NIR SPECTROSCOPY APPLIED TO THE CHARACTERIZATION AND SELECTION OF PRE-TREATED MATERIALS FROM MULTIPLE LIGNOCELLULOSIC RESOURCES FOR BIOETHANOL PRODUCTION

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(Received: June 17, 2013 - Accepted: December 11, 2013)

ABSTRACT

Lignocellulosic biomass (LB) has been recognized as potential raw for bioethanol production. To facility LB bioconversion a pretreatment is applied, followed by simultaneous or separated saccharification and fermentation (SSF or SHF, respectively) steps. Characterization of pretreated materials, needed to evaluate their ethanol yields, involves laborious and destructive methodologies. Therefore, saccharification is also time consuming and expensive step and some pretreated samples have not suitable characteristics to obtain high ethanol yields. Since bioethanol production aims to be a multivariable process respect to lignocellulosic resources, this work attempts to use NIR spectroscopy as alternative to wet chemical analysis to characterize samples from multiple pretreatments and lignocellulosic resources simultaneously and estimate their ethanol yield after a SSF process using multivariate calibration. Selection of suitable samples to obtain high ethanol yields using a classification method is also evaluated. Partial least squares (PLS) and discriminant partial least squares (PLS-DA) were used as calibration and classification techniques, respectively. Results showed ability of NIR spectroscopy to predict the chemical composition of samples and their ethanol yields, even if different lignocellulosic materials were used in the models, with low prediction errors and high correlation coefficients with reference methods ($r > 0,96$) in PLS models and low misclassification rates (20- 30%) in classification models. Use of these models could facility the fast selection of high number of samples with suitable characteristics to obtain high ethanol yields and as predictive tool of these ethanol yields after a SSF process under controlled conditions.

Keywords: NIRS, bioethanol, simultaneous saccharification and fermentation (SSF), lignocellulosic biomass

1. INTRODUCTION

Lignocellulosic biomass (LB) has been recognized as a sustainable alternative to the existing starch and sucrose-based bioethanol production. The processing of LB for ethanol production includes 4 principal steps: 1) A pretreatment to make the raw material amenable to hydrolysis; (2) hydrolysis to break down cellulose and hemicelluloses molecules (saccharification); (3) yeast fermentation of the sugar solution; and (4) distillation to produce ethanol. LB recalcitrance due to cellulose, hemicelluloses and lignin arrangements, is largely responsible for the high cost of LB conversion, especially when wood is used. In order to disrupt the LB structure and increase the yield of the hydrolysis, a pretreatment step is applied. During this pretreatment, a separation of the carbohydrates from the residual materials, mainly lignin and hemicelluloses, takes place. Physical (steam explosion, hydrothermolysis), chemical (Acid or alkaline hydrolysis, solvent solubilization (Organosolv), sulphite alkaline/anthraquinone delignification, ammonia fiber explosion) and biological (such as fungi biodegradation) processes are used as pretreatments¹. They have specific advantages and disadvantages and the cost and performance in the subsequent hydrolysis and fermentation is heavily influenced by this pretreatment. Cellulose and hemicelluloses can be converted to ethanol by separate hydrolysis and fermentation (SHF) or by a simultaneous saccharification and fermentation process (SSF)¹. The SSF processes have some advantages over the SHF processes: end-product inhibition in the process is removed, resulting in better ethanol yields from lignocellulosic materials²⁻⁴. Several structural and compositional factors such as cellulose crystallinity, residual lignin and hemicelluloses, the degree of hemicelluloses acetylation and the accessible surface area in the pulps affect the enzymatic digestibility of lignocellulosic materials. To evaluate the substrate quality and its ethanol yield, residual lignin and hemicelluloses, as well glucans in the pretreated material are measured. Traditionally, chemical analyses of the lignocellulosic components are performed by acid hydrolysis followed by gravimetric determination of lignin and chromatographic determination of sugars⁵. These methods provide highly precise data, but they are laborious, time-consuming and expensive; consequently, sample throughput is limited.

Near infrared spectroscopy (NIRS) has been reported in diverse applications concerning to biofuels production. Some of these works include glucans and ethanol determination in fermentation of substrates from wheat and rye⁶⁻⁷, prediction of degradability and ash content of wheat straw⁸, discrimination of biomass for energy purposes⁹, ethanol yield determination from sugar beet pulps¹⁰ and digestibility of sugars through enzymatic hydrolysis on *Eucalyptus* and *Poplar* species¹¹. Recently, we have demonstrated that NIRS is also able to be used as predictive tool to determine the ethanol yield (g/L) from reflectance

measurements of the organosolv pulps of *Eucalyptus*¹². In most of these works, analyses are related to a specific lignocellulosic material or pretreatment. Some global analysis has been reported by using diversity of woody and non woody materials¹³⁻¹⁴; however, chemical composition and wood properties are the principal objectives of analysis and these works are not focused in bioethanol production applications. In our knowledge there are not reports over a possible ethanol yield prediction from LB materials after a SSF process using NIR data from different lignocellulosic bioresources and pretreatments.

In view the bioethanol production prospective aims to be a multivariable process respect to the lignocellulosic feedstock, this work attempts to use NIR spectroscopy and multivariate calibration to characterize simultaneously, in a fast way, samples from multiple pretreatments and lignocellulosic species and estimate their ethanol yield after a SSF process in order to select future suitable samples for acquisition of high ethanol yields. Chemical characterization included determination of glucans, lignin and hemicelluloses content of pulps. Selection of samples as suitable to obtain high ethanol yields were carried out through of discriminant partial least squares (PLS-DA). Models were constructed under controlled conditions of SSF and attempts to use NIR spectra as patterns to model the ethanol yield of a SSF process and chemical characterization of samples.

2. MATERIALS AND METHODS

Figure 1 summarizes the experimental procedures followed in this work for bioethanol production from the lignocellulosic materials. Pretreated samples were submitted to a SSF process and analyzed by NIRS in parallel. A detailed experimental procedure for each step is described below.

Lignocellulosic materials and pretreatments

Samples included *Eucalyptus globulus* chips pretreated by steam explosion^{15,1} (EG-SE) and Organosolv process (EG-O)¹⁶⁻¹⁷, *Pinus radiata* wood chips pretreated by sulphite alkaline/anthraquinone delignification (PR-ASA) and wheat straw pretreated by steam explosion¹⁵ (WS-SE). EG-SE and WS-SE were obtained using 100 g of LB (dry weight) in a 4 L steam explosion reactor at temperatures and time conditions ranged at 200 - 220°C, 5 - 10 min and 172 - 230°C, 3 - 17 min, respectively. EG-O were obtained in a 1L Parr reactor using 100 g of wood and 700 mL of pulping liquor containing ethanol : water mixtures from 25:75 to 75:25% (v/v) and 0.83 - 2.00% H₂SO₄ (w/w of dry wood) ranged at 155 - 205°C and 26 - 70 min. PR-ASA were obtained in a Parr 4843 using 100 g of wood and 400 mL of pulping liquor containing 25 g of Na₂SO₃/NaOH (50/50, 60/40 and 70/30 w/w) and anthraquinone 0.1-0.3% w/w of wood, at 120 - 185°C and 30 - 120 min.

Figure 1. Experimental procedures for the acquisition of pre-treated samples and NIR predictive models from multiples lignocellulosic resources.

Pretreated materials were washed with distilled water, filtered at vacuum and the solid residues were stored at 4°C for posterior analysis. All the conditions of the pretreatments were selected from experimental designs and optimal conditions are included in the data set.

Chemical characterization of pretreated materials

Chemical composition of the pretreated materials was determined by the Puls method⁵. Pulps (300 mg) were hydrolyzed with 3 mL 72% (v/v) H₂SO₄ at 30°C for 60 min followed by a dilution until 3% (v/v) H₂SO₄. The hydrolyzed material was heated at 121°C in an autoclave by 60 min. The resultant product was filtered. The glucose and hemicellulosic sugars (quantified as a mixture of xylose, mannose and galactose) in the filtrate were measured by normal phase HPLC using a Merck Hitachi equipment with refractive index detector, an Aminex HPX-87H column at 45°C, mobile phase H₂SO₄ 5mM and a flow rate of 0.6 mL min⁻¹. The solid fraction, insoluble lignin, was dried and weighted. Soluble lignin in the filtrate was analyzed by UV-VIS spectrophotometry at 205 nm. Total lignin was calculated as the sum of soluble and insoluble lignin. All the analyses were carried out in triplicate.

SSF process and ethanol quantification

SSF was carried out following the technical report NREL/TP-510-42630¹⁸ with little modifications¹⁹⁻²⁰, using a consistence of 10% of the pretreated materials (w/v), a thermal acclimatized (40°C) *Saccharomyces cerevisiae* IR2-9a (6.0 gL⁻¹) and cellulases (Celluclast; Novozymes, NC, USA) supplemented with β-glucosidase (Novozymes, NC, USA) enzymes. Enzymatic loadings were 20 FPU of cellulases and 20 CBU of β-glucosidase per g of pretreated material. SSF was performed at 40°C for 72 h. Samplings were carried every 12 hours. The content of the released ethanol was analyzed by gas chromatography (GC) on a Perkin-Elmer autosystem XL Headspace using a FID detector and a column HPSMS 30m. The GC program was: 50°C×3min; 10°C/min, 100°C×1min; 25°C/min, 125°C×1min. Temperature of the injector and detector were 200 and 300°C, respectively. Samplings were performed at 12, 36, 48 and 72 hours of reactions. Ethanol concentration was expressed in grams of produced ethanol by liter of the SSF solution (gL⁻¹) and as the percentage of the obtained ethanol respect to the theoretical ethanol content in pulps (yield (%)):

$$\text{Ethanol yield (\%)} = \left(\frac{\text{Ethanol (g)}}{\text{Potential glucos e (g)} \times 0.51} \right)$$

Maximal ethanol concentration of samplings was used for the construction of NIR models.

NIR spectra measurements

Pulps were milled using a Moulinex A5052HF (sieving between 40 and 60 mesh). Spectra were acquired using a Perkin Elmer Identichack FT-NIR spectrometer in the range of 1000 to 2500 nm with 2 nm intervals and 32 scans per spectrum in reflectance mode and converted to Kubelka-Munk units. Mean centering was used as pre-processing technique and transformation by multiplicative scatter correction (MSC) and/or derivatives were applied for each model. Two spectra were acquiring per sample and an average spectrum of them was calculated for the construction of the models.

Chemometric analysis

Principal component analysis (PCA) was applied over the spectra in order

to evaluate possible clustering of samples by species or pretreatment. Partial Least Squares (PLS) was used to build predictive models to quantify glucans, hemicellulosic sugars, lignin and ethanol (gL⁻¹ and yield (%)). Predictive models were validated by cross validation (one leave out criterion) and by external validation using exclusion set. Errors parameters of calibration and validation of PLS models were expressed as RMSEC (root mean of the standard error of calibration), RSECV (root mean of the standard error of cross validation) and RMSEP (root mean of the standard error of external validation), which were calculated according to:

$$RMSEC = \sqrt{\frac{PRESS}{n-k}} \quad (1)$$

$$RMSECV = \sqrt{\frac{PRESS}{n_{cv}-k}} \quad (2)$$

$$RMSEP = \sqrt{\frac{PRESS}{n_e}} \quad (3)$$

Where n is the number of samples of calibration, n_{cv} is the number of samples in the cross validation, n_e is the number of samples in the external validation data set and PRESS the prediction residual error sum of squares calculated by:

$$PRESS = \sum_{i=1}^n (y_i - \hat{y}_i)^2 \quad (4)$$

Discriminant partial least squares (PLS-DA) was used to classify the pulps according their ethanol concentration after the SSF process. Classifications rates were evaluated through the percentage of misclassification samples in modeling and external validation sets of two models: a) model based on ethanol concentration, and b) model based on ethanol yield percentage in wood base. For model a), class 1 was assigned for samples with ethanol concentration less to 30 gL⁻¹, while class 2 was assigned to samples with ethanol concentration equal or superior to 30 gL⁻¹. For model b), class 1 was assigned to samples with yield percentage less to 65% while class 2 was assigned to samples with percentages equals or superior to 65%. Chemometric analyses were carried out using Pirouette 4.0 software (Infometrix Inc).

3. RESULTS AND DISCUSSION

Exploratory analysis

Figure 2 shows the raw NIR spectra of all the samples. The analysis trough of PCA showed principally a separation of *P. radiata* samples from the others samples and lightly groupings by pretreatments, as is shown in the Figure 3. These differences are expected, even on the same species, since the pretreatment produces different chemical characteristics of the pretreated materials. Besides, chemical composition of *P. radiata* is big different to the chemical of wheat straw and *E. globulus*, who show a little more similarities

as in lignin type and hemicelluloses composition²¹. The three first principal components (PCs) explain the 94.8% of variance of the data and these results suggest that methods based on PCs can be used to get most of the variability of data. Multivariate calibration models using PLS were obtained using simultaneously all the pretreated samples as is shown in the Figure 4, where measured and NIR predicted chemical composition and ethanol yields are

highly correlated. A graphical evaluation of the chemical characterization models (Figure 4) show that glucans, lignin and hemicelluloses have a good variability (range) since each sample was obtained at different conditions of each pretreatment; however, high ethanol yields are clearly shown for EG-O, suggesting that this pretreatment was the best of the used pretreatments for ethanol production.

Figure 2. Raw NIR spectra of pretreated materials.

Figure 3. PCA scores plot of pretreated lignocellulosic materials: ●WS-SE, ▼EG-SE, ■PR-ASA, ▲EG-O.

Figure 4. Measured versus the NIR predicted values for the chemical characterization and ethanol concentrations. ●WS-SE, ▼EG-SE, ■PR-ASA, ▲EG-O. Empty figures denotes external validation samples.

Quantitative models for prediction of ethanol and chemical composition of pulps

Parameters of the PLS models are shown in the Table 1. These results show low errors of calibration (RMSEC) and validation (RMSECV, RMSEP) and high correlation coefficients between the NIR predictions and the measured values by the reference methods ($r > 0.9$) for all the cases, indicating robustness of the models and their capacities to predict the chemical composition and the released ethanol from the different pretreated samples, even when different

species are used. Since RMSECV and RMSEP have the same magnitude order, validation of models can be considered realistic and the predictive abilities of these models could be considered excellent, excepting the case of the ethanol yield (%), where the yield percentage based in the LB weight used in the pretreatment should be improved. Values of RPD are relatively high (RPD value more than 3 is considered adequate for analytical purposes in most of the NIR applications for agricultural products)²² and refer low errors of the models compared with the used reference method errors.

Table 1. PLS parameters of NIR predictive models for chemical characterization and ethanol yield quantification of pulps.

Model	Glucans (%)			Hemicellulosic sugars (%)			Lignin (%)		
Pretreatment	MSC			MSC			MSC		
	Sample set			Sample set			Sample set		
Parameter	Cal	CV	Ext Val	Cal	CV	Ext Val	Cal	CV	Ext Val
n	38	38	14	23	23	7	26	26	10
Range	42.5-84.3	42.5-84.3	50.1-84.2	0.4-22.2	0.4-22.2	0.7-21.0	8.2-40.2	8.2-40.2	7.4-35.3
Mean	61.1	61.1	64.5	9.2	9.2	7.0	18.9	18.9	22.2
SD	10.7	10.7	11.5	6.4	6.4	6.6	9.4	9.4	8.6
PLS Comp	5	5	5	6	6	6	5	5	5
RMSEC	2.8	-	-	1.3	-	-	1.9	-	-
RMSECV	-	3.4	-	-	2.1	-	-	2.7	-
RMSEP	-	-	3.6	-	-	0.9	-	-	2.1
R	0.9692	0.9457	0.957	0.9845	0.9441	0.9940	0.9831	0.9571	0.9686
RPD	-	3.1	3.2	-	3.0	7.3	-	3.5	4.1
Model	Ethanol (gL ⁻¹)			Ethanol yield (%)					
Pretreatment	MSC-1 st derivative (25 points)			MSC-1 st derivative (25 points)					
	Sample set			Sample set					
Parameter	Cal	CV	Ext Val	Cal	CV	Ext Val			
n	19	19	10	19	19	9			
Range	4.7 - 46.8	4.7 - 46.8	7.9-45.7	20.4-96.9	20.4-96.9	20.5-90.6			
Mean	24.3	24.3	24.4	63.0	63.0	61.7			
SD	12.7	12.7	13.2	20.8	20.8	24.6			
PLS comp	9	9	9	12	12	12			
RMSEC	1.0	-	-	1.7	-	-			
RMSECV	-	3.1	-	-	7.7	-			
RMSEP	-	-	3.7	-	-	10.2			
R	0.9984	0.9690	0.9568	0.9989	0.9258	0.9002			
RPD	-	4.1	3.6	-	2.7	2.4			

n: number of samples used in the calibration (Cal), cross validation (CV) or external validation (Ext Val). SD: standard deviation of samples obtained by the reference method. PLS Comp: number of principal components in PLS model. RPD: ratio of standard error of prediction (or calibration) to SD predicted.

It is important to mention that ethanol yield (%) value is directly related to the conversion of potential glucose to ethanol, i.e., high yield (%) values means high conversion of the glucose of pulps but not necessarily represent samples with high glucose content and indicate basically performance of the SSF process and this value can be influenced by the presence of enzymatic inhibitors or enzyme adsorption to the lignin part of the substrate. On the other hand, the ethanol concentration of SSF substrates measured in gL⁻¹, is proportional to both, the glucose content obtained in the pretreatment and to its conversion to ethanol, simultaneously. Thus, ethanol (gL⁻¹) model could be useful to predict a possible ethanol concentration from pulps after the SSF process under controlled conditions.

An identification of the important wavelengths for the developed models was carried out using their correlation spectra (Figure 5). Since LB are made up of lignin, cellulose, hemicelluloses and extractives, increases in biomass directed to one of these components will mean less biomass directed toward the

other components and clear negative correlations among them was observed. In order to know which wavelength have correlation with each chemical constituents of pulps and with their ethanol yields in gL⁻¹, the highest R² values for these variables are summarized in the Table 2. Although region 1668-1672 nm and wavelengths at 1154, 2154, 2388 nm have been associated with lignin content in both, pines and eucalyptus wood and 2384 nm with cellulose¹³; in this study these wavelengths have not high correlation with either and wavelengths at 1548, 2078 and 2384 nm were strongly correlated with lignin. Some of these absorptions are inversely correlated with the lignin content (1478, 1548, 1564, 1624 nm). The highest values of R² for glucans model were obtained at 2094 and 2422 nm. These bands can be attributed to O-H stretching and C-H aryl stretching combination bands, respectively²³. Hemicellulosic sugars model show high R² value at 1410 and 1918 nm. These bands could be assigned to the first overtone of fundamental stretching O-H band²³. Ethanol model (gL⁻¹) shows highest R² at 2366, 1624, 2054 and 1478 nm. Most of these bands

have inverse correlation with lignin bands (2366, 1624 and 1478) and not high values of R^2 were found at these wavelengths for the others chemical components as is viewed in the case of lignin. It can sustain that the lignin content have considerable influence over the ethanol yields of the pretreated materials, as is suggesting by some authors²⁴⁻²⁶.

Figure 5. Correlation spectrum of PLS models.

Table 2. Principal bands of models correlation spectrum.

Wavelength (nm)	R^2 of correlation vector			
	Glucans	Lignin	Hem. Sug.	Ethanol (g/L)
1410	0.05(-)	0.20(-)	0.68	0.00
1478	0.34	0.81(-)	0.01	0.60
1548	0.38	0.84(-)	0.01	0.10(-)
1564	0.34	0.82(-)	0.02	0.16(-)
1624	0.06	0.64(-)	0.17	0.64(-)
1918	0.13(-)	0.04(-)	0.69	0.00
2054	0.01	0.51(-)	0.25	0.61
2078	0.36	0.85(-)	0.00	0.23
2094	0.74	0.57(-)	0.24(-)	0.04
2366	0.01(-)	0.41	0.07(-)	0.65(-)
2384	0.27(-)	0.83	0.00	0.33
2422	0.69(-)	0.40	0.46	0.00

(-) indicates an inverse correlation of the NIR absorptions and the PLS dependent variables

Classification by PLS-DA

Results of PLS-DA classification of the pulps is summarized in the Table 3, where only was possible to find acceptable classifications using a threshold of 65% of ethanol yield owing to a few number of samples with high yield (%), at differences of the results of the g/L data, where an excellent classification of samples was obtained in both, modelling and external validation. For future improvements of the classification results, a major number of samples are proposed.

Table 3. PLS-DA misclassification matrix for ethanol concentration and ethanol yield prediction.

Data set		Model a (MSC/2 nd derivative (25p))		Model b (MSC/1 st derivative (25 p))	
		Predicted class		Predicted class	
		Real class			
Mod. Set	1 (<30g/L ⁻¹)	9(100%)	0	8(80%)	2(20%)
	2 (≥30g/L ⁻¹)	0	3(100%)	4(44%)	5(56%)
Ext. Set	1 (<65%)	7(100%)	0	4(80%)	1(20%)
	2 (≥65%)	0	3(100%)	1(14%)	6(86%)

4. CONCLUSIONS

NIR models for chemical characterization, ethanol yield determination and classification of samples from multiple pretreatments and lignocellulosic resources were obtained. The results shown that multivariate analysis of NIR spectroscopical data can be used for ethanol concentration estimation in the bioethanol production process under SSF controlled conditions and consequently ethanol yield from pulps showed to be a controllable and predictable variable. Rapid and less expensive analysis can be applied to high number of samples in a bioethanol production process trough of near infrared spectroscopy and multivariate calibration. The obtained predictive models could facility the fast selection of samples with suitable characteristics to obtain high ethanol yields or could avoid the SSF time consuming and expensive process of non suitable pulps coming from different pretreatments and lignocellulosic biomasses.

5. ACKNOWLEDGMENTS

Authors thanks to Fondecyt Postdoctoral 3100078, INNOVA Bio Bio 05B1416L8 and PBF27(PCS011) projects, as well to Bioenercel S.A. Chile. This work is dedicated in memoriam of Professor Jaime Baeza H. (R.I.P) for his great contribution and advisement.

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