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Multivariable Analysis Reveals the Key Variables Related to Lignocellulosic Biomass Type and Pretreatment before Enzymolysis

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Abstract: In this study, partial least square (PLS), a multivariable analysis, was used to simultaneously quantitatively evaluate the effects of variables related to three pretreatments (alkaline, hot water and acid) and the biomass properties of poplar, salix and corncob. The results showed that biomass type was the most important variable influencing enzymolysis reducing sugar yield (ERSY). The biomass compositions affected the ERSY more than the pretreatment conditions, among which hemicellulose and lignin played vital roles. The alkaline pretreatment had a more positive effect on the ERSY than the acid and hot water pretreatments, in which alkaline content had more influence than temperature. This work provides a deeper understanding of the material properties and the pretreatment conditions in different complex systems before enzymolysis, which might be a guidance to future study.

Keywords: lignocellulosic biomass; pretreatment; enzymolysis reducing sugar; partial least squares (PLS); quantitative evaluation

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1. Introduction

Lignocellulosic biomass, an abundant and renewable resource, has attracted much attention for its great potential to mitigate the fossil energy crisis [1,2]. However, the natural composition and structure of lignocellulosic biomass impede its effective utilization and commercialization [3]. In this regard, the pretreatments of raw materials that aim to conquer those difficulties are particularly necessary and needed to transform the lignocellulosic biomass to the readily enzymatic substrates [4]. Nowadays, kinds of physical, chemical, biological and co-pretreatment are promising approaches [5,6], among which acid, alkaline and liquid hot water pretreatments are the typical methods [5,7–9].

Pretreatment conditions might be the influential factors to the enzymolysis efficiency of lignocellulosic biomass [10,11]. However, most efforts were taken to analyze the responses and behavior of these factors without evaluating their interactions, making it hard to adopt problem-oriented strategies. Partial least square (PLS), a multivariable analysis method, is not only suitable to dig information on the contribution of a single variable to the whole enzymatic saccharification process quantitatively, but it could also gather valuable information from the whole process and identify the most important variable to the complex multivariate systems [12–14]. Li et al. [15] and Xu et al. [16] revealed that the main factors that influenced the enzymatic hydrolysis were the alkaline dosage and the lignin removal rate in the pretreatment process by PLS. Additionally, Xu et al. [2] reported that the acid steam explosion pretreatment was a key factor that enhanced the total saccharification efficiency of corn stover according to PLS. The characters of raw materials that are mainly different in cellulose, hemicellulose and lignin content were also important influential factors to enzymolysis efficiency [17]. But to our knowledge, few analyses simultaneously evaluate the materials and pretreatment variables in multivariate systems, which might lead to a mismatch material to pretreatment.

In order to evaluate the effects of different materials and pretreatments variables on the enzymolysis reducing sugar yield (ERSY), experiments based on three raw materials (poplar, salix and corncob) subjected to the acid, alkaline and hot water pretreatments were set up, respectively. The key influential factor to the ERSY was identified by PLS. This study provides new insights in the optimization of lignocellulosic biomass and pretreatments to enhance the enzymolysis effects.

2. Results and Discussion

2.1. Effects of Three Pretreatments on the Sugar Yield in the Enzymolysis

After being pretreated, some components are hydrolyzed into a liquid part, and the filtered solid is seen as pretreated residues. The liquid part contained reducing sugar, which is readily utilized by the microorganism to ferment [18]. Since the focus of this study was reducing sugar from raw material in the enzymolysis process, the ERSY was seen as the evaluation index for the potential of sugar production of raw material in the enzymolysis.

The changes of the ERSY of the three biomasses under the different pretreatments are visualized in Figure 1 and the experiment data are summarized in Supplementary Material (Table S1). The unpretreated poplar, salix and corncob produced 156.45 mg/g, 184.87 mg/g and 342.23 mg/g of the ERSY, respectively. After the alkaline pretreatment, the ERSY of poplar and corncob significantly increased by 3.45~49.38% and 15.80~98.51% ($p < 0.01$). Moreover, the increasing temperature had little influence on the ERSY of corncob. When the alkaline content was 1% at 30 °C, 80 °C and 121 °C, the similar ERSY of corncob were 643.64 mg/g, 635.82 mg/g and 610.04 mg/g, respectively. Under the hot water pretreatment, the ERSY of poplar and salix significantly increased by 1.14~23.78% and 23.15~41.06% ($p < 0.01$), but the ERSY of corncob significantly decreased ($p < 0.05$). One plausible explanation was that the hot water pretreatment usually increases the crystallinity index of some lignocellulosic biomass [19,20], and the other might be that the microsphere formation of some condensed lignin during the hot water pretreatment process may also inhibit the enzymolysis of corn stover [21,22]. Despite all those above, the hot water pretreatment enhanced the enzymolysis of poplar and salix probably due to their different compositions and structures from the corncob. For the acid pretreatment, the ERSY of poplar, salix and corncob significantly decreased ($p < 0.01$) compared with those in the unpretreated samples. Moreover, the ERSY decreased with the increasing temperature and acid content. Dilute acid destroyed the structure of the hemicellulose fraction, and more polysaccharides hydrolyzed to monosaccharides or disaccharides at elevated temperature [23,24], affecting strongly on the mass balance of the materials.

Generally, the alkaline pretreatment improved the ERSY of the lignocellulosic biomass while the acid pretreatment did not. The hot water pretreatment improved the potential of saccharification for salix and poplar. Regardless of the pretreatment methods and conditions, corncob had the highest ERSY, followed by salix and poplar.

2.2. Influence and Estimation of Factors on Enzymolysis

2.2.1. Establishment of PLS Models for the Analysis of Enzymolysis

The sugar yield in the enzymolysis depends on multiple factors, such as types of lignocellulosic biomass and pretreatments [3,25], which could be explored by the PLS models. Herein, the PLS models were applied to identify the key variables influencing the ERSY. The biomass types (poplar, salix and corncob), pretreatment methods (acid, alkaline, hot water), pretreated residues and pretreatment reducing sugar yield (PRSY) were set as independent variables (X variables), and the result of the ERSY was set as a dependent variable (Y variable). R2 (cum) and Q2 (cum) suggests the fitness degree and the predictive ability for the dataset [2]. The values of R2 and Q2 that exceed 0.5 indicate good data fitting and predictive ability for the model. Biplot served to co-chart score vectors plot and loading scatter plot together to simultaneous display samples and interpretation of variables. The closer the X variable is to the Y variable, the closer relationships they have. By contrast, the variables that are close to the plot origin were poorly described by the model. The coefficient plot and the variable importance degrees (VIP) plot indicate the quantitative contribution and importance degree of the variables on the ERSY [26].

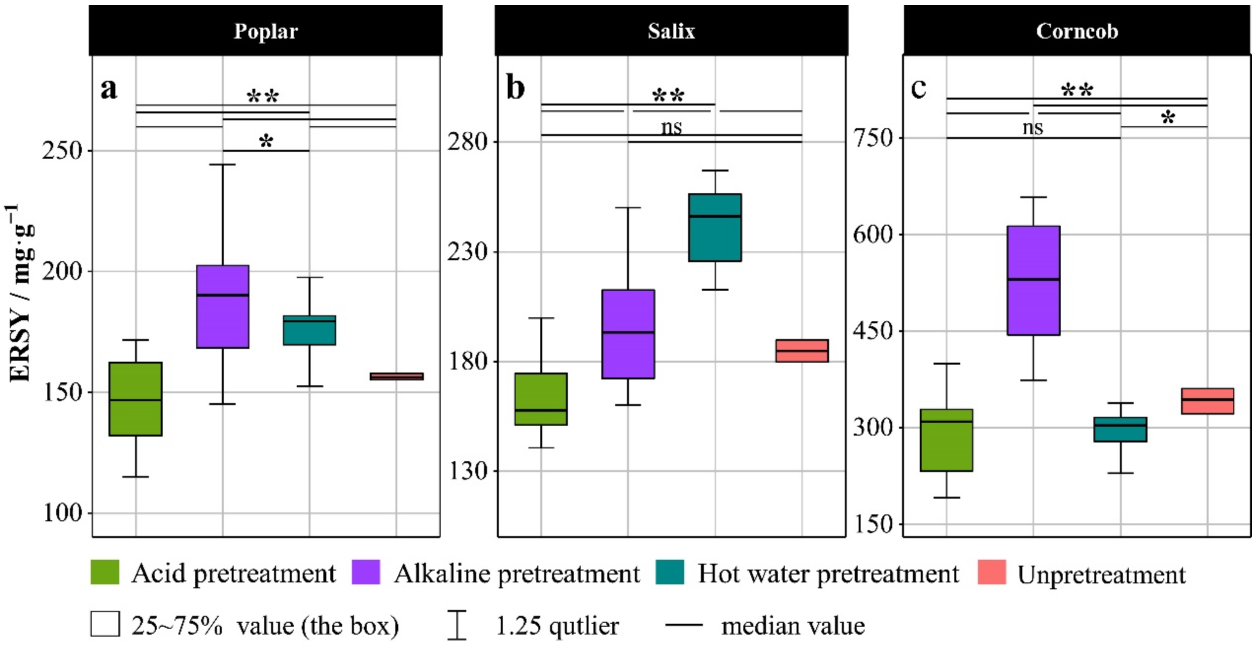

Figure 1. Distribution of enzymatic reducing sugar yield (ERSY) under different pretreatment methods. (**a**). Poplar. (**b**). Salix. (**c**). Corncob. All boxplot distributions are tested using Independent-Samples t-test at the 95% level compared with each other; *, $p < 0.05$; **, $p < 0.01$. Note: Each box represents a distribution of one pretreatment method containing a nine-condition dataset.

2.2.2. Comparison of Different Lignocellulosic Biomass and Pretreatment Methods on ERSY

The effects of different biomass types and pretreatment methods on the ERSY were analyzed by PLS model 1 (Figure 2). The R2X (cum) was 0.530, and the R2Y (cum) was 0.766. The Q2 (cum) was 0.724. It showed that this model explained the 76.6% information of the ERSY and had 72.9% predictive ability of variables. As is shown in Figure 2a, the corncob and alkaline pretreatments were close to the ERSY, indicating that corncob pretreated by alkaline was more capable of producing the ERSY than others. Moreover, corncob had a closer distance to the alkaline pretreatment, while poplar and salix had a closer distance to the hot water pretreatment, suggesting the suitable pretreatment methods for the different types of biomasses.

Figure 2b showed the contributions of the different independent variables. The corncob (0.58) and alkaline pretreatments (0.13) had positive contributions to the ERSY. The other X variables had negative coefficients, which indicated they were negatively related to the ERSY. The negative coefficients of poplar (−0.35) and salix (−0.23) are in line with their lower performance in the ERSY. The acid pretreatment of those three materials had negative contributions and a lower ERSY than the unpretreated samples, which was consistent with that in Figure 1. As shown in Figure 2c, the importance values of the corncob (2.00) and poplar (1.23) were higher than other variables. The pretreated residues (0.83) also played a greater role in the ERSY. The VIP of alkaline pretreatment (0.91) and acid pretreatment (0.88) were higher than the hot water pretreatment (0.27) and the unpretreatment (0.13). In summary, for the different lignocellulosic biomass and pretreatment methods, the biomass type was the most important variable for the enzymolysis. Corncob was a better biomass for enzymolysis, and the alkaline pretreatment was suitable for the three materials to increase the ERSY.

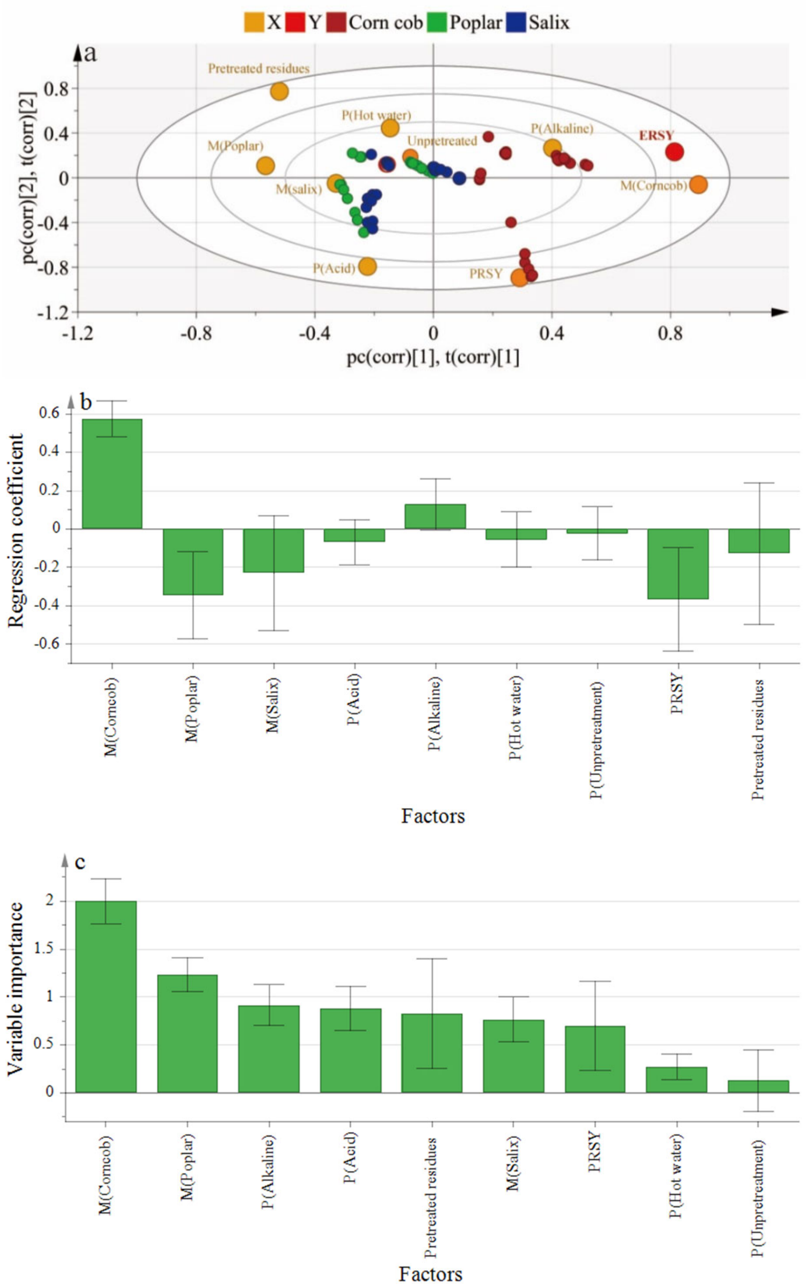

Figure 2. PLS model 1 analysis for the effects of biomass type and pretreatments on the ERSY. (**a**) Biplot. (**b**) Regression coefficients of X variables on the Y variable. (**c**) Importance of X variables on the Y variables for the projection. Note: (**a**) The poplar, salix and corn cob were the pretreated samples under different experimental conditions. The scores t[1] and t[2] summarize the X variables, which are orthogonal and completely independent of each other. The score t[1] (first component) explains the largest variation of the X space, followed by t[2]. The regular loading values *p* correspond to the covariances between the X variables and the score vectors (t) in question, which mean that the magnitude (numerical range) of each X variable is reflected in the loading value. The weights of the variables were indicated by pc[1] (for the first component) and pc[2] (for the second component). The error bars mean the confidence intervals of the coefficients, which indicate the influence degree of each variable, and the influence is significant when the confidence intervals do not include zero.

2.3. PLS Analysis for the Effects of the Biomass Compositions and Conditions on the ERSY under Three Pretreatments

To better understand the effects of the biomass properties on the enzymolysis under the three pretreatments, three PLS models were established based on alkaline, hot water and acid pretreatment conditions and biomass properties, respectively. Each pretreatment had nine pretreatment conditions to pretreat the three biomasses. Thus, each PLS model had 27 samples and 8 X variables.

PLS model 2 was developed to study the effects of the biomass compositions and the alkaline pretreatment conditions on the enzymolysis reducing sugar. Five compositions, including neutral detergent fiber (NDF), hemicellulose, cellulose, lignin, ash, alkaline content, temperature and PRSY were set as X variables and the ERSY of the alkaline pretreatment was set as Y variable. The R2X (cum) was 0.740. The R2Y (cum) was 0.942. The Q2 (cum) was 0.912. Results showed that this model perfectly explained the information (94.2%) of the whole process and had good predictive capability (91.2%). As is shown in Figure 3a, the samples were divided into three groups, and the distances between X variables and the ERSY indicated that the biomass compositions had stronger inter-related connections with the ERSY than the alkaline pretreatment conditions. The coefficient values in Figure 3b showed that hemicellulose (0.25) and alkaline content (0.25) had positive contributions to the ERSY, while the lignin (−0.20) and cellulose (−0.20) had negative contributions. The lowest coefficient value of temperature at the 95% level contained "0", suggesting it had little contribution to the ERSY. Figure 3c sorted the variable importance from high to low. Hemicellulose (1.27) and lignin (1.22) were more important variables for the ERSY than others. The alkaline content (0.71) had a greater impact than temperature (0.13). When the alkaline contents were 0.25%, 0.50% and 1.00% at 30 °C, the ERSY of corncob were 375.45 mg/g, 464.26 mg/g and 643.64 mg/g, respectively, which were similar to those under the same alkaline contents at 121 °C. The alkaline pretreatment had advantages over the improvement of lignocellulose, which removed the lignin and preserved the carbohydrates at room temperature or moderate temperature [27]. High alkaline content also improved the hydrolysis of lignin to enhance the subsequent enzymolysis [28].

In Figure 4, the effects of the biomass properties and the hot water pretreatment on the ERSY are described by PLS model 3. Five compositions, ratio of solid to liquid (S/L), temperature and PRSY were set as the X variables and the ERSY of the hot water pretreatment was set as the Y variable. The R2X (cum) was 0.750. The R2Y (cum) was 0.949. The Q2 (cum) was 0.908. According to Figure 4a, NDF was the closest variable related to the ERSY. Temperature had little influence on the ERSY, since it had a close distance to the plot origin. According to Figure 4b,c, compositions had a greater effect on the ERSY than the hot water pretreatment conditions. The coefficients of NDF (0.38) and cellulose (−0.23) illustrated that the two compositions were the main variables affecting the ERSY. Additionally, the importance degrees of temperature (0.40) and S/L (0.044) were lower than 0.5 and the confidence intervals for the VIP values at the 95% level contained "0", indicating that they were insignificant X variables. Variations of the hot water pretreatment conditions hardly contributed to the significant differences of the ERSY, probably due to the limitation of the temperature gradients. The hot water pretreatment usually treated materials at elevated temperatures (160–240 °C) and this process could remove hemicellulose and lignin to enhance the enzymatic hydrolysis [29].

As shown in Figure 5, PLS model 4 described the effects of the acid pretreatment conditions and the biomass properties on the ERSY. Five compositions, acid content, temperature and PRSY were set as the X variables and the ERSY of the acid pretreatment was set as the Y variable. The R2X (cum) was 0.758. The R2Y (cum) was 0.883, and the Q2 (cum) was 0.851. In Figure 5a, all the X variables were far away from the plot origin, indicating their great influences on the ERSY. Comparing the distances between the Y variable of the ERSY and the X variables, hemicellulose and NDF had closer relationships with the ERSY than other variables. The coefficients in Figure 5b showed that lignin, acid content, temperature and PRSY had negative contributions to the ERSY, and the hemicellulose and NDF had positive contributions. The VIP of the X variables are shown in Figure 5c. Among all the variables, hemicellulose (1.25) and lignin (1.24) were the most important variables. The temperature (0.69) had a greater effect than the acid content (0.52) on the the ERSY. Combining the results from Figure 5b showed that a higher temperature in the acid pretreatment would lead to a lower ERSY. Jung et al. [30] found that the increasing temperature promoted the degradation of hemicellulose and cellulose in the acid pretreatment and decreased the recovery of the pretreated solid residues, which is in line with our study.

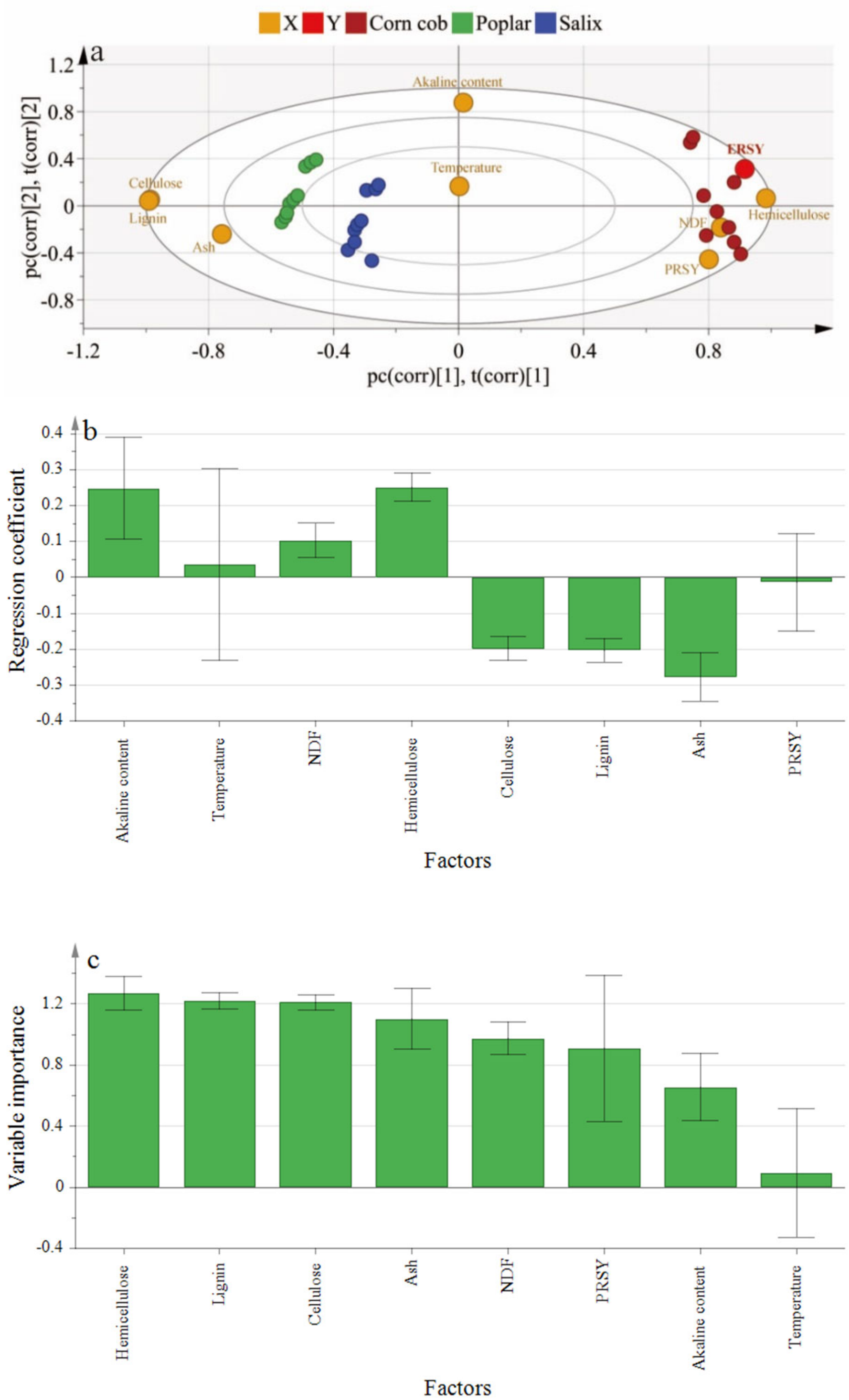

Figure 3. PLS model 2 analysis for the alkaline pretreatment followed by enzymolysis. (**a**) Biplot. (**b**) Regression coefficients of X variables on the Y variable. (**c**) Importance of X variables on the Y variables for the projection.

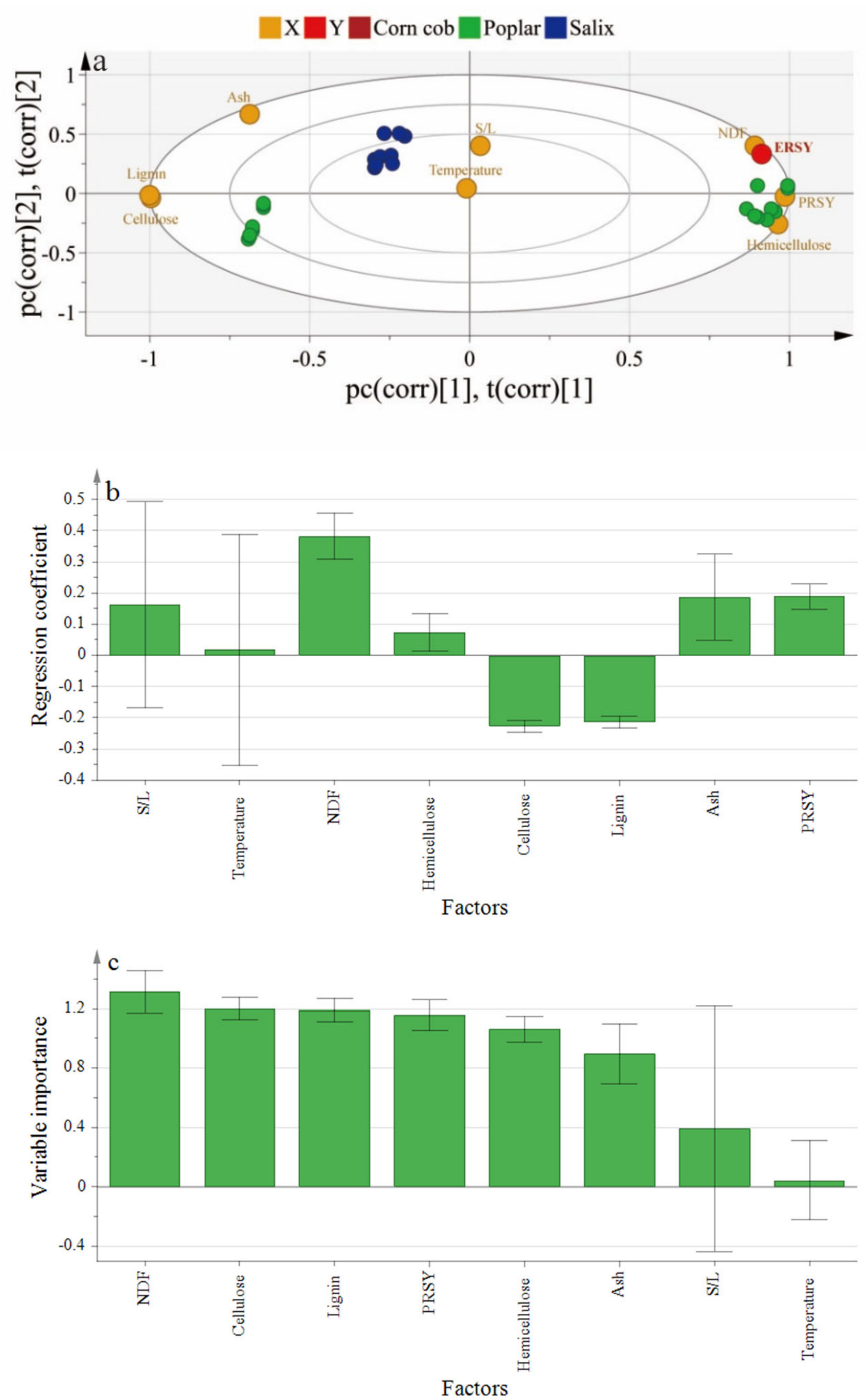

Figure 4. PLS model 3 analysis for the hot water pretreatment followed by enzymolysis. (**a**) Biplot. (**b**) Regression coefficients of X variables on the Y variable. (**c**) Importance of X variables on the Y variables for the projection.

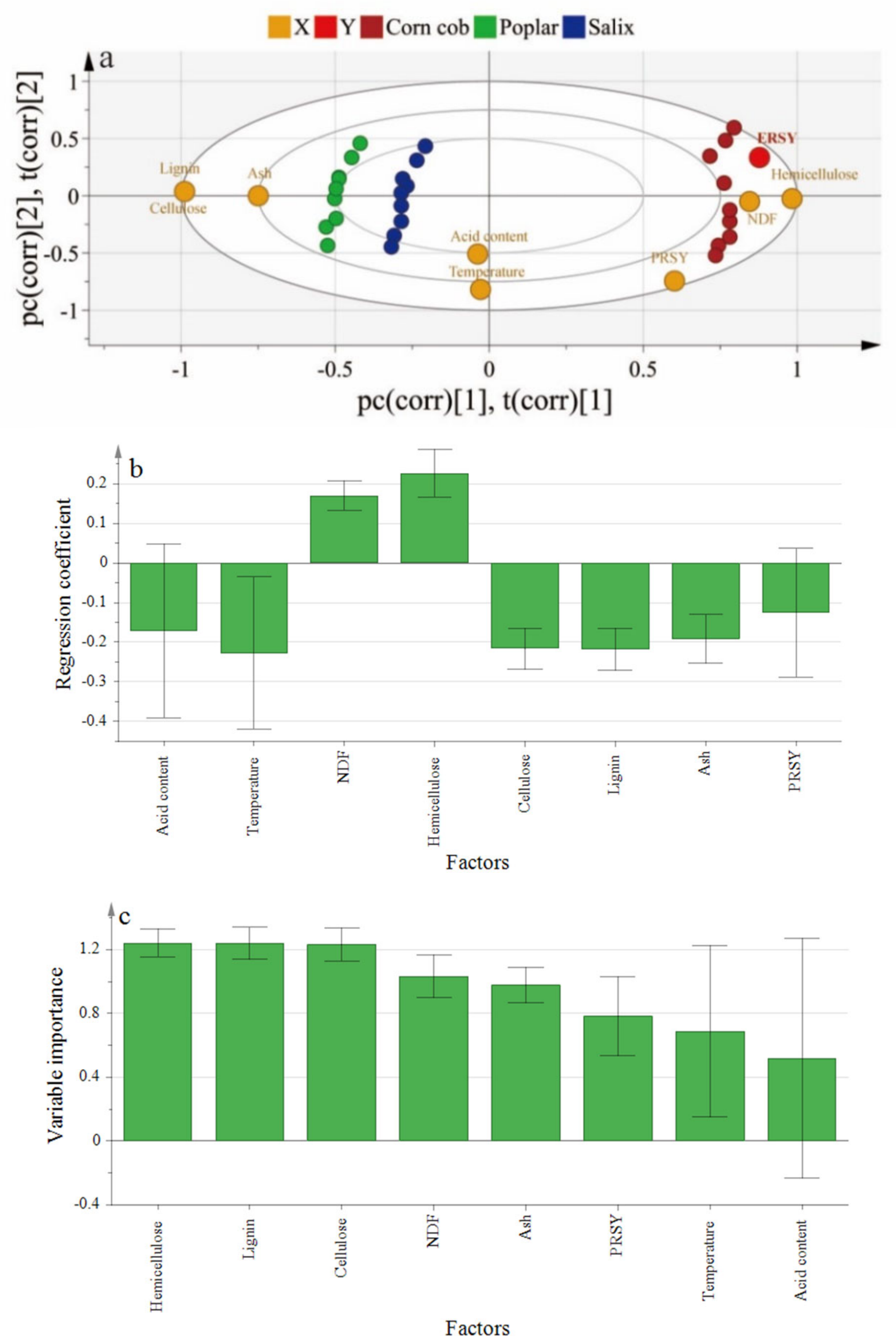

Figure 5. PLS model 4 analysis for the acid pretreatment followed by enzymolysis. (**a**) Biplot. (**b**) Regression coefficients of X variables on the Y variable. (**c**) Importance of X variables on the Y variables for the projection.

Overall, the PLS models showed the biomass compositions had greater influence on the ERSY than the variables of the pretreatments. They also suggested that the type of lignocellulosic biomass was the most important factor influencing the enzymolysis, which was consistent with the previous studies [31]. The common types of lignocellulosic biomass are usually woody plants and herbaceous plants, and the herbaceous species are more readily hydrolyzed by enzymes than the woody species [32]. The corncob is a suitable herbaceous biomass for enzymolysis, due to its low lignin content (7.29%) and high potential for saccharification. Meanwhile, hemicellulose can be easily hydrolyzed in the acid or alkaline environment, which could increase the accessibility of cellulase [33]. Therefore, the corncob after the pretreatments produced more ERSY than poplar and salix. The high lignin content in poplar and salix may lead to the dense combination that is resistant to degrading, causing a negative effect on the enzymatic saccharification. Though high cellulose content was found in poplar and salix, the ERSY of these two materials were lower than the corncob. Cellulose usually presents as a crystalline form or an amorphous form [3]. The crystalline form is more difficult to hydrolyze, and it was probably the main form of cellulose that existed in poplar and salix. Moreover, the effective pretreatment could further attain a better goal of the sugar yield. The alkaline pretreatment was the best method to pretreat the lignocellulosic biomass, and high alkaline content at room temperature can be used to further improve the ERSY.

3. Materials and Methods

3.1. Materials and Preparation

Corncob is produced by herbaceous corn (*Zea mays*.), with the high energy density for enzymatic hydrolysis [34]. Poplar (*Populus euphratica*.) is a tree species and salix (*Salix psammophlia*.) is a shrub species, which commonly grows in Western China.

The poplar, salix and corncob were collected locally in Hohhot, China (the poplar and salix picked the branches without leaves). Three biomass types were milled and screened using a 60-mesh sieve, and then dried in the oven at 60 °C to a constant weight. The composition analysis of NDF, hemicellulose, cellulose, lignin and ash were measured (Table 1) based on Van Soest. All reagents and chemicals are analytical grade.

Table 1. Compositions of lignocellulosic biomass.

Lignocellulose Biomass	NDF (%)	Hemicellulose (%)	Cellulose (%)	Lignin (%)	Ash (%)
Poplar	22.45 ± 0.62	25.72 ± 0.93	34.89 ± 0.32	16.59 ± 0.09	0.34 ± 0.02
Salix	28.43 ± 1.13	25.43 ± 0.17	31.54 ± 0.47	13.94 ± 1.25	0.65 ± 0.06
Corncob	31.32 ± 0.71	37.23 ± 0.54	24.09 ± 0.12	7.29 ± 0.26	0.07 ± 0.01

3.2. Pretreatment

The poplar, salix and corncob were pretreated by acid, alkaline, and hot water. The specific operation methods were as follows. Each condition was performed in triplicate.

Acid pretreatment: The milled materials (5.0 g) and 50 mL of H_2SO_4 (0.25%, 1.00% and 2.13% v/v, respectively) were put into the micro-polymerization reactors (250 mL). Then the samples were performed at 30 °C, 105 °C and 121 °C for 30 min.

Alkaline pretreatment: The milled materials (5.0 g) and 50 mL of NaOH (0.25%, 0.50 and 1.00% w/v, respectively) were put into the micro-polymerization reactors (250 mL). Then the samples were performed at 30 °C, 105 °C and 121 °C for 30 min.

Hot water pretreatment: The milled materials (5.0 g) and the deionized water (the ratio of solid to liquid (S/L) set 1:10, 1:20 and 1:30 (w/v), respectively) were put into the micro-polymerization reactors (250 mL). Then the samples were performed at 30 °C, 105 °C and 121 °C for 30 min.

After being treated, the mixtures were filtered through a piece of gauze (500 mesh) to separate the solid residues and liquid fractions. The solid residues were washed to neutral pH and dried at 80 °C to a constant weight for subsequent enzymolysis. The liquid stored at −20 °C to determine the reducing sugar concentration.

3.3. Enzymolysis

The dryly pretreated residues that were obtained from the above pretreatment processes and unpretreated samples (raw materials) were applied for enzymolysis. The digestion system contained 2.0 g samples, 200 mL of acetate buffer solution (pH 4.8) and 0.5 g cellulase which equaled to 100 U/mL (Zhaodong Sun Shine Enzyme Co., Ltd., Heilongjiang, China), and it was performed at 50 °C in an orbital shaker at 135 rpm. To prevent the interference of microorganisms, 100 mg/L of ampicillin and 100 mg/L of kanamycin were added in the system. The enzymolysis processes lasted seven days. The samples of enzymatic mixtures were collected and put in the boiling water bath for 5 min, and then stored at −20 °C.

3.4. The Reducing Sugar Analysis and Calculation

The pretreated liquid and enzymatic liquid were centrifuged at 12,000 rpm for 5 min. Then the supernatant samples of 1 mL were used to test the reducing sugar concentration using the 3,5-dinitrosalisylic acid (DNS) method [35].

The reducing sugar obtained from the pretreatment and enzymolysis was divided into two parts. The PRSY was calculated by the reducing sugar produced in the pretreatment processes. The ERSY was evaluated by the enzymatic saccharification efficiency multiplied by the ratio of pretreated residues to raw materials used. The specific formulas are as follows:

$$\text{PRSY (mg/g)} = \frac{\text{Reducing sugar concentration (mg/L)} \times \text{Pretreatment reaction volume (L)}}{\text{unpretreated samples (g)}} \quad (1)$$

$$\text{ERSY (mg/g)} = \frac{\text{Reducing sugar concentration (mg/L)} \times \text{Reaction volume (L)}}{\text{Enzymatic sample weight (mg)}} \times \frac{\text{Pretreated residues (mg)}}{\text{unpretreated samples (g)}} \quad (2)$$

3.5. Multivariate Data Analysis and Statistical Analysis

The PLS models were developed by SIMCA-P (Umetrics, version 14). The datasets were normalized by the unit variance scaling (UV) and screened differences of variables by the coefficient plot and variable importance for the projection (VIP) plot. The amount of the ERSY for each pretreated sample was determined via the Independent-Samples t-test, compared with unpretreated materials to test the significant difference at the 95% level by SPSS (USA, version 24).

4. Conclusions

The ERSY produced from corncob was greatly improved after the alkaline pretreatment with a maximum of 98.51%, and the alkaline pretreatment was more advantageous over the hot water and acid pretreatments. PLS analyses indicated that the biomass types and compositions had greater impacts on the production of the ERSY than the pretreatments. To be specific, hemicellulose and lignin were the key variables for the alkaline and acid pretreatments. NDF was too critical to produce the ERSY under the hot water pretreatment. PLS analysis has great applicability in the analysis of the process variables for the effective pretreatment of the various lignocellulosic biomasses.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/catal12101142/s1. Table S1: The datasets during the different pretreatment processes and enzymolysis processes.

Author Contributions: Conceptualization, J.X.; methodology, D.F.; software, D.F. and X.W.; validation, X.W.; formal analysis, D.F. and Y.H.; investigation, D.F. and X.W.; resources, J.X.; data curation, Y.H.; writing—original draft preparation, D.F.; writing—review and editing, J.X. and X.W.; visualization, D.F. and Y.H.; supervision, J.X.; project administration, J.X.; funding acquisition, J.X. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data presented in this study are available upon request from the corresponding author.

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