic hydrolysis

Solid treated or untreated wheat straw (50 mg) was resuspended in solutions (1 mL) of 50 mmol.L^{-1} citrate buffer (pH 5.0) and technical enzymes Viscozyme L (Novozymes, DK). Hydrolysis was performed in shaking (150 RPM) at 30 °C for 24 hours and in the presence of 0.1 % (w/v) sodium azide to prevent microbial contaminant growth. Then samples were placed in a centrifuge tube and centrifuged at 8000 RPM for 5 minutes, filtered and analyzed for reducing saccharide yields.

Reducing saccharide yields

The reducing saccharide yields by enzymatic hydrolysis of wheat straw was determined by 3,5-dinitrosalicylic acid (DNS) method **(Miller, 1959)**. 0.8 mL of DNS reagent was added to 0.1 mL of hydrolysate. Reaction mixture was incubated in boiling water bath for 5 minutes. The mixture was cooled down to room temperature and 8 mL of distilled water was added. The absorbance of the reaction mixture (200 µL) was measured at 540 nm using a microplate reader (BioTek EL 800, Fisher, GE). Reducing saccharide yields were calculated by Equation 2:

$$\text{Reducing saccharide yields} = \frac{c.V}{m},$$

were c is concentration of reducing saccharides determined by DNS method, V is volume of enzymatic hydrolysate and m is mass of hydrolysed plant material.

RESULTS AND DISCUSSION

Selection of experimental ranges

Alkaline pretreatment has generally a stronger effect on lignin than cellulose and hemicelluloses **(Sharma *et al.*, 2002)**. Lignin forms barrier against degradation of cellulose and hemicelluloses by hydrolytic enzymes. The response of reducing saccharide yields to varying pretreatment reaction conditions were modeled using RSM.
First step in optimization of wheat straw pretreatment by lime were selection of experimental ranges. For effective optimization of technological processes, suitable experimental ranges must be used. Efficiency of lignocellulose material pretreatment is affected by multiple parameters such as temperature, time, reaction agent to plant material ratio and among others **(Mosier *et al.*, 2005**; **Carvalheiro *et al.*, 2008**; **Wang, 2009)**.
Generally in chemical reactions, temperature and time are two parameters with evidently dependent. Efficiency of lime treatment at low temperature during long time is comparable with efficiency of lime treatment at high temperature during short time. In the literature, the lime pretreatment of various lignocellulose materials was carried out at temperature varied from 20 to 120 °C during time varied from 20 minutes to 20 weeks **(Kim, 2004**; **Kim and Holtzapple**, **2005**; **Kim and Holtzapple, 2006**; **Xu *et al.*, 2010)**. The dependence of time and temperature during lime pretreatment was studied (Figure 1).

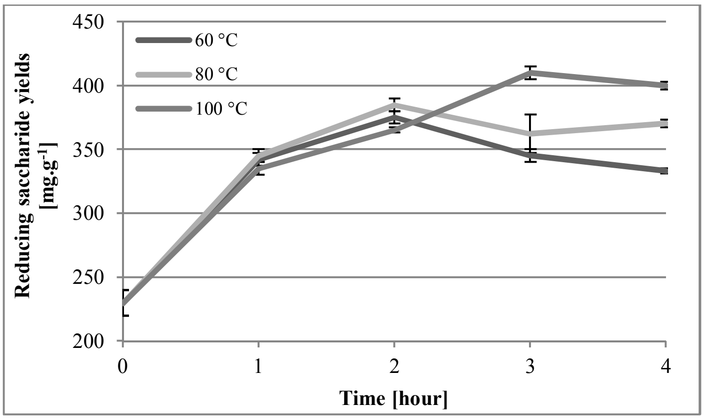

Figure 1 Reducing saccharide yields occurred by enzyme hydrolysis of wheat straw treated by alkaline pretreatment with 1 % (w/v) suspension of calcium hydroxide at 60, 80 and 100 °C during 4 hours at reaction ratio 10 mL to 1 g of dry wheat straw

The results (Figure 1) shown that the highest reducing saccharide yields occurred by enzyme hydrolysis of pretreated wheat straw was recorded at 100 °C after 3 hours (410 mg.g^{-1}). At 80°C, the highest reducing saccharide yields were measured after 2 hours (385 mg.g^{-1}). The lowest reducing saccharide yields were recorded at 60 °C, but these yields were comparable to the reducing saccharide yields at 80 °C (365 mg.g^{-1}). **Chang *et al.* (1998)** found that 1 – 3 hours and 85 – 135 °C were required to reach high saccharide yields and the optimal lime loading was 0.1 (g/g dry mass). Therefore, for optimization of lime pretreatment, parameter temperature was experimentally studied in the range from 50 to 100 °C and parameter time in the range from 1 to 3 hours.
The lime pretreatment is based on the chemical reaction between calcium hydroxide and compounds of lignocellulose. Therefore, the ratio between mass of wheat straw and reaction agent which are on the base our previously study suspension of calcium hydroxide (1% w/v) was studied. In the literature, this ratio was varied from 5 to 20 mL to 1 g of dry wheat straw **(Chang *et al.*, 1997; Chang *et al.*, 1998; Kim and Holtzapple, 2006; Xu *et al.*, 2010)**.

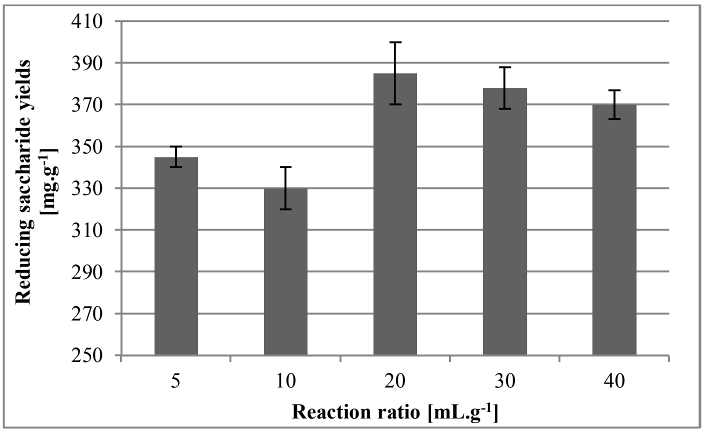

Figure 2 Reducing saccharide yields occurred by enzyme hydrolysis of wheat straw treated by alkaline pretreatment with 1 % (w/v) suspension of calcium hydroxide at 80 °C during 3 hours at various reaction ratios

From the Figure 2, the reducing saccharide yields occurred by enzyme hydrolysis of pretreated wheat straw was increasing to ratio 20 mL to 1 g of dry wheat straw (385 mg.g^{-1}) and then decreased. In comparison with ratio 20 mL to 1 g of dry wheat straw (w/v), the higher ratios not provided increasing of yields. Therefore, for optimization of lime pretreatment, parameter reaction ratio was experimentally studied in the range from 10 to 30 mL to 1 g of dry wheat straw (w/v).

Optimization of extraction by RSM

The effects of selected parameters of lime pretreatment were optimized through the RSM approach. In RSM, natural variables are transformed into coded variables that have been defined as dimensionless with a mean zero and the same spread or standard deviation **(Myers and Montgomery, 2009)**. This method was used in many studies with focusing to optimization of natural compound extraction **(Liyana-Pathirana and Shahidi 2005; Silva *et al.* 2007; Mikulajová *et al.* 2007)**, but it has been successfully used to optimize of different pretreatment methods of plant material **(McIntosh and Vancov, 2011; Avci *et al.*, 2013)**. In our study, the independent variables were temperature, time and reaction ratio. Results of reducing saccharide yields occurred by enzyme hydrolysis of pretreated plant material as dependent variable for all runs are reported in Table 2.

Table 2 Experimental data for the response reducing saccharide yields occurred by enzyme hydrolysis of pretreated plant material by 1 % (w/v) suspension of calcium hydroxide at described conditions

No.	Temperature [°C]	Time [hour]	Reaction ratio [mL.g^{-1}]	Reducing saccharide yields [mg.g^{-1}]
1	90 (1)	2.6 (1)	14 (-1)	361
2	60 (-1)	2.6 (1)	26 (1)	328
3	90 (1)	1.4 (-1)	26 (1)	342
4	60 (-1)	1.4 (-1)	14 (-1)	224
5	75 (0)	2 (0)	20 (0)	348
6	90 (1)	2.6 (1)	26 (1)	386
7	90 (1)	1.4 (-1)	14 (-1)	365
8	60 (-1)	2.6 (1)	14 (-1)	361
9	75 (0)	2 (0)	20 (0)	390
10	60 (-1)	1.4 (-1)	26 (1)	364
11	75 (0)	3 (1.682)	20 (0)	432
12	75 (0)	2 (0)	30 (1.682)	436
13	75 (0)	2 (0)	10 (-1.682)	319
14	75 (0)	1 (-1.682)	20 (0)	389
15	75 (0)	2 (0)	20 (0)	440
16	50 (-1.682)	2 (0)	20 (0)	362
17	100 (1.682)	2 (0)	20 (0)	448

Analyses of the regression coefficients and the response surface

The regression coefficients of the model for reducing saccharide yields obtained by the multiple linear regression are reported in Table 3. Variables in their coded form (Table 2) permitted a direct interpretability of effects (linear, quadratic and interaction) of independent variables, and the surface plot (Figure 3) facilitated the visualization of the statistically significant factors (denoted by the bolded letters in the Table 3) derived from the statistical analysis.

Determination and experimental validation of the optimal conditions

In order to verify the predictive capacity of the model, optimal conditions were determined using the simplex method and the maximum desirability for all dependent variables, and they were used for a validation test of wheat straw pretreatment by lime (Table 4). The measured values lay within a 95% mean confidence interval of the predicted values of dependent variable. These results confirm the predictability of the model for the wheat straw pretreatment by lime in the used experimental ranges.

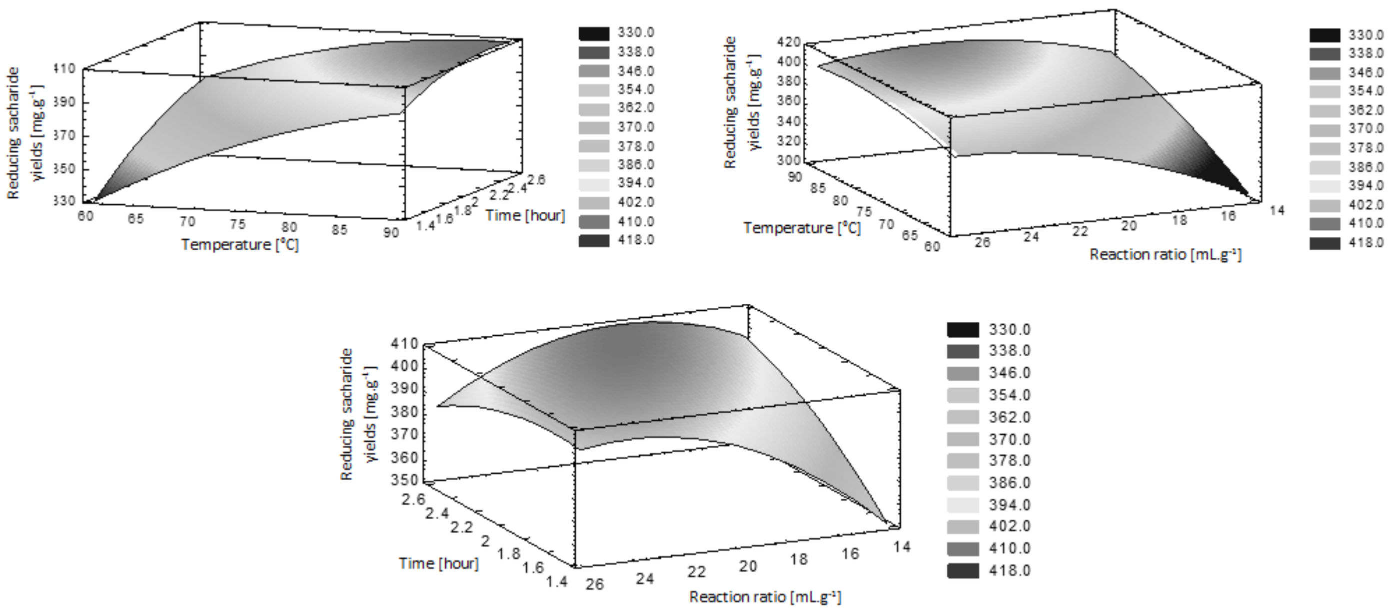

Figure 3 Response surface and contour plots for the effects of time, temperature and reaction ratio for reducing saccharide yields occurred by enzyme hydrolysis of pretreated wheat straw by 1 % (w/v) suspension of calcium hydroxide

Table 3 Regression coefficients of second-order model for dependent variable

Model parameters		Reducing saccharide yields
Constant effect		**-864.2**
Linear effect	Temperature (A)	**12.012**
	Time (B)	**249.75**
	Reaction ratio (C)	**43.48**
Quadratic effect	A x A	-0.03883
	B x B	-17.857
	C x C	**-0.50145**
Interactive effect	A x B	-0.8597
	A x C	**-0.14554**
	B x C	-4.38594

Regression coefficients with a statistically significant at $p < 0.05$ are printed in bold

Table 4 Comparison of predicted and experimental values for the response variable

Parameters	Reducing saccharide yields [mg.g^{-1}]
Temperature [°C]	91.5
Time [hour]	2.4
Reaction ratio [mL.g^{-1}]	19.7
Predicted value	410
Experimental value	432

CONCLUSION

The effects of temperature, time and reaction ratio on pretreatment of wheat straw with 1 % (w/v) calcium hydroxide have been investigated. The experimental ranges of optimization were: temperature 50 – 100 °C, time 1 – 3 hours and reaction ratio 10 – 30 mL to 1 g of wheat straw. Then the response surface methodology was successfully employed to optimize the lime pretreatment of wheat straw. The second-order polynomial model gave a satisfactory description of the experimental data. The optimal conditions for maximum reducing saccharide yields occurred by enzyme hydrolysis of pretreated wheat straw were temperature 91.5 °C, time 2.4 hours and reaction ratio 19.7 mL to 1 g of treated wheat straw. The experimental values agreed with predicted within a 95 % confidence interval.

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