Optimization of Sodium Hydroxide Pretreatment and Enzymatic Hydrolysis of Wheat Straw Powder by Cellulases and Xylanases From *Aspergillus Niger* HQ-1 Using Response Surface Methodology

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Abstract

To maximize fermentable sugars production, response surface methodology (RSM) was adopted to optimize pretreatment and enzymatic hydrolysis of wheat straw powder (WSP) using the crude cellulases preparation containing xylanases from *Aspergillus niger* HQ-1. Factors of pretreatment including sodium hydroxide concentration, pretreatment time and temperature were found to have significant effects on sugars production. Results indicated that WSP with particle size 0.3 mm should be pretreated using 1.8% (w/v) sodium hydroxide solution with 25.0% (w/v) of solid loading at 94.0°C for 46.0 min and the optimized pretreatment conditions could result in 90.9% of cellulose recovery, 54.6% of hemicellulose recovery and 72.7% of lignin removal, respectively. Furthermore, variables of enzymatic hydrolysis including enzyme loading, biomass loading and reaction time were proved to have significant effects on sugars yields. After hydrolysis at 50°C for 44.8 h with 7.1% (w/v) of biomass loading, 8.1 FPU/g of enzyme loading and 0.2% (w/v) of Tween-80, maximum yields of reducing sugar (632.92 mg/g) and xylose (149.83 mg/g) could be obtained, respectively. In addition, holocellulose and hemicellulose conversion were 81.6% and 80.0%, respectively. To the best of our knowledge, this is the first report about systematic optimization of sodium hydroxide pretreatment and enzymatic hydrolysis of WSP using RSM.

Introduction

In the past few decades, bioethanol produced from lignocellulosic substrates has been extensively studied due to increase of population, demand of energy and environmental problems caused by the use of fossil fuels [1]. Compared with food-based biomass, lignocellulosic substrates are more suitable for production of bioethanol as they are abundant, easily available, renewable and relatively low-cost. Furthermore, using lignocellulosic substrates to produce bioethanol could also reduce competition between food and ethanol production [2]. It is well known that lignocellulosic substrates mainly contain three types of components including cellulose, hemicellulose and lignin. Among them, presence of lignin could prevent cellulose and hemicellulose from being hydrolyzed by enzymes to produce fermentable sugars for bioethanol production [3]. Therefore, pretreatment to enhance digestibility of lignocellulosic biomass and remove the lignin in substrates is a primary step during bioethanol production. Among the various pretreatment methods, acid, alkali, hot water, ionic liquids, steam exploration and organic solvent pretreatments are always adopted [4]. It is noteworthy that alkaline pretreatment using sodium hydroxide is one of the most prospective technologies, as it could effectively lead to removal of lignin, swelling of fibers and decrease of crystallinity and polymerization degree of lignocellulosic substrates [5–7]. In addition, sodium hydroxide also had some advantages such as lower price than potassium hydorxide, better solubility than calcium hydroxide and stronger alkalinity than ammonium hydroxide [8]. Whereas, lignin removal and degradation of cellulose and hemicellulose exist simultaneously during pretreatment. Therefore, it is necessary to optimize pretreatment conditions to realize high levels of cellulose and hemicellulose retainance and lignin removal simultaneously.

Cellulose in lignocellulosic substrates can be hydrolyzed through synergistical action of three types of cellulases including endoglucanases, exoglucanases and β-glucosidases [9]. Endoglucanases (EC

3.2.1.4) can hydrolyze glucosidic bonds randomly in the amorphous regions of cellulose to produce oligosaccharides with reducing and non-reducing ends. Exoglucanases, also named cellobio- hydrolases (EC 3.2.1.91) can cleave oligosaccharides to cellobiose. Finally, cellobiose can be hydrolyzed by β-glucosidases (EC 3.2.1.21) to glucose [10, 11], which could be utilized by yeast strains to produce bioethanol. Cellulases could be produced by various microorganisms, in which, filamentous fungi such as *Trichoderma reesei* are mostly adopted to prepare commercial cellulases. Whereas, cellulases from *Trichoderma reesei* lack enough β-glucosidases, which could easily result in feedback inhibition to enzymes. Fortunately, the three types of cellulases could be secreted by cellulolytic *Aspergillus niger* strains efficiently. In addition, during fermentation for cellulases production using lignocellulosic materials as main components in the medium, *Aspergillus niger* strains can also produce xylanases simultaneously. Xylanases including endoxylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37) can hydrolyze xylan, which is a main type of hemicellulose in lignocellulosic substrates. Endoxylanases (EC 3.2.1.8) can cleave β-1, 4 linked xylan back bone and β-xylosidases (EC 3.2.1.37) can convert xylooligomers to monomeric xylose sub unit, which can also be utilized by yeast strains to produce bioethanol [12]. Therefore, enzymatic hydrolysis is also a crucial step during bioethanol production [13] and cellulases preparation containing xylanases from *Aspergillus niger* strains are more suitable for hydrolysis of lignocellulosic substrates to produce fermentable sugars. Whereas, cellulases and xylanases activities could be influenced by some parameters such as reaction temperature, reaction pH, reaction time, biomass loading and so on. Consequently, it is necessary to optimize enzymatic hydrolysis conditions for enhancing fermentable sugars yields.

There are mainly two types of methods including conventional method and response surface methodology (RSM) for optimization of bioprocesses. The conventional method is to deal with one-factor-at-a-time methodology, which is time-consuming and does not detect frequent interactions of variables. On the contrary, RSM is always preferred, as it can reveal the optimum process conditions and interactions of variables by modelling and optimizing multiple variables [14, 15]. In north china, wheat is a common food crop, which makes wheat straw become one type of abundant, easily available and cheaper lignocellulosic substrates. In our previous report, cellulolytic *Aspergillus niger* HQ-1 was isolated and the crude cellulases preparation was successfully applied to hydrolysis of chitosan [16]. However, hydrolysis of wheat straw by cellulases and xylanases from the strain is not finished yet. Therefore, in this work, RSM was adopted to optimize sodium hydroxide pretreatment and enzymatic hydrolysis of wheat straw by the crude enzymes solution containing cellulases and xylanases from *A. niger* HQ-1 for enhancing fermentable sugars yields.

Materials And Methods

Materials and Microbial strain

Wheat straw (WS), corn stover (CS) and wheat bran (WB) were purchased from nearby farm in Liaocheng, Shandong Province, China. After being washed with double distilled water and dried at 85 °C until constant weight, WS and CS were milled to prepare wheat straw powder (WSP) and corn stover powder

(CSP), respectively. CSP with 0.5 mm of particle size and WB were used to prepare medium for enzymes production. WSP was applied to optimization of pretreatment and enzymatic hydrolysis.

Aspergillus niger HQ-1 (Accession number of ITS sequence: HQ891869) was isolated from the degrading paper and maintained at 4 °C on potato dextrose agar (PDA) slants in microbiology laboratory, School of life Sciences, Liaocheng University.

Enzymes production and activities assays

With the use of 250 ml Erlenmeyer flasks, solid state fermentation for cellulases and xylanases production was carried out using the medium containing (g): CSP, 5.0; WB, 5.0; $(NH_4)_2SO_4$, 0.30; KH_2PO_4, 0.06; $MgSO_4 \cdot 7H_2O$, 0.03. Initial pH and moisture content of the medium were 4.7 and 70.3%, respectively. After sterilized at 121 °C for 20 min, the medium was cooled to room temperature. Seed inoculum was prepared according to the methods described in our previous report [16] and was inoculated to the medium at 10% (v/w) of inoculum size. After incubated at 33.7 °C for 72.0 h, fermented substrates were suspended in distilled water at a solid to liquid ratio of 1: 8.54 (g/ml). The mixture was shaken at 162.0 rpm for 106.4 min and filtered. Finally, the filtrate was centrifuged (13, 980×*g*) using a high speed centrifuge (TGL-20 M, China) for 15 min and the clear supernatant was regarded as the crude enzymes preparation for activities assays and hydrolysis. Total cellulase activity (filter paper activity, FPA) and xylanase activity were determined and defined according to the methods described in our previous reports, respectively [16, 17].

Optimization of sodium hydroxide pretreatment of wheat straw powder

Pretreatment was carried out by soaking raw WSP in 100 ml sodium hydroxide solution in screw- capped laboratory bottles (Pyrex bottles) and the bottles were preserved in water bath. After pretreatment, WSP were washed and filtered with double distilled water till neutrality. After being dried in oven at 80 °C for 24 h, the pretreated WSP was hydrolyzed by the crude enzymes preparation and reducing sugar and xylose yields were determined and used as responses, respectively.

At first, significant variables were screened among five variables including sodium hydroxide concentration, solid loading, pretreatment temperature, pretreatment time and particle size of WSP using Plackett-Burman design (PBD). Then, the optimal regions of significant variables were assessed using the method of steepest ascent. Finally, the optimal levels of significant variables including sodium hydroxide concentration, pretreatment temperature and time were determined using central composite design (CCD). All experiments were performed in triplicate. The obtained data were analyzed by the least squares method to fit the second-order polynomial model, given by the following equation in Eq. (1):

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \qquad (1)$$

in which, Y was the predicted response, β_0 the intercept term, β_i linear coefficient, β_{ii} the squared coefficient and β_{ij} the interaction coefficient.

Chemical compositions analyses and calculations

Cellulose, hemicellulose and lignin in WSP were determined according to the methods described by Mamma et al. [18]. Solid recovery, cellulose recovery, hemicellulose recovery and lignin removal were calculated based on the following equations:

Solid recovery (%) = [WSP dry weight recovered after pretreatment (g) / WSP dry weight before pretreatment (g)] × 100% (2)

Cellulose recovery (%) = [$RC_{PT\text{-}WSP}$ × solid recovery / C_{WSP}] × 100% (3)

Where C_{WSP} and $RC_{PT\text{-}WSP}$ are the amount of cellulose in raw WSP and the pretreated WSP expressed in (g/g), respectively.

Hemicellulose recovery (%) = [$RHC_{PT\text{-}WSP}$ × solid recovery / HC_{WSP}] × 100% (4)

Where HC_{WSP} and $RHC_{PT\text{-}WSP}$ are the amount of hemicellulose in raw WSP and the pretreated WSP expressed in (g/g).

Lignin removal (%) = [(L_{WSP} - $RL_{PT\text{-}WSP}$ × solid recovery) / L_{WSP}] × 100% (5)

Where L_{WSP} and $RL_{PT\text{-}WSP}$ are the amount of lignin in raw and the pretreated WSP expressed in (g/g), respectively.

Enzymatic hydrolysis

At first, the pretreated WSP was mixed with 100 ml of sodium acetate buffer (50 mM, pH 4.8) in 250 ml Erlenmeyer flasks at 1.0% (w/v) of biomass loading. Then, Tween-80 was supplemented in the mixtures with final concentration of 0.2% (w/v) and the mixtures were autoclaved at 121 °C for 20.0 min. After being cooled to room temperature, antibiotics tetracycline (40 µg/ml) and cycloheximide (30 µg/ml) were also supplemented in reaction mixtures to prevent microbial contamination according to the methods described by Annamalai et al. [19]. Finally, the crude enzymes preparation was added at 2.0 FPU/g of enzyme loading and hydrolysis was performed at 50 °C for 24.0 h with mild shaking. In addition, filter paper activity and xylanase activity of the crude enzymes solution were 6.13 U/ml and 889.18 U/ml, respectively. After completing hydrolysis, residues were separated by centrifugation at 13, 980×*g* for 10 min and the obtained supernatant was used to determine the released reducing sugar and xylose using dinitrosalicylic acid (DNS) method and phloroglucinol method, respectively [20, 21]. Control of each reaction mixture was performed by replacing the active crude enzymes with heat inactivated (100 °C, 10 min) enzymes.

Optimization of enzymatic hydrolysis

With the use of PBD, significant variables were screened among six variables including enzyme loading, biomass loading, Tween-80 concentration, reaction temperature, reaction pH and reaction time. Then, the method of steepest ascent was used to investigate the optimal regions of the significant factors. Finally, Box-Behnken design (BBD) was employed to determine the optimal values of the significant variables including enzyme loading, biomass loading and reaction time. All experiments were performed in triplicate and mean values of reducing sugar and xylose yields were used as responses, respectively. The obtained data were analyzed by the least squares method to fit the second-order polynomial model, given by the following equation in Eq. (6):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (6)$$

in which, Y was the predicted response, X_1, X_2 and X_3 the coded variables, β_0 intercept, β_1, β_2 and β_3 the linear coefficients, β_{11}, β_{22} and β_{33} the squared coefficients and β_{12}, β_{13} and β_{23} the interaction coefficients.

Statistical analysis

Minitab (14.12) statistical software package and Statistical Analysis System (SAS, 8.0) were used for the experimental design and analysis of the results.

Calculations for hydrolysis

Holocellulose and hemicellulose conversion were calculated according to the following equations:

Holocellulose conversion (%) = [Reducing sugar (mg/g) × 0.9 / cellulose and hemicellulose in pretreated WSP (mg/g)] × 100% (7)

Hemicellulose conversion (%) = [xylose (mg/g) × 0.88 / hemicellulose in pretreated WSP (mg/g)] × 100% (8)

The factor 0.9 and 0.88 were used to convert polysaccharide to monosaccharide accounting for water uptake during hydrolysis of cellulose and hemicellulose (xylan), respectively.

Results And Discussions

Optimization of sodium hydroxide pretreatment of WSP

It was reported that the fixed pretreatment conditions had different effects on cellulose recovery, hemicellulose recovery and lignin removal [22]. Therefore, in our opinion, it was difficult to determine the optimal pretreatment conditions exactly using cellulose recovery, hemicellulose recovery and lignin removal as responses, respectively. In our opinion, reducing sugar and xylose yields were more suitable to be adopted as responses directly during optimization of pretreatment, as the main aim of pretreatment was to enhance sugars yields. As shown in Table 1, yields of reducing sugar and xylose in trials differed

from each other, which indicated that the responses could be influenced by the adopted variables. Results in Table 2 indicated that variables including sodium hydroxide concentration, pretreatment temperature and time had significant and positive effects on responses. The three significant variables were also found to play positive and significant roles during pretreatment of sugarcane bagasse [3] and poplar (*Populus deltoides*) biomass [23], respectively. Whereas, it was reported that pretreatment time had insignificant effect during microwave assisted sodium hydroxide pretreatment of wheat straw [1] and sodium hydroxide concentration had insignificant effect during microwave assisted sodium hydroxide pretreatment of cassava stem [24], respectively. In this work, variables including solid loading and particle size had insignificant effects during pretreatment. However, it was also reported that particle size had significant effect during aqueous ammonia pretreatment of sugarcane bagasse [25] and solid loading had significant effect during microwave assisted sodium hydroxide pretreatment of cassava stem [24]. In our opinion, discrepancy of significant factors for pretreatment in different reports was mainly related with differences of pretreatment methods and lignocellulosic substrates. In this work, particle size had negative effect during pretreatment, as smaller particle could increase surface area of biomass for pretreatment and the following enzymatic hydrolysis [25]. Solid loading had positive effect during pretreatment, as too lower levels of solid loading could lead to too higher sodium hydroxide concentration, which could intensify degradation of cellulose and hemicellulose simultaneously [26]. Therefore, WSP with particle size 0.3 mm was adopted and pretreated at 25% (w/v) of solid loading in the following experiments. As to the three significant variables, the steepest ascent method was used to determine the optimal regions of them (Table 3). Results in Table 3 indicated that reducing sugar and xylose yields reached the plateau while sodium hydroxide concentration, pretreatment temperature and time were 1.8% (w/v), 95.0 °C and 45.0 min, respectively. After the plateau, sugars yields decreased as too severe conditions may result in more lose of cellulose and hemicellulose.

Based on results of the steepest ascent method, CCD (Table 4) was adopted to determine the optimal values of the significant factors. Statistical analysis of CCD (Table 5) indicated that both linear terms (P = 0.000, P = 0.000) and square terms (P = 0.000, P = 0.000) had significant effects on reducing sugar and xylose yields, respectively. P values of the models (P = 0.000, P = 0.000) and lack of fit (P = 0.104, P = 0.122) indicated that the models were also adequate to predict the optimal pretreatment conditions. High values of R^2 (99.6%, 99.5%) and adjusted R^2 (99.2%, 99.1%) also indicated the accuracy of the models. According to canonical analysis, maximal reducing sugar yield (290.99 mg/g) could be obtained after pretreatment using 1.84% (w/v) sodium hydroxide solution at 94.1 °C for 47.0 min. Whereas, maximum xylose yield (52.77 mg/g) could be obtained after pretreatment using 1.76% (w/v) sodium hydroxide solution at 93.8 °C for 43.9 min.

The corresponding regression models for reducing sugar and xylose yields during optimization of pretreatment conditions were given below in Eq. (9) and Eq. (10):

$$Y_1 = 289.737 + 6.537x_1 - 3.445x_2 + 6.472x_3 - 24.540x_1^2 - 20.085x_2^2 - 16.468x_3^2 - 0.103x_1x_2 + 1.365x_1x_3 + 0.180x_2x_3 \quad (9)$$

$$Y_2 = 52.5066 - 1.6063x_1 - 1.0720x_2 - 1.4076x_3 - 5.6265x_1^2 - 4.6030x_2^2 - 6.2700x_3^2 + 0.3262x_1x_2 - 0.1138x_1x_3 - 0.1013x_2x_3 \quad (10)$$

in which, Y_1 and Y_2 were predicted reducing sugar and xylose yields, x_1, x_2 and x_3 were codes of sodium hydroxide concentration, pretreatment temperature and time, respectively.

To validate the predicted conditions, after adjustment, WSP was pretreated using 1.8% (w/v) sodium hydroxide solution at 94.0 °C for 46.0 min. After enzymatic hydrolysis, reducing sugar (291.91 mg/g) and xylose (53.20 mg/g) (average of three replicates) were obtained, which were in close proximity with the predicted values of models. Furthermore, cellulosic compositions of raw and pretreated WSP were determined and the results were shown in Table 6. After calculation, values of solid recovery (74.2%), cellulose recovery (90.9%), hemicellulose recovery (54.6%) and lignin removal (72.7%) were obtained, respectively. Comparisons of the above parameters in this work with those in some previous literature about pretreatment of wheat straw and rice straw were shown in Table 7.

Among different values of cellulose recovery in Table 7, cellulose recovery (90.9%) in this work was relatively lower than 97.9% described by Qiu et al. [27] and 96.0% described by Jaisamut et al. [28], whereas relatively higher levels of lignin removal (72.7%), hemicellulose recovery (54.6%) and solid loading (25.0%, w/v) in this work compared favorable. Though cellulose recovery (92.59%) and lignin removal (95.2%) described by Li et al. [29] were also higher than those (90.9%, 72.7%) in this work, relatively higher levels of hemicellulose recovery (54.6%), shorter pretreatment time (46.0 min) and easier operation in this work were still competitive. As to hemicellulose recovery, only 64.8% described by Qi et al [30] was higher than that (54.6%) in this work. Whereas, relatively lower levels of cellulose recovery (74.7%) and lignin removal (65.54%) still existed in that work [30]. Of course, values of hemicellulose recovery (53.38%) and lignin removal (73.17%) described by Tsegaye et al. [31] were approximate with those (54.6%, 72.7%) in this work, whereas higher cellulose recovery (90.9%) in this work was also competitive. In addition, it was obvious that sodium hydroxide pretreatment of wheat straw was also performed by Tsegayea et al. [1] and the adopted lower sodium hydroxide concentration and shorter pretreatment time were more competitive than those in this work. However, disadvantages including lower solid loading, too higher temperature, relatively lower levels of recovery of cellulose and hemicellulose and lower lignin removal still existed in that work [1]. In our opinion, the optimized pretreatment conditions in this work could simultaneously guarantee satisfactory levels of cellulose recovery, hemicellulose recovery and lignin removal by adopting moderate levels of pretreatment temperature and pretreatment time with higher solid loading and uncomplicated operation. Therefore, the pretreated WSP could be applied to the following optimization of enzymatic hydrolysis.

Statistical optimization of enzymatic hydrolysis conditions

Tween-80, one type of surfactant, could prevent enzymes from being absorbed to lignin and allow more cellulases to catalyze enzymatic hydrolysis of substrates more effectively [32]. Therefore, Tween-80 was directly applied to enzymatic hydrolysis in this work. Similar operation existed in enzymatic hydrolysis

described by Jin et al. [33]. As shown in Table 8, yields of reducing sugar and xylose varied from each other, which indicated that the adopted variables could influence the responses. Results in Table 9 indicated that variables including enzyme loading, biomass loading and reaction time have positive and significant effects on reducing sugar and xylose yields. Similar results were also found during hydrolysis of paddy straw [34], sugarcane tops [35] and rice straw [36]. In addition, enzyme loading and reaction time were also found to have significant effects during hydrolysis of wheat straw described by Singh and Bishnoi [37]. It was also reported that enzyme loading and biomass loading had significant effects on sugars production during hydrolysis of sweet sorghum bagasse described by Saini et al. [2]. In this work, reaction temperature, pH and Tween-80 concentration had insignificant effects on sugars yields. Whereas, reaction temperature was reported to have significant effect on sugar yields during hydrolysis of corn cob described by Gaiai et al. [12]. As to Tween-80 concentration, it was also reported to have insignificant effect on sugars production during hydrolysis of wheat straw described by Singh and Bishnoi [37]. However, it was mentioned that Tween-80 concentration had significant effect on sugars production during hydrolysis of pine foliage [38] and oil palm empty fruit bunch [39], respectively. In addition, reaction pH was also found to have significant effect on hydrolysis of cotton stalk [40]. In our opinion, variance of significant variables during enzymatic hydrolysis among different reports was related with lignocellulosic substrates types and hydrolytic enzymes sources. Therefore, the optimal regions of the three significant variables were investigated using the steepest ascent method by increasing the levels of them. The corresponding hydrolysis was carried out at 50 °C, pH 4.8 with 0.2% (w/v) of Tween-80, respectively. As shown in Table 10, sugars yields could be improved obviously by increasing the levels of the three significant parameters and yields of reducing sugar and xylose reached the plateau while enzyme loading, biomass loading and reaction time were 8.0 FPU/g, 7.5% (w/v) and 45.0 h, respectively. It is well known that enzymatic hydrolysis should need enough enzyme loading and reaction time to reach the maximum sugars yields. Furthermore, increment of solid loading could also promote enzymatic hydrolysis by improving enzymes accessibility to substrates due to a fixed number of active sites in enzymes to bind the substrates [39]. Whereas, sugars yields decreased after the plateau, which was probably related with feedback inhibition caused by end product, poor stirring caused by too higher biomass loading and attachment of enzymes on amorphous regions of cellulose [38, 41].

Subsequently, the optimal values of the significant factors were further investigated using BBD (Table 11). Statistical analysis of BBD in Table 12 indicated that linear terms and square terms had significant effects on sugars yields. According to P values of the models (P = 0.000, P = 0.000) and lack of fit (0.118, 0.109) along with high values of R^2 (99.4%, 98.7%) and adjusted R^2 (98.3%, 96.3%), it was obvious that the models were adequate to predict reducing sugar and xylose yields, respectively.

According to canonical analysis, maximal yield of reducing sugar (633.13 mg/g) could be obtained while enzyme loading, solid loading and reaction time were 8.45 FPU/g, 7.26% (w/v) and 45.9 h, respectively. Whereas, maximal xylose yield (150.29 mg/g) could be obtained while enzyme loading, solid loading and reaction time were 7.76 FPU/g, 6.85% (w/v) and 43.6 h, respectively. The corresponding regression models for reducing sugar and xylose yields were given below in Eq. (11) and Eq. (12):

$$Y_3 = 630.583 + 8.521X_1 - 12.395X_2 + 11.434X_3 - 18.507X_1^2 - 68.759X_2^2 - 30.477X_3^2 - 3.413X_1X_2 - 3.285X_1X_3 - 0.167X_2X_3 \quad (11)$$

$$Y_4 = 148.617 - 3.217X_1 - 5.786X_2 - 5.101X_3 - 16.270X_1^2 - 10.522X_2^2 - 8.702X_3^2 + 0.455X_1X_2 + 2.010X_1X_3 - 1.337X_2X_3 \quad (12)$$

in which, Y_3 and Y_4 were predicted reducing sugar and xylose yields, X_1, X_2 and X_3 were codes of enzyme loading, biomass loading and reaction time, respectively.

In order to determine the accuracy of the models and verify the optimization results, experiments were repeated three times under the adjusted optimized conditions, i.e., enzyme loading 8.1 FPU/g, solid loading 7.1% (w/v) and reaction time 44.8 h. Yields of reducing sugar (632.92 mg/g) and xylose (149.83 mg/g) could be obtained, which were very in close with the predicted values. Compared with the initial yields of reducing sugar (291.91 mg/g) and xylose (53.20 mg/g) under unoptimized conditions, optimization lead to 1.17-fold for reducing sugar yield and 1.82-fold for xylose yield, respectively.

Comparisons of hydrolysis conditions and sugars yields in this work with those in other previous reports were shown in Table 13. Though reducing sugar yield in this work (632.92 mg/g) was lower than 778.30 mg/g described by Annamalai et al. [19] and 772.72 mg/g descirbed by Gupta and Parkhey [36], relatively higher biomass loading (7.1%, w/v) and shorter reaction time (44.8 h) used in this work compared favorable. Though xylose yield (149.83 mg/g) in this work was lower than 156.91 mg/g described by He et al. [42], adoption of higher biomass loading (7.1%, w/v), shorter reaction time (44.8 h) and higher reducing sugar yield (632.92 mg/g) in this work was still competitive. Of course, as shown in Table 13, sugars yields in this work were also higher than those in the other three reports described by Xie et al. [7], Singh and Bishnoi [37] and Patel et al. [43], respectively. In addition, comparisons of enzyme loading among the different reports were not available, as enzymes assay conditions differed from each other. Even so, enzyme loading (8.1 FPU/g) in this work was at the moderate level. Therefore, in our opinion, the optimized hydrolysis conditions could result in considerable sugars yields by adopting moderate levels of enzyme loading, shorter reaction time and higher biomass loading.

Comparisons of pretreatment conditions among different reports were also shown in Table 13. It was obvious that pretreatment temperature (94.0 °C) in this work was higher than that (85.0 °C) during sodium hydroxide pretreatment of rice straw descirbed by Annamalai et al. [19]. However, relatively shorter pretreatment time (46.0 min) and lower sodium hydroxide concentration (1.8%, w/v) in this work were still competitive. Of course, values of pretreatment time (20.0 min, 22 min and 20 min) described by Gupta and Parkhey [36], Singh and Bishnoi [37] and Patel ea al. [43] were shorter than that (46.0 min) in this work. Whereas, we believe that adoption of microwave in the above three reports [36, 37, 43] could enhance equipment input cost for pretreatment and restrict large-scale application of the pretreatment conditions. Furthermore, adoption of lower pretreatment temperature, relatively easier operation and higher solid loading during pretreatment in this work compared favorable to those during pretreatment of wheat straw described by Xie et al. [7]. In addition, compared with pretreatment of corn stover powder

described by He et al. [42], levels of sodium hydroxide concentration and temperature in this work were relatively higher. However, relatively higher solid loading and shorter pretreatment time in this work were still advantageous. In general, the optimized pretreatment conditions in this work had some advantages such as relatively higher solid loading, easier operation and lower equipment requirement.

Conclusions

Even though literature about optimization of sodium hydroxide pretreatment or hydrolysis of WSP for enhancing sugars yields could be available, research about systematic optimization of the two important processes using RSM is still few. Therefore, this work will provide a new reference in this field. The obtained results indicated that RSM was an efficient tool for optimization of the two crucial processes during bioethanol production. The optimized pretreatment conditions could simultaneously guarantee high levels of retention of holocellulose and lignin removal as much as possible. Furthermore, the optimized hydrolysis conditions could also improve production of fermentable sugars using higher levels of biomass loading and shorter reaction time. Certainly, adoption of the crude cellulases preparation from *A. niger* HQ-1 could also reduce enzymes cost for hydrolysis. In order to make full use of hydrolysate from the pretreated WSP, optimization of fermentation for ethanol production by yeast strains should be carried out in future research.

Declarations

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Compliance with Ethical standards

Competing Interest The authors declare that they have no competing interests.

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Tables

Table 1 Codes and levels of five variables in Plackett-Burman design (PBD) along with reducing sugars and xylose yields during optimization of pretreatment

Trials	A	B	C	D	E	RS (mg/g)	XY (mg/g)
1	1 (1.2)	-1	1 (70.0)	-1 (15.0)	-1 (0.3)	146.65 ± 1.79	19.25 ± 0.23
2	1	1 (25.0)	-1 (60.0)	1 (25.0)	-1	145.36 ± 1.67	18.76 ± 0.21
3	-1 (0.8)	1	1	-1	1 (0.5)	123.98 ± 1.56	15.54 ± 0.28
4	1	-1	1	1	-1	160.32 ± 2.23	23.96 ± 0.33
5	1	1	-1	1	1	142.52 ± 1.85	18.01 ± 0.23
6	1	1	1	-1	1	145.73 ± 1.72	19.79 ± 0.24
7	-1	1	1	1	-1	137.98 ± 1.58	18.93 ± 0.25
8	-1	-1	1	1	1	135.23 ± 1.64	19.11 ± 0.28
9	-1	-1	-1	1	1	126.74 ± 1.49	14.07 ± 0.19
10	1	-1	-1	-1	1	122.46 ± 1.52	14.36 ± 0.24
11	-1	1	-1	-1	-1	111.33 ± 1.36	11.11 ± 0.22
12	-1	-1 (15.0)	-1	-1	-1	108.18 ± 1.34	10.75 ± 0.23
13	0	0	0	0	0	133.63 ± 1.55	17.38 ± 0.28
14	0	0	0	0	0	131.29 ± 1.58	17.67 ± 0.31
15	0	0	0	0	0	132.78 ± 1.67	17.47 ± 0.32

A: Sodium hydroxide concentration (%, w/v); B: Solid loading (%, w/v); C: Pretreatment temperature (°C); D: Pretreatment time (min); E: Particle size (mm); RS: Reducing sugar; XY: Xylose

Table 2 Coefficients of regression for reducing sugar and xylose yields during optimization of pretreatment

Terms	RS	XY
Constant	133.873	16.9700
Sodium hydroxide concentration	9.967**	2.0517**
Solid loading	0.610	0.0533
Pretreatment temperature	7.775**	2.4600**
Pretreatment time	7.485**	1.8367**
Particle size	-1.097	-0.1567

Outline criterion: 0.05; ** Significant at 1% level; RS: Reducing sugar; XY: Xylose

Table 3 Design of the steepest ascent method along with reducing sugar and xylose yields during optimization of pretreatment

Steps	(Sodium hydroxide concentration, %, w/v)	Pretreatment temperature (°C)	Pretreatment time (min)	RS (mg/g)	XY (mg/g)
1	1.2	75.0	25.0	172.89 ± 1.93	26.45 ± 0.32
2	1.5	85.0	35.0	220.25 ± 2.56	40.07 ± 0.46
3	1.8	95.0	45.0	288.34 ± 3.39	52.38 ± 0.61
4	2.1	105.0	55.0	242.63 ± 2.94	32.60 ± 0.38
5	2.4	115.0	65.0	184.16 ± 2.94	20.59 ± 0.25

RS: Reducing sugar; XY: Xylose

Table 4 Codes and levels of variables in CCD along with reducing sugar and xylose yields during optimization of pretreatment

Runs	x_1 (Sodium hydroxide concentration, %, w/v)	x_2 (Pretreatment temperature, °C)	x_3 (Pretreatment time, min)	RS (mg/g)	XY (mg/g)
1	-1 (1.5)	-1 (85.0)	-1 (35.0)	220.36 ± 2.89	40.01 ± 0.52
2	1 (2.1)	-1	-1	231.48 ± 3.18	36.59 ± 0.46
3	-1	1 (105.0)	-1	212.83 ± 2.63	37.09 ± 0.57
4	1	1	-1	223.85 ± 2.58	35.58 ± 0.45
5	-1	-1	1 (55.0)	232.36 ± 2.47	37.93 ± 0.62
6	1	-1	1	249.25 ± 2.97	34.66 ± 0.46
7	-1	1	1	225.86 ± 2.63	35.21 ± 0.55
8	1	1	1	242.03 ± 2.79	32.64 ± 0.47
9	-1.682 (1.30)	0 (95.0)	0 (45.0)	208.63 ± 2.45	39.62 ± 0.58
10	1.682 (2.30)	0	0	228.89 ± 3.76	32.98 ± 0.53
11	0	-1.682 (78.18)	0	236.76 ± 2.75	40.97 ± 0.64
12	0 (1.8)	1.682 (111.82)	0	225.96 ± 2.66	37.42 ± 0.54
13	0	0	-1.682 (28.18)	233.44 ± 2.78	37.57 ± 0.62
14	0	0	1.682 (61.82)	249.74 ± 3.26	31.39 ± 0.51
15	0	0	0	288.13 ± 3.64	53.11 ± 0.74
16	0	0	0	291.35 ± 4.25	52.66 ± 0.82
17	0	0	0	288.24 ± 4.39	52.19 ± 0.71
18	0	0	0	291.46 ± 3.96	51.97 ± 0.73

19	0	0	0	288.25 ± 4.19	52.08 ± 0.81
20	0	0	0	291.53 ± 4.36	53.13 ± 0.78

RS: Reducing sugar; XY: Xylose

Table 5 Estimated regression coefficients for reducing sugar and xylose yields during optimization of pretreatment conditions

Terms	RS	XY
Constant	289.737	52.5066
Sodium hydroxide concentration (x_1)	6.537**	-1.6063**
Pretreatment temperature (x_2)	-3.445**	-1.0720**
Pretreatment time (x_3)	6.472**	-1.4076**
Sodium hydroxide concentration × sodium hydroxide concentration ($x_1 \times x_1$)	-24.540**	-5.6265**
Pretreatment temperature × pretreatment temperature ($x_2 \times x_2$)	-20.085**	-4.6030**
Pretreatment time × pretreatment time ($x_3 \times x_3$)	-16.468**	-6.2700**
Sodium hydroxide concentration × pretreatment temperature ($x_1 \times x_2$)	-0.103	0.3262
Sodium hydroxide concentration × pretreatment time ($x_1 \times x_3$)	1.365	-0.1138
Pretreatment temperature × pretreatment time ($x_2 \times x_3$)	0.180	-0.1013
R^2	99.6%	99.5%
Adj-R^2	99.2%	99.1%
Lack of fit	0.104	0.122

Outline criterion: 0.05; ** Significant at 1% level; RS: Reducing sugar; XY: Xylose

Table 6 Contents of cellulosic compositions of wheat straw powder on dry basis

Substrates	Weight (g)	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Ash (%)
Raw WSP	20.00	43.52 ± 0.38	22.41 ± 0.30	9.80 ± 0.18	6.60 ± 0.05
Pretreated WSP	14.83	53.31 ± 0.56	16.49 ± 0.19	3.61 ± 0.05	2.65 ± 0.04

WSP: Wheat straw powder

Table 7 Comparisons of pretreatment conditions along with recovery and removal of main components in wheat straw and rice straw

Substrates	Pretreatment conditions	Cellulose recovery (%)	Hemicellulose recovery (%)	Lignin removal (%)	References
Wheat straw	Sodium hydroxide 1.5% (w/v), solid loading 10.0% (w/v), 160 °C, 15 min in microwave oven.	74.15	38.34	69.49	[1]
Wheat straw	Mixture solution containing H_3PO_4 (79.6%, w/w) and H_2O_2 (1.9%, w/w) , solid loading 10.0% (w/v), 40.2 °C, 2.9 h.	97.9	0	71.8	[27]
Wheat straw	Mixture solution of H_2SO_4 (1.0%, w/v) and Na_2SO_3 (2.4%, w/v), solid loading 10.0% (w/v), 180 °C, 30 min.	96.0	14.0	10.0	[28]
Wheat straw	First step: alkaline hydrogen peroxide 3.0 % (w/v), solid loading 4.0% (w/v), 70 °C, 3.0 h; Second step: lithium chloride/N, N-dimethylacetamide (LiCl/DMAc) solution (8.0%, w/w), solid loading 2.5% (w/v), 110 °C, 2.5 h and room temperature for 12.0 h.	92.59	31.39	95.2	[29]
Wheat straw	Ammonium sulfite solution 10.0% (w/v), solid loading 10.0% (w/v), 140 °C for 100 min in electric oven reactor.	74.7	64.8	65.54	[30]
Rice straw	Mixture of gacetic acid (69.85%, v/v) and formic acid solution (69.85%, v/v) at the ratio of 1:1 (v/v), solid loading 10.0% (w/v), 75.41 °C, 29.68 min.	74.09	53.38	73.17	[31]
Wheat straw	Sodium hydroxide solution 1.8% (w/v), solid loading 25.0% (w/v), 94.0 °C, 46.0 min.	90.9	54.6	72.7	This work

Table 8 Codes and levels of six variables and Plackett-Burman design (PBD) along with reducing sugar and xylose yields during optimization of enzymatic hydrolysis

Trials	A	B	C	D	E	F	RS (mg/g)	XY (mg/g)
1	1 (4.0)	-1 (1.0)	1 (0.2)	-1 (50.0)	-1 (4.4)	-1 (24.0)	315.62 ± 3.53	58.78 ± 0.74
2	1	1 (2.5)	-1 (0.1)	1 (55.0)	-1	-1	335.96 ± 3.48	71.80 ± 1.03
3	-1 (2.0)	1	1	-1	1 (4.8)	-1	317.63 ± 3.32	62.41 ± 0.96
4	1	-1	1	1	-1	1 (30.0)	342.36 ± 3.91	67.93 ± 0.94
5	1	1	-1	1	1	-1	338.13 ± 3.59	72.95 ± 1.16
6	1	1	1	-1	1	1	363.65 ± 3.85	83.09 ± 1.38
7	-1	1	1	1	-1	1	326.74 ± 3.43	69.89 ± 0.94
8	-1	-1	1	1	1	-1	290.13 ± 3.19	51.13 ± 0.85
9	-1	-1	-1	1	1	1	309.25 ± 3.65	60.05 ± 0.95
10	1	-1	-1	-1	1	1	344.25 ± 3.92	71.24 ± 1.28
11	-1	1	-1	-1	-1	1	324.33 ± 3.43	71.25 ± 1.19
12	-1	-1	-1	-1	-1	-1	293.45 ± 3.29	53.91 ± 0.86
13	0	0	0	0	0	0	324.23 ± 3.53	67.33 ± 0.88
14	0	0	0	0	0	0	321.32 ± 3.67	67.97 ± 0.92
15	0	0	0	0	0	0	323.55 ± 3.56	68.29 ± 0.97

A: Enzyme loading (FPU/g); B: Biomass loading (%, w/v); C: Tween-80 concentration (%, w/v); D: Reaction temperature (°C); E: Reaction pH; F: Reaction time (h); RS: Reducing sugar; XY: Xylose

Table 9 Coefficients of regression for reducing sugar and xylose yields during optimization of enzymatic hydrolysis

Terms	RS	XY
Constant	325.125	66.2025
Enzyme loading	14.870**	4.7625**
Biomass loading	9.282**	5.6958**
Tween-80 concentration	0.897	-0.6642
Reaction temperature	-1.363	-0.5775
Reaction pH	2.048	0.6092
Reaction time	9.972**	4.3725**

Outline criterion: 0.05; ** Significant at 1% level; RS: Reducing sugar; XY: Xylose

Table 10 Design of the steepest ascent method along with reducing sugar and xylose yields during optimization of enzymatic hydrolysis

Steps	Enzyme loading (FPU/g)	Biomass loading (%, w/v)	Reaction time (h)	RS (mg/g)	XY (mg/g)
1	4.0	2.5	35.0	407.41 ± 5.12	98.83 ± 1.23
2	6.0	5.0	40.0	501.69 ± 6.32	121.98 ± 1.39
3	8.0	7.5	45.0	630.11 ± 6.89	149.94 ± 1.69
4	10.0	10.0	50.0	491.34 ± 5.48	105.17 ± 1.47
5	12.0	12.5	55.0	386.12 ± 4.66	78.49 ± 1.03

RS: Reducing sugar; XY: Xylose

Table 11 Codes and levels of variables and BBD along with reducing sugar and xylose yields during optimization of enzymatic hydrolysis

Runs	X_1	X_2	X_3	RS (mg/g)	XY (mg/g)
1	-1 (6.0)	-1 (5.0)	0 (45.0)	545.68 ± 6.23	131.54 ± 1.86
2	1 (10.0)	-1	0	570.23 ± 6.15	125.76 ± 1.58
3	-1	1 (10.0)	0	523.23 ± 6.35	116.98 ± 1.49
4	1	1	0	534.13 ± 6.84	113.02 ± 1.43
5	-1	0 (7.5)	-1 (40.0)	555.38 ± 6.87	135.98 ± 1.97
6	1	0	-1	578.31 ± 6.79	123.96 ± 1.89
7	-1	0	1 (50.0)	591.46 ± 7.12	119.31 ± 1.77
8	1	0	1	601.25 ± 7.23	115.33 ± 1.73
9	0 (8.0)	-1	-1	533.22 ± 6.29	136.68 ± 1.66
10	0	1	-1	513.25 ± 6.15	129.86 ± 1.85
11	0	-1	1	549.78 ± 6.94	131.60 ± 1.96
12	0	1	1	529.14 ± 7.18	119.43 ± 1.68
13	0	0	0	632.77 ± 7.28	148.80 ± 2.08
14	0	0	0	630.93 ± 7.39	147.53 ± 2.19
15	0	0	0	628.05 ± 7.41	149.52 ± 2.28

X_1: Enzyme loading (FPU/g); X_2: Biomass loading (%, w/v); X_3: Reaction time (h); RS: Reducing sugar; XY: Xylose

Table 12 Estimated regression coefficients for reducing sugar and xylose yields during optimization of enzymatic hydrolysis

Terms	RS	XY
Constant	630.583	148.617
Enzyme loading (X_1)	8.521**	-3.217*
Biomass loading (X_2)	-12.395**	-5.786**
Reaction time (X_3)	11.434**	-5.101**
Enzyme loading × enzyme loading ($X_1 \times X_1$)	-18.507**	-16.270**
Biomass loading × biomass loading ($X_2 \times X_2$)	-68.759**	-10.522**
Reaction time × reaction time ($X_3 \times X_3$)	-30.477**	-8.702**
Enzyme loading × biomass loading ($X_1 \times X_2$)	-3.413	0.455
Enzyme loading × reaction time ($X_1 \times X_3$)	-3.285	2.010
Biomass loading × reaction time ($X_2 \times X_3$)	-0.167	-1.337
R^2	99.4%	98.7%
Adj-R^2	98.3%	96.3%
Lack of fit	0.118	0.109

Outline criterion: 0.05; * Significant at 5% level; ** Significant at 1% level; RS: Reducing sugar; XY: Xylose

Table 13 Comparisons of reducing sugar and xylose yields along with pretreament and hydrolysis conditions of different substrates

Substrates	Enzyme sources	Pretreatment conditions	Hydrolysis conditions	RS (mg/g)	XY (mg/g)	References
Wheat straw	Commercial cellulase	Potassium hydroxide solution 15.0% (w/v), anthraquinone 0.1% (w/v), sodium lignosulfonate 2.0% (w/v) , solid loading 16.7% (w/v), 121 °C, 40 min.	Enzyme loading 30 FPU/g-glucan, biomass loading 2.0% (w/v), 72 h.	NA	117.5	[7]
Rice straw	*Bacillus carboniphilus* CAS 3	Sodium hydroxide solution 2.0% (w/v), solid loading 25% (w/v), 85 °C, 60 min.	Enzyme loading 125 CMCU/g, biomass loading 2.0% (w/v), 168 h.	778.30	NA	[19]
Rice straw	*Lysinibacillus sphaericus*	Sodium hydroxide solution 4.8% (w/v), solid loading 10.0% (w/v), 20min in microwave (350W).	Enzyme loading 40 FPU/g, biomass loading 1.84% (w/v), Tween-80 0.0325% (w/v), 57.4 h.	772.72	NA	[36]
Wheat straw	*Trichoderma reesei* MTCC 164	Sodium hydroxide solution 2.75% (w/v), solid loading 10.0% (w/v), 100 °C, 22 min in microwave.	Enzyme loading 10.8 FPU/g, biomass loading 1.0% (w/v), 54.1 h.	468.07	NA	[37]
Corn stover powder	*Trichoderma reesei*	Sodium hydroxide solution 1.0% (w/v), solid loading 10.0% (w/v), room temperature, 60 min.	Enzyme loading 7.0 FPU/g, biomass loading 1.0% (w/v), 120 h.	467	156.91	[42]
Wheat straw	Commercial cellulase SIGMA and crude cellulases by *Aspergillus niger* ADH-11	Sodium hydroxide solution 0.5% (w/v), solid loading 10.0% (w/v), 20min in microwave.	Enyzme loading (5 FPU/g of SIGMA cellulase and 5 FPU/g of crude	610.35	NA	[43]

			cellulase), solid loading 2.5% (w/v), Tween-80 0.1% (v/v), 72 h.			
Wheat straw	*Aspergillus niger* HQ-1	Sodium hydroxide solution 1.8% (w/v), solid loading 25.0% (w/v), 94.0 °C, 46.0 min.	Enzyme loading 8.1 FPU/g, biomass loading 7.1% (w/v), Tween-80 0.2% (w/v), 44.8 h.	632.92	149.83	This work

RS: Reducing sugar; XY: Xylose

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryinformation.doc